Prey-mediated effects of imidacloprid on *Laricobius nigrinus* (Coleoptera: Derodontidae) and *Sasajiscymnus tsugae* (Coleoptera: Coccinellidae), two predators of hemlock woolly adelgid

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

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Entomology

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Prey-mediated effects of imidacloprid on *Laricobius nigrinus* (Coleoptera: Derodontidae) and *Sasajiscymnus tsugae* (Coleoptera: Coccinellidae), two predators of hemlock woolly adelgid

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ABSTRACT

Prey-mediated effects of imidacloprid were evaluated for *Laricobius nigrinus* Fender and *Sasajiscymnus tsugae* Sasaji and McClure after feeding on hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae). Two methods were evaluated for detecting imidacloprid in hemlock tissues: a commercially available enzyme linked immunoassay (ELISA) kit and a high performance thin-layer chromatography technique for detecting and quantifying imidacloprid residues in hemlock wood and needle tissues. ELISA is advantageous because of its cost, sensitivity, and ease of use. However, matrix effects in the form of false positives and overestimated imidacloprid concentrations were evident in hemlock wood and needle tissue extracts. Matrix effects could be reduced by dilution with water, effectively raising the lower detection range of the kit from 0.2 to 200 ppb. High performance thin-layer chromatography was accurate, quick, easy to use, and matrix effects were not evident. However, the technique is sensitive in the lower ppm range and tissue samples from field-treated hemlocks are often in the ppb range, making this technique less desirable than more sensitive analytical methods.

Lethal and sublethal effects on both predators were evident after eastern hemlock branches infested with HWA were spiked with imidacloprid in the laboratory. HWA mortality increased with dosage and time, and its 30 d LC$_{50}$ was determined to be 242 ppb. Both predator species exhibited reduced survivorship and fitness parameters after feeding on HWA from the treated branches. In a topical application bio-assay, 6 d imidacloprid LD$_{50}$ values for *L. nigrinus* and *S. tsugae* were 2.43 and 1.82 µg/g, respectively. Imidacloprid and its major metabolites in hemlock tissues were analyzed by liquid chromatography-tandem mass spectrometry. Imidacloprid recovery from beetle cadavers was correlated with beetle mortality from feeding on treated hemlock branches. Olefin was the primary imidacloprid metabolite recovered from hemlock wood tissues.
When predators fed on HWA from field-treated trees, impacts on survivorship and fitness were variable. In 2005, significantly higher proportions of both species of beetles were affected by feeding on control branches compared with treated branches. In 2006, beetles feeding on HWA from some of the trees treated in the field exhibited longer fliptimes compared with beetles feeding on controls, although beetle mortality was not significant among treatments. In the field, imidacloprid controlled HWA populations 1-3 years post-treatment. Hemlock health improved in the highest dosage group, with significantly greater lengths of new shoots compared with shoots from control trees. Eastern hemlock trees primarily metabolized imidacloprid into the olefin metabolite, which can have increased insecticidal toxicity compared with imidacloprid. Imidacloprid was detected in beetle cadavers after feeding on HWA from treated branches, suggesting that prey-mediated impacts of systemic imidacloprid are possible on nontarget predators. However, because of HWA’s sensitivity to imidacloprid, in field situations predators are more likely to be affected by reduced adelgid density and quality.
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In memory of Warren Mays and Olen Sharron.
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Preface

Each chapter is presented as an independent manuscript intended for publication, resulting in some repetition of information. Appendix A was not included as a chapter because the imidacloprid concentrations recovered from the hemlock branches were exponentially higher than concentrations that are expected in hemlock tissues from imidacloprid field applications (see Chapter 4). Appendix B was not included as a chapter because it is not directly associated with control of hemlock woolly adelgid. Appendices C – F are included to supplement Chapters 3 and 4.
Chapter 1
Introduction and Literature Review

1.1 Hemlock woolly adelgid in the United States

Hemlock woolly adelgid (HWA), Adelges tsugae Annand (Hemiptera: Adelgidae) is an introduced pest of eastern hemlock, Tsuga canadensis (L.) Carriere, and Carolina hemlock, T. caroliniana Engelmann, two hemlock species that are native to the eastern U.S. This adelgid is a piercing-sucking feeder that takes nutrients and sap from the hemlock’s parenchyma cells (McClure 1987a, 1991a, Shields et al. 1995, Young et al. 1995). Infestation causes needle desiccation and discoloration and can inhibit production of new growth (McClure 1987a, McClure et al. 2001). Eastern hemlocks are particularly sensitive to the stress of adelgid attack, and can die in as few as 4 years after infestation (McClure 1991b, McClure et al. 2001).

In western North America, HWA infests both western hemlock, T. heterophylla Raf. Sarg. and mountain hemlock T. mertensiana Bong., but does not reach a lethal level of density on either species as it does on eastern species (McClure et al. 2001). HWA is considered native to Asia, where it is found in India, Japan, China, and Taiwan on other Tsuga species at generally low densities (McClure 1987a, 2001). Mitochondrial DNA analysis of HWA in eastern North America confirms a recent introduction with the initial population exhibiting a close evolutionary relationship to HWA infesting T. sieboldii Carriere in southern Japan (Havill et al. 2006). However, the multiple halotypes present in the western north American HWA population suggests an earlier time of establishment with lineage more closely related to mainland Chinese populations infesting T. chinensis Franchet (Havill et al. 2006). The low HWA densities observed in its native environment are possibly the result of predation by natural enemies and host plant resistance (McClure and Cheah 1999).

HWA was first observed in the Pacific Northwest in the 1920s (Annand 1924) and on the east coast in Richmond, Virginia in the 1950s (McClure 1989, Souto et al. 1996). Possibly dispersed by Hurricane Gloria in 1985, HWA was found in Connecticut in 1986 (McClure 1987a, Souto et al. 1996, Souto and Shields 1999). Once established in the northeast, HWA spread rapidly
throughout the native range of the eastern hemlock. Dispersed by wind, birds, deer, and humans, (McClure 1987a, 1990) HWA has been reported as far south as Georgia and as far north as Maine. The main front of infestation is moving as quickly as 10-15 miles per year and isolated colonies are dispersing even more rapidly throughout the southeast (Souto et al. 1996, McClure 2001). Left unchecked, HWA threatens to significantly diminish hemlock populations throughout the entire east coast (Orwig and Foster 1998).

The Adelgidae are members of the Aphidoidea (Hemiptera), and their evolution and speciation has been closely linked to the speciation of their tree hosts (Havill and Foottit 2007). HWA has a polymorphic life cycle and completes two generations each year on eastern hemlock (McClure 1987a, 1995). In the eastern U.S., the sistentes, or the overwintering generation, hatch in early summer and undergo aestival diapause, resuming development in the fall (McClure 1987a, McClure et al. 2001, Salom et al. 2001). Nymphs feed and develop at the base of the hemlock needles throughout the winter. HWA has a low lethal temperature and is able to survive at temperatures below -20° C (Parker et al. 1999, Gouli et al. 2000), although cold tolerance will vary spatially and temporally (Skinner et al. 2003). Sistentes nymphs reach maturity in early spring and begin to lay eggs. From March to May each adult lays about 48 eggs in a protective cotton-like white ovisac (McClure 1989). The eggs hatch in April and May as one of two types of progeny, either as sexual sexuparvae or asexual progredientes. Progredientes are morphologically analogous to the sistentes. They have four instars and develop at increasing rates from 4 to 22°C (Salom et al. 2002). In late spring to early summer the progredientes lay approximately 22 eggs per ovisac (McClure 1989). The eggs hatch as sistentes in late June and begin the cycle again. Members of the sexual generation are called sexuparvae; they are alate and migrate to spruce trees to lay eggs, resulting in sexual progeny called sexuales (McClure 1989). However, the 15 species of spruce found in eastern North America are not suitable hosts and the sexuparvae cannot develop past the first instar (McClure 1987b, McClure 1989). The proportion of progredientes and sexuparvae is a density-dependent relationship, with higher sistentes densities leading to higher proportions of sexuparvae (McClure 1991b).
1.2 Eastern hemlocks

Eastern hemlocks are found throughout the northeastern U.S., extending from Maine and southward through the Appalachian Mountains into northern Georgia and Alabama (Godman and Lancaster 1990). Carolina hemlock is less prevalent than eastern hemlock, and is found primarily in isolated stands in the southern-most Appalachian mountains (Evans 2004). A pollen analysis of two sites in Massachusetts revealed that Eastern hemlock was a dominant taxon for 8000 years, with its numbers decreasing after disturbances, and then gradually resurgence over 300-1200 years to its former abundance (Foster and ZebravkJ 1993). Eastern hemlock is slow-growing, shade-tolerant, and long-lived; the record holder was measured to be 988 years old (Godman and Lancaster 1990). Eastern hemlock is a late-successional species with the ability to thrive in shade and extremely low light levels (Hadley 2000a). Hemlock stands rely on a positive feedback system, as they create an environment with low light and poor soil that encourages hemlock seedling germination and growth (Catovsky and Bazzaz 2000). Eastern hemlocks are able to germinate in deep shade on highly organic soils. Seedling germination begins at temperatures above 7 °C, and hemlock seedlings face heavy competition with spruce and fir seedlings in the northern-most areas of its range (Vostral et al. 2002).

Eastern hemlock maintains year round transpiration rates with the highest rates measured in the spring (Ford and Vose 2007). Its photosynthesis rate does not drop with the onset of winter but slows down when air temperatures drop to below -10 °C (Burkle and Logan 2003). Rates slow when air temperature falls below 4 °C or rise above 20 °C, and increasing net photosynthetic rates are seen as temperatures rise from 4-20 °C (Martin and Lewis 2004). Eastern hemlock saplings in the understory are little affected by temperature from late spring through early fall, with net photosynthetic rate increasing between 11 and 15 °C but not changing between 15 and 30 °C (Hadley 2000b). Carbon storage and annual photosynthesis are aided by mild winters with fewer frosts and extended thaws (Hadley 2000b), with greatest rates occurring in May (Hadley 2000a). In a subsequent study in Massachusetts, carbon storage was highest from April through July and in October, with maximum rates in April and May. Carbon exchange in autumn and winter is most affected by daily minimum air temperature, and in the spring and fall it is most affected by time of day, water vapor pressure deficit, and air temperature. Carbon storage was
negligible from December through March, and was reduced by nocturnal frost and sub-zero temperatures (Hadley and Schedlbauer 2002).

HWA breaks aestivation and begins to feed in early fall, and continues throughout the winter. In the spring, HWA sistentes lay eggs and give rise to a second generation. Thus, HWA biology is well synchronized with hemlock phenology, feeding when the trees are active and entering aestivation in the summer in parallel with the trees becoming dormant. Cellular nutritional chemistry may also play a role in hemlock susceptibility, with higher nitrogen and potassium concentrations increasing hemlock palatability by HWA and high calcium and phosphorous concentrations discouraging heavier infestations (Pontius et al. 2006). Terpenoids are naturally occurring tree chemicals that can inhibit acetylcholinesterase and thereby repel or display toxicity against feeding insects. Terpenoid composition plays a role in tree susceptibility to HWA infestation, with myrcene and germacrene D in the leaf cushion deterring settling and feeding by HWA and isobornyl acetate acting as a potential chemical attractant for HWA feeding (Lagalante and Montgomery 2003). The adelgid lifecycle on eastern hemlock closely parallels changing terpenoid levels within the leaf cushion as HWA avoids variable levels of terpenoids by entering a non-feeding aestivation in the summer. This corresponds to unstable and variable levels of terpenoids in hemlock leaf tissues, while adelgids preferentially reproduce and feed during the spring on new growth tissue that has a relatively stable and low concentration of terpenoids in the parenchyma cells (Lagalante and Montgomery 2003). Thus, the chemical makeup of hemlock foliage seems to be a primary component affecting adelgid population densities on hemlock branches. The composition of volatile emissions of terpenoids from eastern hemlocks is affected by the presence or absence of HWA infestation (Broeckling and Salom 2003).

Northwestern American and Asian *Tsuga* species are resistant to HWA and infestations are often found at lower densites compared with eastern U.S. *Tsuga* species, possibly as a consequence of differences in tree terpenoid chemistry (Lagalante and Montgomery 2003, Lagalante et al. 2006) and tree nutritional components (Pontius et al. 2006), tree host resistance (Cheah and McClure 1995), genetic differences between the different geographic populations of HWA (Havill et al.
2006), and the presence of a complex of natural enemies in the pacific-northwest and Asia (McClure et al. 2000, Wallace and Hain 2000, Mausel 2005).

Hemlocks play many important roles in the forested ecosystems, and their widespread mortality has been ecologically traumatic. They are critical components of habitats for many species including birds, turkey, rodents, rabbits, and many plant species (Yamasaki et al. 1999, McClure et al. 2001, Tingley et al. 2002). Hemlocks create winter shelter for white-tailed deer (Reay 1999) and provide shade for aquatic organisms (Yamasaki et al. 1999, McClure et al. 2001). Along streambeds they help sustain cool water temperatures vital to brook trout (Evans et al. 1995), an important game fish that is more common and abundant in streams draining hemlock forests than hardwood forests (Snyder et al. 2005). Widespread hemlock decline could lead to higher nitrification in the forest floor and to water pollution from nitrate leaching (Jenkins et al. 1999). Hemlocks play an important role in hydrological processes in forest ecosystems. Their decline and subsequent replacement by rhododendron (*Rhododendron maximum* L.) in many southern Appalachian riparian communities will impact nutrient and carbon cycling, riparian vegetative transpiration, and will reduce soil moisture and seasonal transpiration rates (Ford and Vose 2007).

The disappearance of hemlocks would be comparable to the loss of elms and chestnuts, with a loss of vegetative diversity and establishment (Foster 1999, Orwig et al. 2002, Evans 2004) and alterations of forest micro-environments and vegetation (Kizlinski et al. 2002). Hemlock decline and death from HWA is leading to transformations in canopy biomass and distribution, such as the increasing prevalence of early successional species black birch (*Betula lenta* L.) (Orwig and Foster 1998, Stadler et al. 2005), and late succesional species such as black gum (*Nyssa sylvatica* L.) and yellow-poplar (*Liriodendron tulipifera* L.) (Ford and Vose 2007). Other species gaining from hemlock decline include white pine (*Pinus strobes* L.) and the exotic invasives tree-of-heaven (*Ailanthus altissima* (P. Mill.) Swingle), Japanese barberry (*Barberis thunbergii* DC), Asiatic bittersweet (*Celastrus orbiculatus* Thunb.), and Japanese stiltgrass (*Microstegium vimineum* (Trin.) A. Camus.) (Orwig and Foster 1998, Evans 2004). Regeneration of eastern hemlocks may be hindered since it cannot sprout or re-foliate after defoliation and rely on seeds and seed banks to re-propagate; seeds are viable for only 1 to 4 years (Orwig and Foster 1998).
Heavy deer-browse pressure may also impede hemlock regeneration (Orwig and Foster 1998). Hemlocks are often pre-emptively logged before deterioration from HWA infestation begins (Orwig et al. 2002), a process that can lead to nitrogen loss and other detrimental environmental changes compared with naturally deteriorating stands (Kizlinski et al. 2002).

Eastern forests contain enough hemlock wood fiber for 1.5 million conventional homes and 15 billion newspapers Rhea (1995). Hemlock can be used for pulpwood and lumber, and plantings are often used in ornamental settings and landscapes (McClure et al. 2001). As an ornamental, hemlock is valued for its landscape appeal and shade qualities, and nursery industries have invested millions of dollars in this species (Rhea 1995). In urban landscapes, declining hemlock health contributes to deterioration of residential property values (Holmes et al. 2005).

1.3 Biological control of HWA

Applications of any one of a variety of available insecticides are effective approaches for HWA control. Use of insecticides, however, is not suitable in large or inaccessible forest settings. Foliar sprays require mechanized sprayers, and hemlocks must be covered on all sides to achieve adequate protection. Currently, chemical control is more practical in ornamental landscapes where trees are of high value and are easily accessible (Rhea 1995). Because of the impracticalities of using insecticides in the forest, alternative approaches are needed. In Asia and in the Pacific Northwest, HWA infests hemlock species but the trees are rarely killed or injured, suggesting that host resistance and natural enemies are important factors keeping HWA at sub-injurious levels (Cheah and McClure 1995, McClure 1996, 2001). Biological control offers the most promising options for keeping HWA densities below damaging levels on T. canadensis and T. caroliniana (McClure 1987a, Wallace and Hain 2000). There are no known parasitoids of Adelgidae (Montgomery and Lyon 1996), so biological control efforts have focused on predators. Establishment of an individual predator species can have beneficial impacts against pests, however, a complementary complex of predators is a likely requirement to achieve some level of population regulation of HWA (Cheah et al. 2004).
Sasajiscymnuss tsugae (= Pseudoscymnus tsugae) Sasaji and McClure, is a predator associated with HWA in Japan (Cheah and McClure 1995, Sasaji and McClure 1997). It displays characteristics that make it amenable to mass rearing in the laboratory (Cheah and McClure 1998), as well as displaying seasonal synchrony with HWA, including an ability to overwinter in Connecticut hemlock stands (Cheah and McClure 2000, 2002, Cheah et al. 2005). Between 1995 and 2002 more than 627,000 S. tsugae beetles were released in 11 eastern U.S. states (McClure and Cheah 2002). Releases since 2002 have brought the numbers to over 3,000,000 (Cheah et al. 2004, Mausel 2007). Mass releases of the beetle into infested stands in the northeastern U.S. resulted in a greater HWA density reduction than control stands (McClure and Cheah 1999, McClure 2001). In Connecticut and New Jersey, hemlock crowns exhibited improved foliage transparency in S. tsugae release sites than in comparable stands where no beetles were released, although relatively few individuals were recovered (Cheah et al. 2005). However, another study in Massachusetts concluded that S. tsugae may be inadequate to control high densities of HWA, in part because the beetle failed to produce progeny in the field (Butin et al. 2003).

Another predator being studied as a biological control agent is Laricobius nigrinus Fender (Coleoptera: Derodontidae). L. nigrinus is found on HWA in western North America where both larvae and adults have been observed feeding on HWA (McClure 2001, Zilahi-Balogh et al. 2003a). The beetle shows good synchrony with HWA’s lifecycle, and strong host specificity (Zilahi-Balogh et al. 2002, 2003b). Adults are univoltine and feed on sistentes throughout winter before the females oviposit into adelgid ovisacs in spring. Following eclosion of L. nigrinus eggs, the larvae feed on adelgid eggs until their fourth instar at which time they drop from the hemlock branches and seek a soil site for pupation (Zilahi-Balogh et al. 2003a). After pupation, adults remain in the soil and enter aestival diapause in the early summer. They break diapause and emerge to feed in the fall. The timing of aestivation and emergence is synchronized with the life cycle of HWA (Zilahi-Balogh et al. 2003a). Field tests showed the ability of L. nigrinus to survive southwestern Virginia winter temperatures and the beetle’s capacity to reduce the density of HWA sistentes by at least 50 percent in sleeve cages (Lamb et al. 2005a). Predator field releases began in 2003. L. nigrinus is currently being mass reared at Virginia Tech, the University of Tennessee and Clemson University. In Virginia, HWA densities on branches where L. nigrinus adults were released in cages were lower than HWA densities on control branches,
and F₂ beetle progeny were recovered in the field one year after release (Lamb et al. 2006). F₃ generation progeny were recovered from 13 of 22 sites in eight states on the eastern U.S. (Mausel 2007). Releases through 2008 have brought the total to over 50,000 individuals released at over 80 sites.

Many species of predators are found in association with HWA in Asia. Fifty-four generalist ladybird species were collected from infested hemlocks in China (Montgomery et al. 1999, Yu et al. 2000). Scymnus (Neopulus) sinuanodulus Yu and Yao (Coleoptera: Coccinellidae), has been imported from China and is being evaluated as a biological control agent for HWA (Lu et al. 2002). Laricobius baoxingensis Zilahi-Balogh and Jelinek, and L. kandingensis Zilahi-Balogh and Jelínek are associated with Adelges species on T. chinensis (Franchet) Pritzel in southwestern China (Zilahi-Balogh et al. 2007). Another coccinellid predator from China, Scymnus ningshanensis, Yu et Yao, reduced HWA populations in sleeve cages and was able to lay successful progeny in the field in Massachusetts (Butin et al. 2003). Tetraphleps galchanoides Ghauri (Hemiptera: Anthocoridae) found on Chinese Tsuga spp. display desirable characteristics for a biological control agent, and is currently being studied in quarantine in the U.S. (McAvoy et al. 2006).

Scymnus suturalis Thunberg (Coleoptera: Coccinellidae) and Laricobius rubidus LeConte (Coleoptera: Derodontidae) are predators of pine bark adelgid, Pinea strobi Hartig (Hemiptera: Adelgidae), and are found in association with HWA in the eastern United States (Montgomery and Lyon 1996) although current densities are insufficient to provide a significant reduction of the pest in eastern forests (Zilahi-Balogh et al. 2005). Generalist predators found in association with HWA in the eastern U.S. include ladybeetles (Coleoptera: Coccinellidae), lacewings (Neuroptera: Chrysopidae and Hemerobiidae), gall gnats and midges (Diptera: Cecidomyiidae), and flower flies (Diptera: Syrphidae), although their impacts are insufficient for control (McClure 1987a, Wallace and Hain 2000).

The releases of multiple predators with overlapping lifecycles and habitats are likely to interact in the field as they establish and jointly occupy niches of HWA-infested hemlock stands. In a study investigating conspecific and interspecific competitive interactions among S. tsugae, L.
nigrinus, and H. axyridis, heterospecific interactions were not of consequence while conspecific interactions resulted in negative competitive interactions in both a laboratory setting (Flowers et al. 2005) and in a field setting in southwestern Virginia (Flowers et al. 2006). Differing behavior patterns among L. nigrinus, S. tsugae and H. axyridis suggest that high density releases of multiple predator species combinations would generally be the most effective release strategy for impacting HWA populations (Flowers et al. 2007).

1.4 Imidacloprid and chemical control of HWA

Imidacloprid, 1-(6-chloro-3- pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine, one of the best selling and widely used insecticides in the world (Nauen and Bretschneider 2002) is registered and commonly used for HWA control. It is a neonicotinoid and is an agonist of insect post synaptic nicotinic acetylcholine receptors (nAChRs) (Elbert et al. 1991). Binding activates nAChRs and initiates nervous system stimulation along the synapse (Lind et al. 1999, Silcox 2002, Tomizawa and Casida 2003). Imidacloprid is relatively safe for vertebrates because of binding properties that are more compatible with insect nAChRs (Tomizawa and Casida 1999, 2003, Tomizawa et al. 2000), and are unable to bind with muscarinic receptors (Buckingham et al. 1997). The physiological differences between mammalian and insect nAChRs give imidacloprid selectivity towards insect systems and a high level of mammalian safety (Tomizawa and Casida 1999, 2003). Poisoning symptoms mimic nicotine poisoning with loss of coordination, twitching, tremors, and eventually paralysis.

Imidacloprid has physical characteristics that result in effective systemic properties that make it effective as a foliar, seed, or soil treatment (Pflüger and Schmuck 1991, Placke and Weber 1993, Silcox 2002). It is not considered active in the vapor stage (Elbert et al. 1991, Boiteau and Osborn 1997). Imidacloprid provides protection in growing plants as it is especially prevalent in new growth and has shown efficient acropetal translocation after foliar application (Elbert et al. 1991). Cotton plants transformed imidacloprid into the olefin metabolite, which has 10-fold the insecticidal properties of the parent compound against some insects (Nauen et al. 1998a, Nauen et al. 1999). Imidacloprid is completely degradable in organic soils, and it degrades and disappears under temperate climate conditions (Krohn and Hellpointner 2002). It has been
effective in pest systems where insecticide resistance is common against other compounds (Nauen and Denholm 2005), although brown planthopper, *Nilaparvata lugens* Stål, populations in China have been reported to have a nAChR mutation with target-site resistance to imidacloprid (Liu et al. 2006). Imidacloprid displays a positive temperature coefficient of toxicity (Elbert et al. 1991) and is a strong systemic that is used as a seed treatment, foliar spray, or soil drench against many agricultural pests, including Lepidopterans, Coleopterans, Hemipterans (Nauen and Bretschneider 2002), Isoptera (Ramakrishnan et al. 2000, Thorne and Breisch 2001), Blattaria (Appel and Tanley 2000), Diptera (Stelinski and Liburd 2001, Barry et al. 2004, Liburd et al. 2004, Paul et al. 2006), and Siphonaptera (Jacobs et al. 2000, Rust et al. 2002).

Currently, chemical control is the most effective way to control HWA infestations in accessible stands and on individual trees. Use of imidacloprid as a control of HWA is well documented and endorsed because of its effectiveness as a systemic insecticide for control of sucking pests. Imidacloprid can be applied by foliar sprays or administered systemically through trunk or soil injections (McClure et al. 2001, McClure and Cheah 2002, Doccola et al. 2003). Foliar applications of diazinon, ethion, fluvalinate, and malathion resulted in 99 to 100 percent mortality of HWA (McClure 1987a).

Foliar sprays of insecticidal soap and horticultural oil kill by suffocation and are common treatments for HWA. These compounds have been shown to kill significant percentages of HWA populations, with mortality ranging from 95 to 100% (McClure 1987a, 1991a, Rhea 1995, McClure et al. 2001). Cowles and Cheah (2001) applied imidacloprid to hemlocks as a foliar spray using a CO₂ pressurized sprayer, resulting in greater than 96% adelgid mortality. Despite such good results, foliar sprays are less appropriate in forest settings because of the need to cover the tree completely, the inaccessibility of some trees, tall trees requiring power spray equipment, and a tendency for aerial drift (Doccola et al. 2003). Foliar sprays also have a broad spectrum, killing all insects on the tree indiscriminately including any natural enemies.

For systemic application, stem injections work more quickly and require less pesticide volume than soil injection because they are injected directly into the vascular system. One drawback to
stem injection is the necessity to wound the tree by drilling into or puncturing the bark or vascular bundle, which can lead to wounding and girdling (Doccola et al. 2003, Smith and Lewis 2005). Uptake in conifers is slow but efficient and uniform, and healthy trees have greater and faster uptake rates than unhealthy trees (Sánchez-Zamora and Fernández-Escobar 2004). Conifer xylem is constructed from tracheids, which are less adept at water movement than the large straw-like xylem tissues found in hardwoods. Some coniferous species also exhibit a defensive wound response, producing resin and sap to discourage boring pests or invading pathogens (Sánchez-Zamora and Fernández-Escobar 2004). Successful uptake using trunk injection depends upon the volume of material injected, plant species, environmental conditions, tree health and size, water movement and availability, and the molecular properties of the product injected (Sánchez-Zamora and Fernández-Escobar 2000, 2004). Imidacloprid is a amenable to trunk injections, and has been proven efficacious when trunk injected against gall wasps, *Callirhitis cornigera* Osten and Sacken, on pin oak, *Quercus palustris* Muench-hausen (Eliaison and Potter 2000).

The Mauget® method (J. J. Mauget Company, Arcadia, California) uses holes drilled into the vascular system so the chemical can be applied to the xylem and the tree passively takes up the chemicals. Self-pressurized capsules (3 mL of formulated (10%) Imicide® per 15 cm trunk circumference) deliver the product through holes drilled through the bark. Tattar et al. (1998) treated eastern hemlocks with imidacloprid in June using both trunk injections with the Mauget® system and soil injections with a hydraulic sprayer. They found soil-injected imidacloprid took 8-12 weeks to reach a concentration of approximately 0.15 parts per million (ppm) in eastern hemlock foliage, while trunk-injected imidacloprid only took 4 weeks to reach the same concentrations. Cowles et al. (2006), using the same method, observed variable control with a fall injection, and poor uptake and control with early summer injections. Poor uptake from summer injections may have been due to the season and associated physiology of the tree of that time of year.

Arborjet VIPER (Arborjet, Inc., Winchester, Massachusetts) is a specialized self-pressurized injection device that can inject Imicide [10% formulation, 2 mL injected every 2.5 cm of total diameter at breast height (dbh)], or Imajet® (5% formulation, 6 mL injected every 24 cm of

Bayer Advanced Tree and Shrub Insect Control® [68 mL (1 gram active ingredient (a.i.) per 2.5 cm dbh] is an effective option for homeowners to treat infested trees (Cowles et al. 2005). Webb et al. (2003) used a soil drench (Merit® 75 WP) to treat hemlocks and found that it curbed infestation regardless of HWA density levels and tree health. All treated trees were healthier than control trees after treatment. Rhea (1995) used imidacloprid (Merit® 75 WP) as a soil drench and found that it took 4-6 months to reach lethal concentrations. Soil injections of imidacloprid (Merit 2f) resulted in 95.9% HWA mortality compared with 56.5% control mortality (Steward and Horner 1994).

The Kioritz® (Kioritz Corp., Tokyo Japan) soil injector is a handheld injector that injects pesticides in aqueous solution into the root zone of the tree approximately 15 cm under the surface (Steward et al. 1998). The Kioritz injects its contents with hand powered pneumatic pressure, and uses a low volume of water, which is advantageous for forest applications or locations where sufficient water resources are not available. The Kioritz is suitable for eastern hemlocks and imidacloprid application for HWA control. Steward et al. (1998) and Cowles et al. (2005) used the Kioritz to apply recommended doses of imidacloprid (Merit® (Bayer) 75% wettable powder, 1 gram a.i. per 2.5 cm DBH) to hemlocks infested with HWA, resulting in 98% adelgid mortality up to one year after treatment. One drawback to soil application is problems with certain soil types. Use of the Kioritz can be a problem in some areas because rocky or clay soils can make application difficult and restrict distribution of the insecticides in the soil for the root system to take up (Doccola et al. 2003, Cowles et al. 2005).

1.5 Insecticides and natural enemies

While control of HWA is the long-term goal of biological control programs and releases, insecticides are currently heavily relied-upon to control HWA in ornamental settings and high-value hemlock stands in State Parks and National Forests. Insecticides can potentially be a factor negatively influencing the establishment, reproduction, and spread of biological control agents.
The impact of insecticides on pest-natural enemy complexes needs investigation, as the overlap of biocontrol agents on trees treated with insecticide is likely to occur. Impact of insecticides on insect natural enemies can be influenced by active ingredient, insecticide formulation, timing, rate, application method, as well as pest stage, and natural enemy fecundity, genetic diversity, sex ratio, mobility, host searching ability, life stage, and size. (Stark and Bamfo 2002).

Predators can be exposed to pesticides through either direct or indirect exposure (De Cock et al. 1996, Johnson and Tabashnik 1999). Direct effects result from contact with the pesticides or their residues and results are most obviously expressed in terms of acute mortality within the first 24 hours. This is often seen with foliar sprays, where the natural enemy and/or their habitats are directly sprayed. Lethal effects can be obvious to the investigator, but sub-lethal effects such as reduced longevity, fecundity, mobility, and searching ability, (Wiedl 1977, Yokoyama and Pritchard 1984, Elzen 1989) are all direct effects that can be present but can be difficult to observe.

Indirect effects are consequences of the pesticide that affect the natural enemy by affecting their hosts or prey (Waage 1989). Pesticides can be so efficacious against pests that they can reduce a population so drastically that it cannot support a natural enemy population, causing them to either starve, disperse or find new food sources (Johnson and Tabashnik 1999). Predators can be exposed by ingesting affected pests (Ahmed et al. 1954, Roach and Moore 1988, Johnson and Tabashnik 1999, Grafton-Cardwell and Gu 2003) or by drinking contaminated water (De Cock et al. 1996). Parasitoids tend to be more sensitive to pesticides than their hosts, and parasitoid immatures can be exposed while feeding on or emerging from affected hosts (Elzen 1989, Symington 2003, Oliver et al. 2005, Saber et al. 2005). Natural enemies who supplement their diet with plant products can be exposed through contaminated nectar or pollen (Smith and Krischik 1999, Stapel et al. 2000).

Predicting pesticide impacts on natural enemies can be very difficult. Banken and Stark (1998) discussed complications of predicting field impacts in toxicological studies. Laboratory conditions often do not fully represent environments and situations present in the field, such as multiple routes of exposure. Laboratory tests for toxicity to natural enemies usually involve
Topical application, an appropriate test if the insects could be exposed to direct spray. Natural enemies in the field are more likely to have multiple routes of exposure, such as residue contact on plants and prey and ingesting contaminated prey or water. Pesticides can interfere with pest-enemy synchrony, changing timing of emergence and viable population size (Stark and Wennergren 1995).

Nontarget beneficials can come into contact with systemic toxicants by feeding on plant parts or materials, feeding on insects, or through the environment such as contaminated soils (Groot and Dicke 2002). Predators can be exposed to Bacillus thuringiensis (Bt) toxins in a tritrophic context by feeding on contaminated prey (Hilbeck et al. 1998, Dutton et al. 2002, Harwood et al. 2005, Bai et al. 2006, Ferry et al. 2006, Obrist et al. 2006a,b). Predators that supplement their diet with plant products can be exposed to toxins from direct feeding on plant materials (Harwood et al. 2005). In Bt systems, while uptake of toxins through feeding on contaminated prey is possible, impacts on predator survivorship and fitness vary among different toxins, plants, pests, and predators. The effects of ingesting toxins vary from no effect, to having a positive effect to negatively impacting fitness. For example, predator ingestion of Bt toxins heightened maturation rates (Lundgren and Wiedenmann 2002, Zhang et al. 2006), while in other systems their development was reduced (Bell et al. 2003), or no impacts on fitness were observed (Lundgren and Wiedenmann 2005, Bai et al. 2006, Obrist et al. 2006a). When negative impacts on predators were observed, they were usually linked to a reduction in prey quality (Bell et al. 2003, Romeis et al. 2004, Obrist et al. 2006a). When Frankliniella tenuicornis Uzel reared on Bt maize were fed to Chrysoperla carnea Stephens, predator exposure to Bt toxin was dependant upon the prey stage fed on, and predators were exposed from feeding on honeydew (Obrist et al. 2005). Using low doses of insecticides may be beneficial for manipulating tritrophic interactions between plant, pest, and natural enemies (Wright and Verkerk 1995).

Systemic applications of insecticides act directly on sucking pests, minimizing effects on natural enemies that could be directly affected through sprays (Mizell and Sconyers 1992). Smith and Krischik (1999), however, found that Coleomegilla maculata DeGeer exhibited reduced mobility, increased flip time, and reduced survivorship and oviposition after exposure to systemic-imidacloprid-treated sunflowers and other ornamentals. Similarly, imidacloprid treated
sunflower pollen and syrup had sub-lethal effects on bumblebees, *Bombus terrestris* L. (Tasei et al. 2000). When fed imidacloprid-sucrose solutions, honeybees, *Apis mellifera* L., ingested less food as imidacloprid concentrations increased either as a result of dose-related sublethal effects or antifeedant responses (Nauen et al. 2001). In another experiment, however, bees feeding for 39 d on sunflower honey fortified with 0.002-0.02 ppm imidacloprid had no adverse effects to bee colonies. (Schmuck et al. 2001). When imidacloprid granules were applied to turf for control of grubs, no adverse effects were observed on bumblebees, *Bombus impatiens* Cresson, feeding on white clover nectar on treated plots (Gels et al. 2002).

Other experiments have investigated various routes of predator exposure to insecticides. For example, cotton plants growing from seeds treated with imidacloprid before planting had no negative effects on the natural enemy complex associated with cotton pests (Kannan et al. 2004). Similarly, imidacloprid sprays did not negatively affect the natural enemy complex in beans (Marquini et al. 2002). In other systems, however, extrafloral nectar of treated cotton plants negatively affected parasitoids (Stapel et al. 2000) and predators (Smith and Krischik 1999). Foliar treatments reduced populations of beneficial coccinellid and neuropteran larvae (but not spiders or parasitoids) (James and Vogele 2001), and reduced parasitoid populations in vegetables (Simmons and Jackson 2000). Soil treatments with imidacloprid had no effect on population growth rates of a parasitoid feeding on exposed pea aphids, *Acythosiphon pisum* Harris (Kramarz and Stark 2003). Carabids, *Harpalus pennsylvanicus* DeGeer, feeding on imidacloprid-contaminated food displayed poisoning effects (Kunkel et al. 2001). Vedalia beetle, *Rodolia cardinalis* Mulsant, larvae were negatively affected when fed cottony cushion scale, *Icerya purchasi* Williston, feeding on systemically treated citrus, *Pittosporum tobir* Thunb., and adults displayed similar effects when feeding on cottony cushion scale sprayed with foliar imidacloprid (Grafton-Cardwell and Gu 2003). When *C. maculata* was exposed to potato leaflets and Colorado potato beetle, *Leptinotarsa decemlineata* Say, egg masses dipped in imidacloprid, both larvae and adults exhibited >40% mortality beginning only 24 hours after treatment (Lucas et al. 2004). * Macrosiphum euphorbiae* Thomas aphids that infested lettuce seedlings treated by an imidacloprid-drench were considered highly toxic to predacious brown lacewings, *Micromus tasmaniae* Walker, for up to 4 weeks after application (Cole and Horne 2006). Imidacloprid reduced the numbers of beneficial coccinellids feeding on green peach

Some insects can metabolize imidacloprid rapidly. In the honeybee, no imidacloprid was detected six hours after ingesting 50 μg kg⁻¹ as it was metabolized into 5-hydroxyimidacloprid and olefin metabolites 4 h after ingestion (Suchail et al. 2003). When radiolabelled imidacloprid distribution was tracked *in vivo* in the honeybee, total radioactivity half-life was eliminated 25 hours after oral treatment with 100 μg imidacloprid (Suchail et al. 2004). Another imidacloprid characteristic is its tendency to be metabolized by plants when introduced systemically, giving rise to metabolites that can provide complementary control and residual activity alongside the parent compound (Nauen et al. 1998b, 1999). In cotton plants, metabolites have shown insecticidal properties against aphids, sometimes with properties more toxic than imidacloprid itself and protecting the plant even after parent compound concentrations declined (Nauen et al. 1998b). Oral ingestion of the olefin metabolite is ten times more active than imidacloprid against a susceptible strain of cotton whiteflies, *B. tabaci* (Nauen et al. 1999). Suchail et al. (2004) hypothesized that in bees imidacloprid causes the immediate symptoms of neuro-toxicity, whereas the eventual mortality is from the 5-hydroxyimidacloprid and olefin metabolites.

At low concentrations, imidacloprid has been shown to influence antifeedant effects in aphids as in green apple aphid, *Aphis pomi* De Geer (Lowery and Smirle 2003). Concentrations of 16 parts per billion (ppb) reduced honeydew in *Myzus persicae* Sulzer excretion by 50% compared with controls (Nauen 1995, Nauen and Elbert 1997), however, in another study, imidacloprid showed no antifeedant effect (Elbert et al. 1991). The tobacco aphid, *M. nicotiana*e Blackman, exhibited antifeedant responses in dip tests, contact, and oral treatments using imidacloprid (Nauen and Elbert 1997, Nauen et al. 1998a, c). The antifeedant response could be a result of feeding mechanism impairment by imidacloprid, or by a behavioral response to the insecticide as
evidenced by feeding avoidance of treated food materials (Nauen 1995, Nauen and Elbert 1997, Nauen et al. 1998c). Subterranean termites, *Reticulitermes virginicus* Banks (Isoptera: Rhinotermitidae), exposed to 10 or 100 ppm imidacloprid for 4 h affected tunneling behavior and exposed termites showed no avoidance of imidacloprid-treated surfaces after recovering from the initial dose (Thorne and Breisch 2001). Potato aphids, *Macrosiphum euphorbiae* Thomas, had no antifeedant response feeding on imidacloprid treated potato leaflets in choice tests (Boiteau and Osborn 1997). *Bemisia tabaci* Gennadius preferred untreated leaf discs over discs systemically treated with imidacloprid, but with topically treated discs no preference was observed (Nauen et al. 1998a). In the same study, after oral ingestion of systemic imidacloprid, the EC$_{50}$ values (a 50% reduction in honeydew excretion) for the three tested *B. tabaci* strains ranged from 0.027-0.048 ppm, concentrations which were 16 to 852 times lower than observed LC$_{50}$ values for mortality. Pollen and syrup treated with 6 -10 ppb had no effect on food consumption by bumblebees, but significantly affected worker survival rate by 10% (Tasei et al. 2000). Imidacloprid displays two dose-dependant modes of action on *M. persicae*. The first is irreversible neurological poisoning symptoms such as tremors and uncoordinated leg movements found at higher doses, and the other is an antifeedant effect found at lower concentrations that is evidenced by a reversible starvation response, possibly as a result of either inhibition of feeding by affecting mouthpart muscle systems or by changing the physical qualities of plant sap that inhibits ingestion (Nauen 1995).

Impacts on fecundity vary with different organisms. Sub-lethal (ppb range) effects for *M. persicae* and *M. nicotianae* in aphid dip and leaf dip bioassays included reduced fecundity and viability of offspring (Devine et al. 1996). Cotton aphids, *Aphis gossypii* Glover produced a significantly higher proportion of alate offspring and a significantly lower proportion of offspring per adult when feeding on cotton plants sprayed with imidacloprid (Conway et al. 2003). Imidacloprid treatment of cotton plants induced increased wing formation in the cotton aphid either as a result of declining plant quality or as a result of direct exposure to the insecticide (Conway et al. 2003). Imidacloprid’s lethal and sublethal effects may stimulate reproduction in some arthropods, as shown when imidacloprid increased egg production in the two-spotted spider mite, *Tetranychus urticae* Koch (James and Price 2002). A higher fecundity resulting from stimulation of oviposition was observed in adult Asiatic rice borers, *Chilo*
suppressalis Walker, after developing from larvae feeding on Zizamia lalifolia Turcz galls on rice plants, *Oryza sativa* L., treated systemically with imidacloprid (Yu et al. 2007). Similarly, *Tryporyza incertulas* Walker larvae feeding on rice (*O. sativa*) plants treated with imidacloprid resulted in fourth instar weights higher than controls as well as increased fecundity after developing into adults (Wang et al. 2005a).

**1.6 Imidacloprid residue analysis**


Thin-layer chromatography (TLC) and high performance thin-layer chromatography (HPTLC) are used for pesticide analysis in a variety of systems (Sherma 2005). HPTLC has been used to quantify imidacloprid from liver and crop samples from birds (Berny et al. 1999) and Chinese cabbage, *Brassica rapa* (Cao et al. 2005). Thin-layer chromatography (TLC) was used for purification of avocado tree tissue samples before ELISA analysis (Byrne et al. 2007) and for qualitative analysis of imidacloprid residues in date palm, *Phoenix dactylifera*, before HPLC analysis (Kaakeh 2006). TLC has also been used to quantify radiolabeled (¹⁴C) imidacloprid in sugar beets, *Beta vulgaris* L. (Westwood et al. 1998), whiteflies, *B. tabaci* (Rauch and Nauen 2003), and honey bees (Suchail et al. 2004).

Immunoassays such as enzyme linked immuno-sorbent assays (ELISA) have become an increasingly popular tool for imidacloprid quantification from agricultural samples and has been
shown to be compatible for detecting imidacloprid residues in the tissues of many different types of plants: including vegetables and fruits (Wanatabe et al. 2001, Watanabe et al. 2004a, b), citrus (Castle et al. 2005), grapevines (Byrne et al. 2005a), avocados (Byrne et al. 2005b) and ash trees (Lewis et al. 2004, Harrell 2006). ELISA has been the technique of choice because it is relatively quick, easy, and cheap and does not require expensive and sophisticated analytical equipment or techniques. It is also advantageous because of its high sensitivity; the kit has a detection range of 0.2-6 ppb (Envirologix 2006). ELISA has been used to qualify and quantify imidacloprid residues within treated hemlock tree fluids and tissues, however one drawback is that the resulting matrix effects are an obstacle in accurately quantifying imidacloprid concentrations in hemlock sap and tissues (Cowles et al. 2006). Extracts from hemlock xylem fluid, wood and needle tissues affect ELISA kit performance by causing false positives and overestimated imidacloprid concentrations.

ELISA matrix effects have been reported for ELISA kits designed for herbicides (Watts et al. 1997), fungicides (Cairoli et al. 1996), insecticides such as diazinon (Sullivan and Goh 2000) and imidacloprid (Watanabe et al. 2004a, b, Byrne et al. 2005a, b). Matrix effects are caused by nonspecific binding between nontarget compounds and kit antibodies (Sullivan and Goh 2000) and investigators have dealt with them using different methods. Their effects have been accounted for by creating an index factor that adjusts for them (Cairoli et al. 1996) by generating a threshold which adjusts for interference in controls and subtracts the values from treated samples of interest (Cowles et al. 2006), and by comparing curves in a control matrix of water with calibration curves generated within the matrix under investigation (Sullivan and Goh 2000). In some systems, a simple dilution with water is all that is necessary to overcome matrix effects (Byrne et al. 2005a), although the necessary degree of dilution varies between plant species. For example, grape juice required a 20-fold dilution to absolve matrix effects while for orange juice a 100-fold dilution was necessary (Watanabe et al. 2007). Lettuce required a 400-fold dilution while green pepper required a 800-fold dilution (Watanabe et al. 2004b). A 20-fold dilution was necessary for both grapevines (Byrne et al. 2005a) and avocados (Byrne et al. 2005b).
1.7 Research rationale and objectives

Chemical controls with imidacloprid-based products are widely used in ornamental, landscape, and forest settings. Trunk and soil injections are the primary control components for HWA in large-scale injection programs in National Forests and State Parks. The Great Smoky Mountains National Park in northeastern Tennessee is an example of an initiative currently underway to limit hemlock decline from HWA infestation. In the park more than 164,000 beetles (predominantly *S. tsugae*) have been released at over 66 sites and more than 75,000 hemlocks have been treated via soil or trunk injections of imidacloprid (K. Johnson, pers. comm.). In settings such as the Great Smoky Mountains where chemical and biological controls are jointly applied, it is important to determine the compatibility of the two control options. High-value hemlock stands are often the first candidates for chemical control, and these same stands are desired as sites for biological control predator releases. Often predator releases are in close proximity to chemically treated sites, occupying the same valleys or mountain ridges. As predators are released into the environment, it is important to analyze factors that could affect their establishment (Mausel 2007). Reproduction, subsequent relocation of predators from release sites, and dispersal make it possible for predators to come into contact with hemlocks that have been chemically treated.

Using imidacloprid to control HWA could impact predators either through direct exposure to toxins or indirectly by changing HWA availability, quality, or distribution. In the hemlock system, imidacloprid could potentially be passed on to predators as they feed on contaminated adelgids, especially because both *L. nigrinus* and *S. tsugae* rely on HWA as a primary food source. Adelgids surviving on treated trees may contain sublethal levels of imidacloprid that could be ingested by foraging predators. Predators exposed to systemic imidacloprid through feeding on contaminated prey could be subjected to lethal and sublethal effects that affect their biology and survival. Inhibited flight and dispersal, foraging, or fecundity could result from exposure to imidacloprid residues. For instance, sublethal effects such as paralysis could cause predators to be susceptible to predation themselves (Kunkel et al. 2001).
This study investigates potential ways that systemic imidacloprid in hemlock tissue could impact non-target predators feeding on HWA. This study aims to investigate the relationship between imidacloprid, eastern hemlocks, HWA, and two nontarget predators in order to help determine the most constructive application of chemical and biological control that will benefit HWA management in the eastern United States.
Chapter 2

Evaluation of ELISA for imidacloprid detection in eastern hemlock (*Tsuga canadensis*)
wood and needle tissues

Abstract
A commercially available ELISA kit was evaluated for quantification of imidacloprid in eastern hemlock wood and needle tissues. Matrix effects in the form of false positives and inflated imidacloprid concentrations were observed in both wood and needle extracts. Tissues required a 100-1000 fold dilution with water in order to avoid matrix effects. Standard curves in 1% wood or needle extract were not significantly different from standard curves prepared in water. Matrix effects were more pronounced at concentrations in the lower working range of the kit, with recovery of 5 ppb imidacloprid more accurate than recovery of 0.2 ppb. The cause of matrix effects in the kit is unknown. ELISA remains a valuable tool for semi-quantitative imidacloprid detection within the hemlock system because of its sensitivity, cost, and ease of use. However, to ensure accurate quantification, a 1000-fold dilution is recommended to ensure accurate imidacloprid determinations.

Keywords: Matrix effects; hemlock woolly adelgid; systemic insecticide.
2.1 Introduction

The hemlock woolly adelgid (HWA) *Adelges tsugae* Annand, (Hemiptera: Adelgidae), is an exotic invasive pest from Japan that infests and kills eastern hemlock, *Tsuga canadensis* (L.) Carriére, trees throughout much of their native range in the eastern United States. This adelgid is a piercing-sucking insect that feeds on nutrients from parenchyma cells located at the base of needles (McClure 1987, 1991a, Shields et al. 1995, Young et al. 1995). Infestation causes needle desiccation and discoloration, inhibits production of new growth, (McClure 1987, McClure et al. 2001), killing trees in as little as four years after initial infestation (McClure 1991b, McClure et al. 2001). Hemlocks play many important roles in forested ecosystems, and their widespread mortality has been ecologically traumatic. They are critical components of habitats for many animal species including birds, turkey, rodents, rabbits, numerous plant species and help sustain cool water temperatures by providing shade for aquatic organisms (Evans et al. 1995, Yamasaki et al. 1999, McClure et al. 2001, Tingley et al. 2002).

The neonicotinoid insecticide 1-(6-chloro-3-pyridylmethyl)-N-nitro-imidazolidin-2-ylideneamine (imidacloprid) is the primary insecticide used against HWA, and is currently the only systemic insecticide registered for adelgid control in forest environments. Trunk and soil injections of imidacloprid are the primary methods of control in forest and urban landscapes and can provide protection against infestation for several years after application (Cowles and Cheah 1999, Doccola et al. 2003, Webb et al. 2003). There continues to be wide scale applications of imidacloprid in public and private forests and parks, often geographically close to releases of adelgid predators in a coordinated biological control program. Forest managers and researchers are interested in tracking imidacloprid levels in treated trees and the environment in order to determine appropriate treatment rates, methods and timing, and to investigate if imidacloprid treatments are having any negative impacts on beneficial or nontarget organisms. A simple, inexpensive, accurate, and rapid analytical technique for quantifying imidacloprid residues would benefit studies on the distribution of imidacloprid throughout the tree canopy, its residual activity and hemlock metabolism pathways, and the necessary threshold concentration that will provide control of HWA infestations.

Immunoassays such as enzyme linked immuno-sorbent assays (ELISA) have become an increasingly popular tool for imidacloprid quantification in agricultural samples and have shown their capability in detecting imidacloprid residues in the tissues of many different types of plants including vegetables and fruits (Wanatabe et al. 2001, Watanabe et al. 2004a, b), citrus trees (Castle et al. 2005), avocados (Byrne et al. 2005a), grapevines (Byrne et al. 2005b) and ash trees (Lewis et al. 2004, Harrell 2006). ELISA has been the technique of choice because it is relatively quick, easy, inexpensive and does not require expensive and sophisticated analytical equipment or expertise. It is also advantageous because of its sensitivity; the kit used in this study has a detection range of 0.2-6 ppb (Envirologix 2006). ELISA has been used to detect and quantify imidacloprid residues within treated hemlock tree fluids and tissues. However, one drawback is that the matrix effects resulting from these tissues are an obstacle in accurately quantifying imidacloprid concentrations in hemlock sap and tissues (Cowles et al. 2006, Lagalante and Greenbacker 2007). Extracts from hemlock xylem fluid, wood and needle tissues affect ELISA kit performance by causing false positives and overestimated imidacloprid concentrations.

Matrix effects have been reported for ELISA kits designed for herbicides (Watts et al. 1997), fungicides (Cairolı et al. 1996), insecticides such as diazinon (Sullivan and Goh 2000) and imidacloprid (Watanabe et al. 2004b, Byrne et al. 2005a, b). Matrix effects are caused by nonspecific binding of nontarget compounds with ELISA kit antibodies (Sullivan and Goh
Investigators have dealt with matrix effects using different methods by: creating an index factor that adjusts for matrix effects (Cairol et al. 1996), generating a threshold which adjusts for interference in controls and subtracts the values from treated samples of interest (Cowles et al. 2006), and by comparing curves in a control matrix of water with calibration curves generated within the matrix under investigation (Sullivan and Goh 2000). In some systems, a simple dilution with water (Watanabe et al. 2004b, Byrne et al. 2005a, b) or buffer (PBS; 7.75 mM disodium hydrogen phosphate, 2.25 mM potassium dihydrogen phosphate, 154 mM sodium chloride, pH 7.2) (Watanabe et al. 2007) is all that is necessary to overcome matrix effects although the necessary degree of dilution varies between plant species (Table 2.1). While methods to deal with matrix effects in agricultural crops have been developed, no standardized method has been published to provide guidelines for using ELISA to quantify imidacloprid within the hemlock system. Two publications have reported on the use of ELISA to quantify imidacloprid from systemically treated trees. Harrell (2006) and Cowles et al. (2006) used ELISA for imidacloprid detection in green ash (*Fraxinus pennsylvanica* Marsh.) and hemlock trees, respectively. Both studies reported the presence of matrix effects; however, they were addressed in different ways. Harrell adjusted for control of false positives by averaging the concentrations determined in the controls and treating them as background and subtracting their values from concentrations from treated trees (Harrell M, 2006, pers. comm.). Cowles et al. (2006) diluted all samples at least 10-fold with water and established a threshold for a positive detection from untreated checks by taking the maximum determination from the control group plus twice that group’s standard error. They discussed the evidence of matrix effects within their study, yet did not attempt to quantify them systematically.

This study evaluated a commercially available competitive ELISA technique for quantifying imidacloprid concentrations in hemlock wood and needle tissue. Experiments were designed to investigate matrix effects and to provide standardized methods for researchers to use in order to overcome them and provide accurate and precise quantification of imidacloprid within treated hemlock tissues. Other goals were to evaluate an extraction method and determine percent recovery based on a previously published extraction technique (Cowles et al. 2006). As ELISA continues to be used to detect and quantify imidacloprid residues in hemlock tissues, this project
will provide much-needed insight into quantification of imidacloprid in hemlock wood and needle tissues using ELISA analysis.

2.2 Materials and Methods

ELISA kit procedure
ELISA test kits (QuantiPlate™ kit for imidacloprid, catalog no. EP 006, Envirologix Inc., Portland, ME) were purchased and used according to the manufacturer recommended procedures. In this ELISA, imidacloprid competes with enzyme-labeled imidacloprid for binding sites bound to microtiter well walls. The degree of binding is quantified colorimetrically and the amount of imidacloprid present in each sample is inversely proportional to the degree of color development. For imidacloprid in water samples, the kit has an assay range of 0.2 - 6 µg/kg [parts-per-billion (ppb)], with a limit of detection of 0.07 ppb and a limit of quantification of 0.3 ppb (Envirologix 2006). Plates were read with a Dynex MRX microplate reader (Dynex Technologies Inc., Chantilly, VA) by measuring the optical density (OD) of each well at a wavelength of 450 nm with a reference wavelength of 600 nm. Optical density is inversely proportioned to the imidacloprid concentration in the sample. Since optical density values can vary between measurements they are converted to % Bound values (% B₀) using the formula:

\[
% \text{B}_0 = \left(\frac{\text{average OD of calibrator or sample} \times 100}{\text{average OD of negative control}}\right)
\]  

A standard curve is created by plotting the % B₀ of the kit standards against their imidacloprid concentration on a semi-log scale. Imidacloprid concentrations from samples were calculated from the x-intercept of their % B₀ value based on the slope of the kit calibrator standard curve. Kit calibrators consistently produced standard curves with \(r^2\) values > 0.99.

Tissue preparation
HWA-infested eastern hemlock trees in and around Marion, Virginia (Smyth County) were selected in spring 2006. Each tree had never been treated with insecticides and all were located in a forest setting. Trees were approximately the same diameter at breast height (diameter at 1.5 m) with a mean of 30 cm. All trees were lightly infested with HWA, but appeared to be in good
health with new shoots and little dieback. The distal 30 cm of 4-6 branches were cut from the lower crown of each tree in March and April 2006. Branches were cut, sealed in Ziploc® plastic bags, and transported to Virginia Tech in Blacksburg, Virginia for sample preparation and ELISA analysis. Branches were frozen until they were dried in an oven at 40°C for 24 h or until needles were easily removed. Tissues were pulverized using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) until pulverized material was able to pass through a 1 mm² mesh screen. Ground tissues were immediately frozen. In preparation for analysis, 100 mg of ground wood or needle tissue from each tree was thawed and placed into a 1.5 mL micro-centrifuge tube and 1 mL of distilled water was added to each sample (approximately a 1:10 (weight:volume) dilution). Each sample was shaken by hand for 1 min and then centrifuged at 10,000 x g for 5 min. Supernatant was removed from each sample with a pipette and placed in a new, clean micro-centrifuge tube. This supernatant hereafter referred to as 100% extract, which has a dilution factor of 10-fold, is the final sample for all ELISA analyses in this report. All samples were freshly prepared, refrigerated at 5°C and analyzed by ELISA within 24 h.

**Matrix effects**
The first series of tests were designed to investigate the compatibility of imidacloprid-free tissue extracts with ELISA. The 100% extract was added to kit wells in duplicate and ELISA was used to determine imidacloprid concentrations according to the procedures described previously. These samples had never been exposed to imidacloprid, so any imidacloprid concentration reported by ELISA was considered to be a false positive. 100% extract from all trees tested showed varying degrees of false positives. To test the amount of dilution necessary to eliminate false positives, 100% extract was diluted 10, 20, and 100-fold in fresh distilled water (total dilution factors of 100, 200, and 1000-fold, respectively) and analyzed by ELISA. A minimum of three independent samples were analyzed in duplicate on separate ELISA plates.

**Recovery from spiked extract**
To test the influences of different matrix dilutions on imidacloprid recovery, a series of stock solutions of technical grade imidacloprid (Bayer, 99.2% purity) were prepared in distilled water. Next, 100% wood tissue extract was diluted with different concentrations of imidacloprid stock solutions to yield a range of samples with a final imidacloprid concentration of 1 ppb in 1%, 5%,
10%, 50%, and 96% extract. Because 100% extract is a 10-fold dilution, these samples had a total dilution factor of 1000, 200, 100, 20, and 10.4-fold, respectively. Based on results from this experiment, it was determined that a minimum 10-fold dilution of the 100% extract (100-fold total dilution) was necessary to overcome matrix effects. Subsequently, in order to test the influence of different extract dilutions on the slopes of imidacloprid standard curves, 100% extract was diluted with imidacloprid in water to create four standard curves: one in water, one in 1% extract, one in 5% extract, and one in 10% extract. Three independent spiked samples with final concentrations of 0.2, 1, and 5 ppb were used to generate each curve.

**Extraction method comparison**

To extract imidacloprid from hemlock wood and needle tissues, Cowles et al. (2006) dried and pulverized tissue, extracted with acetone, evaporated the acetone and re-suspended wood and needle tissue with water. The efficacy of an extraction method adapted from the above procedure was performed. To initially spike the tissue, 1 mL of imidacloprid standards (20, 100, or 500 ppb) prepared in HPLC grade methanol was added to 100 mg of wood tissue in a 1.5 mL micro-centrifuge tube (yielding concentrations of 0.2-5 ppm). The methanol was evaporated under nitrogen stream until dry. One mL of HPLC-grade acetone was added to the tissue and the sample was sonicated for three min. Samples were centrifuged at 10,000 x g for 5 min, and 500 µL of supernatant was removed with a pipette. The acetone was evaporated to dryness under nitrogen stream before 500 µL of distilled water was added to the sample. The sample was sonicated for three min., and centrifuged at 10,000 x g for 5 min. Samples were then diluted 100-fold with distilled water before ELISA analysis, resulting in final concentrations of 0.2, 1, or 5 ppb imidacloprid in 1% tissue extract. Three independent samples for each concentration were prepared and run on separate ELISA plates. Designs for all experiments were based on previously published reports (Watanabe et al. 2004b, Watanabe et al. 2007, Byrne et al. 2005a, b, Cowles et al. 2006).

**Statistical analysis**

Statistical analyses were performed with SPSS 11 for Macintosh. Analysis of variance (ANOVA) was used to test for significant effects of tissue extract dilutions on the recovery of imidacloprid concentrations. Means were separated by Tukey’s honestly significant difference
test. Imidacloprid concentrations were log transformed and standard curves were compared by linear regression.

2.3 Results

Matrix effects
Matrix effects evidenced by false positives and overestimated imidacloprid concentrations were observed in both wood and needle extracts. As the proportion of tissue extract to water in a sample decreased, the degree of false positives determined by ELISA decreased. When imidacloprid was not present, the % Bound values of wood and needle extracts were not significantly different \( F = 2.032; \text{df} = 1, 28; P = 0.223 \). Wood and needle tissue % Bound values were significantly different from water at the 5%, 10% and 100% extract proportion values for both wood \( (F = 12.74; \text{df} = 4, 29; P < 0.0001) \) and needles \( (F = 31.05; \text{df} = 4, 13; P < 0.0001) \). When imidacloprid was not present, 100% extract required a 100-fold dilution in order to have values comparable to water and avoid false positives. The limit of detection (LOD) was calculated as 3 times the standard deviation of the mean absorbance of a blank and the limit of quantification (LOQ) was calculated as 10 times the standard deviation of a blank sample. The manufacturer’s reported kit LOD and LOQ imidacloprid in water were 0.07 and 0.3 ppb, respectively. Our experiments determined the ELISA kit LOD for imidacloprid in water to be 0.099 ppb and the LOQ was 0.33 ppb. The LOD and LOQ for wood and needle tissues in 1 and 5% matrices were calculated (Table 2.2).

Recovery from spiked extract
When imidacloprid concentrations of 1 ppb were compared in different extracts, 1%, 5%, and 10% wood tissue extract means were not significantly different from a 1 ppb sample prepared in water while 50% and 96% extract means were significantly different \( F = 12.2; \text{df} = 5, 24; P < 0.001 \) (Figure 2.1). The standard curves of imidacloprid prepared in water were comparable with identical standards in 1% needle tissue extract (Figure 2.2). Wood tissue, water, 1% and 5% extracts had similar standard curves and their slopes were not significantly different \( F = 1.079; \text{df} = 2, 56; P = 0.347 \), but intercepts were significantly different \( F = 4.046; \text{df} = 2, 58; P = 0.0226 \). Intercepts were statistically equal for water and 1% extract \( F = 0.324; \text{df} = 1, 35; P \).
= 0.573). No significant differences between water and 1% extract slopes \( F = 0.00832; \text{df} = 1, 16; P = 0.929 \) or intercepts \( F = 0.336; \text{df} = 1, 17; P = 0.569 \) were found for needle tissue.

Mean percent recovery values for wood (Table 2.3) and needle tissue (Table 2.4) were dependant upon tissue extract proportion and the amount of imidacloprid in the sample. Mean percent recoveries were more accurate at lower extract dilutions. For wood, percent recovery ranged from 91-150%, and needles ranged from 101-127% in 1% extract. Recoveries from higher tissue concentrations were overestimated and higher than predicted. Mean percent recovery varied between extracts and was dependant upon the amount of imidacloprid present. Recovery efficiency increased with increasing imidacloprid concentration in the sample. Variations between samples (expressed as % CV) were lower when more imidacloprid was present in the sample. When standard curves were created by plotting detected imidacloprid concentration over spiked concentration, curves were most linear and intercepts were closer to the calibrator intercept in 1% extract (Table 2.5). Generally, coefficient of determination values \( (r^2) \) were high, ranging from 0.901 to 0.999, with higher values found at lower extract dilutions. To measure the degree of matrix interference, slopes within tissue matrices were compared with the slope of kit calibrators provided by the manufacturer using the formula:

\[
\text{% Difference} = \frac{\text{Slope}_{\text{control}} - \text{Slope}_{\text{matrix}}}{\text{Slope}_{\text{control}}} \tag{2}
\]

The percent difference of slopes from the various extract dilutions ranged from 5-20% when compared with the calibrator standard curve. Lowest differences were found in the 1% extract curves. Mean percent slope difference was 14% for wood and 11% for needle tissues.

**Extraction method comparison**

Extraction efficiency ranged from 62-110% (Table 2.6). Recovery efficiency (%) was higher at lower imidacloprid concentrations and lower at higher imidacloprid concentrations.
2.4 Discussion

Matrix effects were observed in both hemlock wood and needle tissue extracts and were evidenced by false positives and overestimated imidacloprid concentrations. Matrix effects are caused by nonspecific binding and the nature of the interfering agents in hemlock tissues is unknown at this time. Although not addressed in this report, there appears to be variability of matrix effects between individual trees. Whenever an exact quantification of imidacloprid residue in a particular tree is required, the dilution factor for that particular tree should be explored with preliminary dilution tests similar to those described here. All samples tested thus far have come from HWA-infested trees, using branches cut during the spring. Summer, fall, and winter samples may have different matrix effects than spring samples. Eastern hemlock physiological processes and tissue composition change throughout the year (Lagalante et al. 2006), which may result in differing amounts of interfering plant compounds as the tree physiology changes throughout the seasons. Hemlock wood and needle tissues were used in this report. While other reports have utilized sap, problems remain with using sap for imidacloprid quantification. Sap is primarily water, and at certain times of year, such as when the trees are dormant or during periods of water stress, sap is less available for analysis. Imidacloprid concentrations in sap are highly variable, and can be inconsistent among branches because sap must be collected from one branch at a time (Cowles et al. 2006). HWA is not a sap feeder. Their stylet bundles are found in conjunction with xylem ray parenchyma cells in the wood tissue and they have not been observed feeding on sap (Young et al. 1995). Quantifying imidacloprid from hemlock wood tissue is more likely to better represent concentrations that HWA could be exposed to from feeding.

One source of matrix effects in hemlock tissues where imidacloprid is present could be from imidacloprid metabolites. The ELISA kit does not distinguish between imidacloprid and imidacloprid metabolites, but it is most reactive with imidacloprid and detects the metabolites to varying degrees (Envirologix 2006). Lagalante and Greenbacker (2007) further investigated the cross-reactivity of some of the major imidacloprid metabolites and determined that all of the tested compounds were less reactive than imidacloprid and their degree of cross-reactivity was comparable to the values given by the ELISA manufacturer (Envirologix 2006). Even though metabolites are less reactive, the fact that five of the tested metabolites had cross reactivity above
30% suggests that the presence of these metabolites in a sample would artificially elevate imidacloprid levels in a sample. Thus, the presence of metabolites could be a source of cross-reactivity and complicate the accurate quantification of imidacloprid in treated trees.

To avoid false positives and inflated imidacloprid concentrations due to the matrix, tissue extracts must be diluted a minimum of 100-fold, and a dilution of 1000-fold will provide the most accurate quantification. Any less dilution will provide false positives and incorrect and elevated estimates of imidacloprid within hemlock samples. This raises the practical detection limit of the kit 100-fold, from 0.2 ppb to approximately 20 ppb, resulting in a working range of 20 – 600 ppb. While decreased sensitivity may make the kit less desirable, a working range of this caliber is still sensitive enough for quantification of imidacloprid within the range of biological efficacy of imidacloprid in hemlock tissues against HWA (Cowles et al., 2006).

The extraction method tested had similar results to Watanabe et al. (2004b), who reported percent recovery range of 80-134% and a mean recovery average of 85.3-105.3%. Recovery is more efficient at lower imidacloprid concentrations. To achieve more accurate recovery at concentrations above 1 ppb additional extraction steps are necessary. This study describes an extraction method for recovery from spiked tissues. Extraction from environmental samples such as tissues from field-treated trees may require additional extraction steps and methodology.

HWA is just one of several recent invasive species attacking native American trees. Imidacloprid is used to control other important invasive tree pests such as the emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Harrell, 2006), a pest of native ash (*Fraxinus*) species, and the Asian longhorned beetle, *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) a pest of maple (*Acer* spp.), poplar (*Populus* spp.) and elm (*Ulnus* spp.) (Wang et al. 2005b). Guidelines for using ELISA to determine imidacloprid residues in other tree species would benefit efforts to control the spread of these pests in the eastern United States.

Other residue detection techniques such as HPLC, LC/MS, and GC/MS can provide residue concentrations without matrix effects. The benefit of these techniques is that, unlike ELISA,
metabolites can be qualified and quantified along with the parent compound. Overall, ELISA remains a relatively inexpensive, rapid, and simple resource for imidacloprid detection, and does not require as much solvent, time, or expensive analytical equipment as the previously mentioned techniques. ELISA is a beneficial tool for researchers that lack expensive analytical instrumentation and wish to measure imidacloprid levels using a simple procedure and commonly available instrumentation such as a digital scanner to quantify the results (Cowles et al. 2006). In spite of the presence of matrix effects, ELISA remains a valuable tool for detecting and quantifying imidacloprid within hemlock tissues; however, operators should be aware of the method’s limitations and address its propensity for error.
Table 2.1 Minimum dilution factor necessary to circumvent matrix effects in imidacloprid ELISA kits.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange/Lemon tree xylem fluid</td>
<td>0</td>
<td>Castle et al., 2005</td>
</tr>
<tr>
<td>Grape juice</td>
<td>20</td>
<td>Watanabe et al., 2007</td>
</tr>
<tr>
<td>Grapevine xylem fluid</td>
<td>20</td>
<td>Byrne et al., 2005a</td>
</tr>
<tr>
<td>Avocado</td>
<td>20</td>
<td>Byrne et al., 2005b</td>
</tr>
<tr>
<td>Apple</td>
<td>100</td>
<td>Watanabe et al., 2004a</td>
</tr>
<tr>
<td>Orange juice</td>
<td>100</td>
<td>Watanabe et al., 2007</td>
</tr>
<tr>
<td>Cucumber</td>
<td>100</td>
<td>Watanabe et al., 2004b</td>
</tr>
<tr>
<td>Lettuce</td>
<td>400</td>
<td>Watanabe et al., 2004b</td>
</tr>
<tr>
<td>Green pepper</td>
<td>800</td>
<td>Watanabe et al., 2004b</td>
</tr>
</tbody>
</table>
Table 2.2 Limits of detection and quantification for imidacloprid in hemlock tissue matrices using ELISA.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>LOD(^1) (ppb)</th>
<th>LOQ(^2) (ppb)</th>
<th>Dilution factor</th>
<th>Practical LOD(^3) (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit(^4)</td>
<td>0.07</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>0.099</td>
<td>0.33</td>
<td>0</td>
<td>0.099</td>
</tr>
<tr>
<td>1% wood</td>
<td>0.28</td>
<td>0.93</td>
<td>1000</td>
<td>280</td>
</tr>
<tr>
<td>5% wood</td>
<td>0.26</td>
<td>0.87</td>
<td>200</td>
<td>52</td>
</tr>
<tr>
<td>1% needle</td>
<td>0.29</td>
<td>0.96</td>
<td>1000</td>
<td>290</td>
</tr>
<tr>
<td>5% needle</td>
<td>1.3</td>
<td>4.3</td>
<td>200</td>
<td>260</td>
</tr>
</tbody>
</table>

\(^1\) Limit of detection  
\(^2\) Limit of quantification  
\(^3\) Limit of detection after adjusting for dilution factor  
\(^4\) Kit manufacturer’s (Envirologix) reported limits for detecting imidacloprid in water
Table 2.3 Recovery of imidacloprid in spiked hemlock wood tissue extracts.

<table>
<thead>
<tr>
<th>Wood Extract (%)</th>
<th>Expected concn, (ppb)</th>
<th>Detected concn, (ppb)</th>
<th>CV (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0.50</td>
<td>126.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.30</td>
<td>102.7</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.92</td>
<td>59.6</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.54</td>
<td>23.3</td>
<td>91</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0.37</td>
<td>33.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.48</td>
<td>74.3</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.48</td>
<td>22.9</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.07</td>
<td>18.6</td>
<td>121</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>170</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>1.75</td>
<td>141.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.94</td>
<td>99.1</td>
<td>468</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.36</td>
<td>44.5</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.61</td>
<td>25.2</td>
<td>152</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>319</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>6.81</td>
<td>77.6</td>
<td>-</td>
</tr>
</tbody>
</table>

Means of 3 replications; CV, coefficient of variation.
Table 2.4  Recovery of imidacloprid in spiked hemlock needle tissue extracts.

<table>
<thead>
<tr>
<th>Needle Extract (%)</th>
<th>Expected concn, (ppb)</th>
<th>Detected concn, (ppb)</th>
<th>CV (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.15</td>
<td>63.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.25</td>
<td>37.5</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.01</td>
<td>14.8</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.95</td>
<td>6.35</td>
<td>119</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>116</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0.55</td>
<td>78.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>1.45</td>
<td>81.1</td>
<td>725</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.59</td>
<td>30.9</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.54</td>
<td>1.7</td>
<td>171</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>385</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>2.06</td>
<td>88.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>2.44</td>
<td>67.8</td>
<td>1218</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.72</td>
<td>76.1</td>
<td>371</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9.28</td>
<td>19.3</td>
<td>186</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>592</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>16.78</td>
<td>11.2</td>
<td>-</td>
</tr>
</tbody>
</table>

Means of 3 replications; CV, coefficient of variation.
Table 2.5 Influences of different matrices on imidacloprid ELISA standard curves.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Extract(^a) (%)</th>
<th>Slope(^b)</th>
<th>Intercept(^c)</th>
<th>(r^2)</th>
<th>Difference(^d) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator</td>
<td>0</td>
<td>1.01</td>
<td>-0.032</td>
<td>0.999</td>
<td>0</td>
</tr>
<tr>
<td>Wood Tissue</td>
<td>1</td>
<td>0.91</td>
<td>0.198</td>
<td>0.999</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.13</td>
<td>0.988</td>
<td>0.999</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.21</td>
<td>2.74</td>
<td>0.977</td>
<td>20</td>
</tr>
<tr>
<td>Needle Tissue</td>
<td>1</td>
<td>1.06</td>
<td>0.80</td>
<td>0.989</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.91</td>
<td>3.13</td>
<td>0.919</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.83</td>
<td>4.68</td>
<td>0.901</td>
<td>18</td>
</tr>
</tbody>
</table>

\(^a\) Percent of 100% extract.

\(^b,c\) Slope and intercept from linear regression using ELISA-determined concentrations (Y axis) of standards versus their predicted concentrations (X axis).

\(^d\) Percent difference between the calibrator slope (1.01) and the slope of the standard curve in extract.
Table 2.6 Spiking method efficiency in 1% wood tissue extract.

<table>
<thead>
<tr>
<th>Expected concn (ppb)</th>
<th>Detected concn (ppb)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0.22 ± 0.023</td>
<td>109.82</td>
</tr>
<tr>
<td>1</td>
<td>0.93 ± 0.10</td>
<td>93.02</td>
</tr>
<tr>
<td>5</td>
<td>3.11 ± 0.54</td>
<td>62.29</td>
</tr>
</tbody>
</table>

Means ± SE of 3 independent replications.
Figure 2.1 Influences of extract proportion on the recovery of 1 ppb imidacloprid from hemlock wood tissue. Means ± SE of 5 replicates. Means with same letter are not significantly different (Tukey’s HSD test, $P = 0.05$).
Figure 2.2 Matrix interference from standard curves for imidacloprid in 0% (water), 1, 5, and 10% wood and needle tissue extracts. Percent bound (\%B_o) values on y axis calculated from optical density values of kit wells.
Chapter 3

Imidacloprid impacts on Laricobius nigrinus, Sasajiscymnus tsugae and hemlock woolly adelgid maintained on eastern hemlock branchlets treated in the laboratory

Abstract
Eastern hemlock (Tsuga canadensis) branchlets infested with hemlock woolly adelgid (HWA), Adelges tsugae, were treated systemically with imidacloprid in the laboratory and used to assess its impact on two HWA predators, Laricobius nigrinus and Sasajiscymnus tsugae. Liquid chromatography-mass spectrometry was used to quantify imidacloprid and some of its major metabolites in hemlock wood tissues and in beetles post-mortem. Probit analysis of HWA mortality and imidacloprid concentrations recovered from branch wood tissues determined the 30 d LC₅₀ to be 242 ppb. A topical application of imidacloprid to the ventral abdomen of individual beetles resulted in a 6 d LD₅₀ value of 1.8 and 0.71 ng imidacloprid per beetle for L. nigrinus and S. tsugae, respectively. In no-choice tests, L. nigrinus mortality was significantly higher on the 100 ppm branchlets than on controls. S. tsugae mortality on treated branchlets was greater than controls but was not significantly different. S. tsugae consumed the same number of adelgids on treated branchlets as on controls, but L. nigrinus consumed significantly fewer adelgids from the 100 ppm branchlets than on controls. With no prey choice, both beetle species fed on HWA residing on treated branchlets. In choice tests, beetle mortality and flip times generally increased as imidacloprid dose increased, however, means were not significantly different from control mortality. At times, both beetle species displayed intoxication symptoms from feeding on adelgids. Systemic imidacloprid in HWA-infested eastern hemlock branchlets negatively impacted fitness parameters of two non-target host specific HWA predators.

Keywords: Adelges tsugae; Tsuga canadensis, non-target effects
3.1 Introduction

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an exotic invasive pest of two hemlock (*Tsuga*) species in the eastern United States. Carolina hemlock, *T. caroliniana* Engelmann, is a relatively rare species confined to mountain ridges and isolated stands in the Appalachian highlands of western North Carolina, Tennessee and Virginia. Eastern hemlock, *T. canadensis* L. Carrière, is common along streams and cool mountain slopes throughout eastern forests; its range extends from Maine to the southern extent of the Appalachian chain in northern Georgia and Alabama (Godman and Lancaster 1990). Eastern hemlocks are an important economic (Rhea 1995, Holmes et al. 2005) and ecological species; they provide habitats and nutrients that are beneficial for many aquatic, terrestrial, and avian animal species (Reay 1999, Yamasaki et al. 1999, Snyder et al. 2002, Tingley et al. 2002, Ross et al. 2003).

HWA feeds on fluids and nutrients from parenchyma cells at the base of hemlock needles (McClure 1987, 1991, Shields et al. 1995, Young et al. 1995). Infestation causes desiccation, bud mortality, needle loss and a reduction of new growth (McClure 1987, McClure et al. 2001), which can lead to dieback and tree death in 4-10 years (McClure et al. 2001). HWA has an anholocyclic lifecycle with two parthenogenic generations per year on hemlocks. The asexual sistentes generation is approximately 1 mm in length and secrete a woolly white flocculence or ovisac. Sistentes 1st instar crawlers aestivate on hemlock stems throughout the summer. Breaking aestivation in the fall, they begin feeding and develop through four instars during the winter months. Adults mature and lay eggs in March and April, which hatch as either progredientes or sexuparae. A greater proportion of sexuparae production is initiated by high densities of sistentes and resulting poor host nutritional quality (McClure 1991). Sexuparae are winged and sexual; they do not feed on hemlocks and attempt to migrate to spruce (*Picea*) species in order complete a holocyclic lifecycle (Havill and Foottit 2007). The progredientes generation is similar in body morphology to the sistentes. After hatching, 1st instar crawlers disperse, settle, and begin to feed at the base of needles. The progredientes develop rapidly through four instars, mature and lay eggs. Eggs hatch into sistentes crawlers which migrate to new growth, settle, and aestivate through the summer.
A classical biological control program has been initiated by the USDA Forest Service to combat the spread and establishment of HWA. *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and *Sasajiscymnus (Pseudoscymnus) tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) are two predators that have been reared and released as biological control agents of HWA (Cheah and McClure 2002, Cheah et al. 2005, Lamb et al. 2006). *L. nigrinus* is native to the Pacific Northwest and is a specialist predator of HWA. It is univoltine, host specific and closely synchronized with HWA’s lifecycle (Zilahi-Balogh et al. 2002, 2003b, Mausel 2007). Adults emerge from the soil and begin feeding on sistentes in the fall after the adelgids break aestivation. *L. nigrinus* feeds throughout the winter. The adults are cold hardy and active at temperatures above 0 °C (Mausel 2007). In late winter/early spring adults begin laying eggs; larvae develop through four instars and feed primarily on HWA eggs. In late spring larvae drop from the hemlock branches into the soil where they pupate and aestivate through the summer. *L. nigrinus* can survive, oviposit, and reduce HWA populations in field cages during winter and spring in southwestern Virginia (Lamb et al. 2005a, Flowers et al. 2006, Lamb et al. 2006). *L. nigrinus* F₂ and F₃ adults have been recovered from previous release sites in eastern forests (Lamb et al. 2006, Mausel 2007). *S. tsugae* is a multivoltine predator that is native to Japan where it is found in association with HWA (Cheah and McClure 1995). It can feed and develop to maturity on all stages of HWA, and its life cycle suggests a seasonal synchrony with HWA in the field (Cheah and McClure 1998, 2000). *S. tsugae* is less cold tolerant than *L. nigrinus* and develops most rapidly at temperatures from 20-25 °C (Cheah and McClure 2000). Over 3 million *S. tsugae* and more than 50,000 *L. nigrinus* have been released in eastern forests before 2008.

The insecticide imidacloprid is the primary chemical used against HWA. Imidacloprid is a neonicotinoid and is an agonist of insect nicotinic acetylcholine receptors (nAChRs) (Elbert et al. 1991). Imidacloprid binds with post-synaptic nAChRs, activating them and initiating nervous stimulation along the synapse, which leads to neurotoxicity poisoning symptoms and death (Lind et al. 1999, Silcox 2002, Tomizawa and Casida 2003). Toxicity symptoms in insects include loss of coordination, excessive grooming, twitching, tremors, and paralysis (Kunkel et al. 2001, Decourtye et al. 2004). Imidacloprid is effective against a wide range of insects, and when applied systemically, it is especially successful protecting plants from sucking insects (Mizell
and Sconyers 1992, Lind et al. 1999). Some plant species will biotransform imidacloprid into metabolites that have insecticidal properties (Nauen et al. 1998b, 1999). For instance, in cotton plants imidacloprid metabolites protect the plant from aphid feeding even after parent compound concentrations declined (Nauen et al. 1998b).

Effective control of HWA can be achieved when imidacloprid is applied by foliar sprays or through systemic trunk or soil injections (McClure et al. 2001, Doccola et al. 2003, Webb et al. 2003, Cowles et al. 2006). Chemical controls with imidacloprid-based products are widely used in ornamental, landscape, and forest settings. Trunk and soil injections are the primary control components for HWA in large-scale injection programs in National Forests and State Parks. The Great Smoky Mountains National Park in northeastern Tennessee is an example of an initiative currently underway to limit hemlock decline from HWA infestation. In the park more than 164,000 beetles (predominantly S. tsugae) have been released at over 66 sites and more than 75,000 hemlocks have been treated via soil or trunk injections of imidacloprid (K. Johnson, pers. comm.).

In settings where chemical and biological controls are jointly applied, it is important to determine the compatibility of the two control options. High-value hemlock stands are often the first candidates for chemical control, and these same stands are desired as sites for biological control predator releases. Often predator releases are in close proximity to chemically treated sites, occupying the same valleys or mountain ridges. As predators are released into the environment, it is important to analyze factors that could affect their establishment (Mausel 2007). Reproduction, subsequent relocation of predators from release sites, and dispersal make it possible for predators to come into contact with hemlocks that have been chemically treated.

Using imidacloprid to control HWA could impact predators either through direct exposure or indirectly by changing HWA availability, quality, or distribution. Systemic insecticides do not directly expose predators to the degree that foliar applications do (Mizell and Sconyers 1992), however, nontarget beneficials can come into contact with systemic toxins by feeding on plant parts, contaminated prey, or through environmental factors such as contaminated soil (Groot and Dicke 2002). Predator consumption of sucking pests feeding on plants treated systemically with
imidacloprid can cause predator mortality (Grafton-Cardwell and Gu 2003, Cole and Horne 2006, Walker et al. 2007) and natural enemies who supplement their diet with plant products can be exposed to imidacloprid through contaminated nectar or pollen (Smith and Krischik 1999, Stapel et al. 2000). In the hemlock system, imidacloprid could potentially be passed on to predators as they feed on contaminated adelgids, especially because both *L. nigrinus* and *S. tsugae* rely on HWA as a primary food source. Adelgids surviving on treated trees may contain sublethal levels of imidacloprid that could be ingested by foraging predators. Predators exposed to systemic imidacloprid through feeding on contaminated prey could be subjected to lethal and sublethal effects that affect their biology and survival. Inhibited flight and dispersal, foraging, or fecundity could result from exposure to imidacloprid residues. For instance, a sublethal effect such as paralysis could cause predators to be susceptible to predation themselves (Kunkel et al. 2001).

This study investigates potential ways that systemic imidacloprid in hemlock tissue could impact non-target predators feeding on HWA. This study aims to investigate the relationship between imidacloprid, eastern hemlocks, HWA, and two nontarget predators in order to help determine the most constructive application of chemical and biological control that will benefit HWA management in the eastern United States.

### 3.2 Materials and Methods

**General methods of treating beetles and hemlock branches with imidacloprid**

Eastern hemlock branches infested with HWA were collected from Montgomery, Smyth, and Wythe counties in southwest Virginia during April and May 2007. Trees were approximately 20-50 years old, with substantial new growth and healthy green needles. Distal 60-150 cm sections were cut from the lower and mid-crown of each tree and returned to the laboratory. Ends were re-cut and submerged in water for storage. Distal 20 cm branchlets exhibiting HWA populations greater than 1.5 HWA per cm and healthy new growth were cut from each branch and placed into 8 x 3 cm plastic vials containing 20 mL of 0, 1, 10, or 100 parts-per-million (ppm) imidacloprid in water. Floral foam acted as a barrier in the mouth of the vial; the cut end of each hemlock branchlet main stem was pushed through the floral foam into the vial. The
serial dilutions were created by dilution of a 100 ppm stock solution of technical grade imidacloprid (Bayer, 99.2% purity) with distilled water. Branchlets were stored in a growth chamber (Model Percival Scientific, Boone, Iowa) maintained at 18:16 °C (D:N) with a photoperiod of 14:10 (L:D) h. Two open-topped 50 mL plastic containers were filled with tap water and placed in the chamber to slow desiccation of the branchlets. All vials and containers were re-filled with water as needed over the course of the experiment. After 10 d the branchlets were removed and the top portions that had no contact with the imidacloprid solution were clipped for use in the experiments while the 8 cm bottom portions were discarded. The top portions were placed in 8 x 3 cm vials with 20 mL fresh water for HWA and predator studies.

The experiment was complete block design with 12 blocks (time). There were approximately six 20 cm branchlets per treatment concentration in each block. In each block, all branches were from the same tree. The experiment was begun on April 17th and repeated approximately every 6-10 d.

Beetles
All *L. nigrinus* beetles originated from a colony maintained at Virginia Tech. Adults emerged from summer diapause in Fall 2006 were fed HWA collected in the field throughout the winter of 2006-07. A subset was set aside for these experiments in March, 2007. *S. tsugae* adults originated from a colony maintained by Clemson University in Clemson, South Carolina. Adults 1-2 weeks old were shipped to Blacksburg in March 2007 and the colony was reared on HWA collected from the field in spring 2007. To match normal environmental rearing conditions, *L. nigrinus* was stored at 12:10 °C (D:N) and 14:10 h (L:D) and *S. tsugae* was stored at 20:18 °C and 14:10 h.

Imidacloprid impacts on HWA
The first 4 blocks of the HWA experiment covered the over-wintering sistentes generation and the subsequent 8 blocks (beginning May 14th) covered the progredientes generation. Data were pooled by HWA generation and analysis was carried out on the progredientes generation. For a description of the development of HWA in the 2007 season see Table 3.1. Approximately 30 cm of new growth from each branchlet was sub-sampled 10, 20, and 30 d after treatment.
Adelgids were observed under a microscope and noted as alive or dead at each sampling period. Those that moved when disturbed or exuded fresh aqueous hemolymph when punctured were recorded as alive. Adelgids that did not move or respond when disturbed or were dry and exuded thick black hemolymph when punctured were recorded as dead. After examination, branchlets were placed into Ziploc® freezer bags and frozen until extraction.

**Imidacloprid and metabolite recovery in hemlock tissues**

To extract imidacloprid, branchlets were removed from the freezer and dried in an oven overnight at 40 °C or until needles could easily be removed. Branches were then ground using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA), which mechanically pulverizes the tissue until it was able to pass through a 1x1 mm screen. To extract imidacloprid, 150 mg of the dried, ground wood tissue was added to a 1.5 mL micro-centrifuge tube. Each sample was combined with 1.5 ml of 5% H₂SO₄ and shaken with a tabletop vortexer for 5 s. Samples were placed on a tabletop shaker at room temperature and shaken overnight. The following day, samples were centrifuged at 13,000 x g for 13 min. Supernatant was removed with a 1 mL syringe and filtered through a 0.45 μm PVDF filter before being added to 1.8 ml auto-sampler vials to be analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Samples were then packed in ice and shipped overnight to Villanova University, Villanova, PA., where Dr. A. Lagalante performed the LC/MS/MS procedure. The procedure quantifies imidacloprid, and the olefin, urea, 6-chloronicotinic acid (6-CNA), des-nitro, N-nitroso, des-nitro-olefin and dihydroxy metabolites.

**Impact on predators: No-choice experiment**

A subset of branchlets not used for the HWA experiments was used in a predator no-choice experiment. A branchlet with at least 100 ovisacs taken from 1 of 3 treatment levels (0, 1, or 100 ppm imidacloprid) was inserted into a wetted floral foam block wrapped in parafilm and placed into a 10 cm Petri dish. A 9 cm piece of filter paper was placed at the bottom of each Petri dish and was wetted every 5 d to prevent desiccation.
To measure loss of coordination or mobility due to imidacloprid, a test was initiated based on Bai et al. (2006). Before placement on a treated branchlet, each beetle was placed in the center of a Petri dish with a 9 cm filter paper and was flipped onto its dorsum with a paintbrush. The length of time taken for each beetle to right itself was timed by hand with a stopwatch. Time was measured to the nearest 0.01 s, with a maximum time of 60 s. The amount of time taken by each beetle to right itself was recorded as “flip time.” Flip time has been used to quantify beetle health and mobility (Bai et al. 2006, Smith and Krischik 1999, Lundgren and Wiedenmann 2002, 2005). One beetle was added to each Petri dish containing a hemlock branchlet from 0, 1, or 100 ppm treatment group. Each beetle’s flip time was recorded at each observation event every 5 d. Observations were terminated 20 d after beetles were placed on branchlets (30 d after treatment).

Also recorded at each observation time were beetle mortality and evidence of intoxication from imidacloprid. Imidacloprid intoxication symptoms include twitching, spasms, or paralysis. Beetles were rated at each observation period on an ordinal scale according to the degree of intoxication symptoms observed. Ratings increased in value as intoxication symptoms became more severe (Table 3.2). Beetles that were observed intoxicated or died during the course of the experiment were labeled as “affected.” The number of HWA consumed was recorded at each observation period by counting the number of ravaged ovisacs.

A randomized complete block design with 3 treatment levels and 10 beetles per level was used. For each species there were three blocks (time), resulting in 30 beetles of each species per treatment level. *L. nigrinus* and *S. tsugae* trials were initiated May 16 and 23, 2007, respectively.

**Impact on predators: Choice experiment**

A subset of branchlets not used for the HWA experiments was also used in a predator choice experiment. One branchlet from an untreated control branch was placed in a 10 cm Petri dish along with one branchlet from a 1, 10 or 100 ppm treated branch. Controls received two untreated branchlets. Branchlets were inserted into water-soaked floral foam blocks wrapped in Parafilm. The Parafilm of the floral foam block of each treated branchlet was marked with a permanent marker to distinguish it from the control branchlet. One beetle was added to each Petri dish. Half the replications received a male beetle and the other half received a female
beetle. As gender of L. nigrinus beetles cannot be determined by external physical characteristics, adults were sexed by the observation of oviposition after 72 h as reported in Lamb et al. (2005). L. nigrinus beetles referred to as “male” are non-ovipositing individuals. S. tsugae adults were observed under a microscope with the two sexes identified according to distinguishing morphological characteristics as discussed by Cheah and McClure (1998). L. nigrinus was stored at 12:10 °C (D:N) and 14:10 (L:D). S. tsugae was stored at 20:18 °C (day:night) and 14:10 (L:D).

Beetle mortality, evidence of intoxication, and beetle location in terms of which branch it occupied was recorded at each observation time. Flip time was recorded before beetle placement in the Petri dish and subsequently at each observation event. Beetles were rated on the intoxication scale mentioned earlier. The number of HWA eaten on each branchlet was based on counts of ravaged ovisacs. The experiment was a randomized complete block design, with each block (time) consisting of 6 replications (beetle) per treatment level. There were 5 blocks, resulting in a total of 30 beetles of each species tested for each treatment level. For all blocks, observations were made every 5 d for a total of four observations over 20 d. L. nigrinus trials were begun April 15, 2007 and S. tsugae trials were begun May 25, 2007.

**Predator topical application**

Technical grade imidacloprid was mixed with acetone to create a serial dilution resulting in concentrations of 0 (acetone only), 0.01, 0.1, 1, 10, and 100 ppm. A mechanized micro-applicator applied 0.5 μL of each treatment rate to the ventral abdomen of individual beetles, resulting in 0, 0.05, 0.5, 5, or 50 ng of imidacloprid applied per beetle. Beetles were placed on an untreated 5 cm hemlock twig infested with HWA within a 5 cm Petri dish. Before topical application, each beetle’s flip time was recorded and were subsequently examined every 24 h for 6 d. At each observation, individuals were rated on the level of intoxication (Table 3.2) and flip time. Individuals were recorded as dead when no response was elicited after probing with a paintbrush. Each species was stored according to temperature and light conditions mentioned previously. Approximately 10 L. nigrinus or S. tsugae were tested at each treatment level in 3 independent random complete blocks resulting in 145 and 132 individuals tested, respectively. One S. tsugae block was excluded from analysis because 95% of beetles from all treatment
groups were moribund within 24 h after treatment, possibly because a paintbrush used for manipulating the beetles during dosing may have been contaminated with imidacloprid. Consequently, only 102 S. *tsugae* beetle responses were used in analysis. *L. nigrinus* and *S. tsugae* trials were begun June 3 and 11, 2007, respectively.

**Imidacloprid and metabolite recovery in beetles**
At the conclusion of each experiment, beetles were stored in 1.5 mL microcentrifuge tubes and frozen at -60 °C. To extract imidacloprid, beetles were frozen with liquid nitrogen and ground into a powder. 0.5-1.5 mLs of acetonitrile were added to each sample and shaken overnight at room temperature. The LC/MS/MS procedure was similar to that of the tissue procedure and was carried out by Dr. A. Lagalante at Villanova University.

**Statistical analysis**
Data were analyzed using SPSS® for Macintosh OS X version 11.0.4. Control mortality was corrected using Abbott’s formula (Abbott, 1925). All mortality data were arcsine square-root transformed before analysis; reported means are untransformed. For all tests, *P* ≤ 0.05 was used to separate means.

Probit analysis was conducted for HWA mortality after 30 d based on imidacloprid concentrations recovered by LC/MS/MS. Probit analysis was conducted for beetle mortality 3 and 6 d after topical application. Linear regression was performed comparing imidacloprid concentration on a logarithmic scale versus HWA mortality. Imidacloprid and metabolite concentrations determined by LC/MS/MS were corrected by subtracting any values in the control branchlets (less than 20 ppb) from treated branchlets.

For the no-choice test, the proportion of beetle mortality was averaged within replications for each treatment observation period combination and analyzed using repeated measures ANOVA. Means were separated using Tukey’s honestly significant difference test (HSD). Repeated measures ANOVA tested for flip time differences over time.
For the choice test, a two-way ANOVA compared mortality by beetle sex and treatment. The total number of adelgids eaten by each beetle during the course of the experiment was analyzed using one-way ANOVA, with means separated by Tukey’s HSD test. Repeated measures ANOVA tested for flip time differences over time. The number of eggs laid by each female *L. nigrinus* on control and treated branchlets in the choice test were compared by the Wilcoxon signed rank test. A binomial sign test compared proportions of observations of beetles on treated vs. untreated branchlets with the expected proportion of 50% of total observations on treated branchlets and 50% of observations on untreated branchlets.

Flip time data were transformed to achieve homogeneity of variance using the inverse of the square root. One-way analysis of variance (ANOVA) compared beetle flip time means before placement in the various experiments. Intoxication and affected ratings were compared among treatments and tested for significance using the Kruskal-Wallis test. Differences between treatments were separated by pair-wise comparison of treatment ranks using a Wilcoxon signed rank test. Intoxication ratings were from the ordinal intoxication scale but did not include dead beetles in the analysis. Affected ratings were also from the ordinal intoxication scale but included both intoxicated and dead beetles in the analysis.

### 3.3 Results

**Imidacloprid impacts on HWA**

Imidacloprid affected HWA mortality in a dosage-dependant manner over time (Table 3.3). After 30 d, HWA mortality was 29, 70, 84 and 96 % for the 0, 1, 10, and 100 ppm treatment groups, respectively. Significant differences among dosages were found for all 3 sample periods (Table 3.3). Probit analysis of HWA mortality after 30 d versus detected imidacloprid concentration means determined the LC$_{50}$ of imidacloprid and its 95% confidence limit (CL) to be 242 and 105 - 411 ppb, respectively. After correcting for control mortality, HWA mortality means 30 d after treatment had a strong dependent relationship with the average concentration of imidacloprid in each treatment group determined by LC/MS/MS (Figure 3.1).

**Imidacloprid and metabolite recovery in hemlock tissues**
Imidacloprid and metabolite concentrations in wood tissues generally increased with treatment rate and time after treatment (Table 3.4). Olefin, des-nitro, urea, and 6-CNA were the metabolites detected in the largest proportions. Imidacloprid comprised 64-98% of the total metabolite concentrations detected. Olefin was the most prevalent metabolite, consisting of 2-27% of the total concentrations. Other metabolites combined did not exceed 2% of the total concentration in most samples.

**Impacts on predators: No-choice experiment**

Differences were observed in predator feeding, flip time, mortality, and intoxication symptoms among treatments in the no-choice test (Table 3.5). Predator mortality increased with HWA mortality and imidacloprid concentrations recovered from hemlock wood tissues (Figures 3.2 and 3.3 A).

*Feeding*

*L. nigrinus* consumed significantly less HWA from treated branchlets than from un-treated branchlets ($F = 6.153; \text{df} = 2, 87; P = 0.003$). No differences in consumption of HWA by *S. tsugae* were observed between treated and un-treated branchlets ($F = 0.229; \text{df} = 2, 87; P = 0.796$).

*Flip time*

Before placement on branchlets, there was no difference in flip times between individuals for *L. nigrinus* ($F = 0.40; \text{df} = 2, 87; P = 0.667$) or *S. tsugae* ($F = 0.65; \text{df} = 2, 87; P = 0.523$). Flip times for beetles from the 100 ppm branchlets were significantly longer than beetles from control branchlets ($F = 4.526; \text{df} = 2, 85; P = 0.014$). Flip time means increased for *S. tsugae* as the imidacloprid concentration increased, however, differences were not significant ($F = 0.653; \text{df} = 2, 87; P = 0.523$).

*Mortality*

Treatment had a significant effect on mortality for *L. nigrinus* ($F = 2.952; \text{df} = 2, 6; P = 0.048$), but not for *S. tsugae* ($F = 1.50; \text{df} = 2, 6; P = 0.296$). After correcting for control mortality, *L.
*nigrinus* mortality averaged more than 40%, while *S. tsugae* mortality was less than 7% in the 100 ppm treatment group. Mortality generally increased with time and dose.

**Intoxicated**

The percentage of beetles observed intoxicated or moribund at least once during the course of the experiment ranged from 0-20% and 0-13% for *L. nigrinus* and *S. tsugae*, respectively. For *L. nigrinus*, 25% of the beetles observed intoxicated in the 1 ppm treatment group eventually died, and 50% from the 100 ppm treatment group exhibiting intoxication eventually died. For *S. tsugae*, none of the intoxicated beetles in the 1 ppm group died, but as in *L. nigrinus* 50% of beetles observed intoxicated in the 100 ppm group died. Intoxication ranks were significantly different among treatments for *L. nigrinus* ($\chi^2 = 9.79; \text{df} = 2; P = 0.007$) but differences were not significant for *S. tsugae* ($\chi^2 = 4.44; \text{df} = 2; P = 0.109$).

**Affected**

Affected ranks were significantly different among treatments for both *L. nigrinus* ($\chi^2 = 13.15; \text{df} = 2; P = 0.001$) and *S. tsugae* ($\chi^2 = 7.09; \text{df} = 2; P = 0.029$). Proportions of affected beetles generally increased with time and dose.

**Impacts on predators: Choice experiment**

Differences in predator biology were evident in choice tests (Table 3.6). Predator mortality increased with HWA mortality and imidacloprid concentrations recovered from hemlock wood tissue (Figure 3.3 B).

**Feeding**

In paired t-tests comparing the number of HWA eaten on control and treated branchlets, there was a significant difference between control and 100 ppm for *L. nigrinus* ($P = 0.006$), but no significant differences were found for *S. tsugae* (Table 3.7). The difference in the number of HWA consumed on un-treated branchlets and treated branchlets increased with dose for both species. For both species, there were no significant differences between sexes: *L. nigrinus* ($F = 3.698; \text{df} = 1, 111; P= 0.057$), *S. tsugae* ($F = 2.163; \text{df} = 1, 112; P = 0.144$) or treatment levels: *L. nigrinus* ($F = 0.851; \text{df} = 3, 115; P = 0.469$), *S. tsugae* ($F = 0.217; \text{df} = 3, 116; P = 0.885$).
Flip time
Flip times before beetle placement in the choice arena were not significantly different for L. nigrinus ($P = 0.161$) or S. tsugae ($P = 0.563$), nor between sexes L. nigrinus ($F = 2.674; \text{df} = 1, 119; P = 0.105$) or S. tsugae ($F = 0.083; \text{df} = 1, 112; P = 0.774$) and data for the two sexes of each species were pooled. Flip times increased over time and treatment level but were not significantly different for L. nigrinus ($F = 1.238; \text{df} = 3, 106; P = 0.30$). S. tsugae flip times in the 100 ppm treatment group were significantly longer than controls ($F = 3.634; \text{df} = 3, 113; P = 0.015$).

Mortality
A two-way ANOVA of mortality showed no differences among treatments for L. nigrinus ($F = 1; \text{df} = 3, 32; P = 0.406$) but differences between sexes were significant ($F = 0.937; \text{df} = 1, 32; P = 0.019$). After analyzing mortality within each sex, treatment effects were not significant for females ($F = 1.588; \text{df} = 1, 32; P = 0.231$) and males ($F = 0.940; \text{df} = 3, 16; P = 0.445$). For S. tsugae, a two-way ANOVA revealed no differences in mortality by treatment ($F = 0.667; \text{df} = 3, 32; P = 0.579$) or sex ($F = 2; \text{df} = 1, 32; P = 0.167$). After correcting for control mortality, L. nigrinus mortality was as high as 26% in the 100 ppm treatment group, but S. tsugae mortality was never higher than 3%.

Intoxicated
Over 20% of L. nigrinus beetles showed signs of intoxication from feeding on treated branchlets and 25, 57 and 43% of them died from the 1, 10 and 100 ppm treatment groups, respectively. Signs of intoxication for S. tsugae were less prevalent, ranging from 7% to 13%. From these intoxicated beetles, 25 and 33% eventually died from the 10 and 100 ppm treatment groups, respectively. Intoxication ranks were significantly different among treatments for both L. nigrinus ($\chi^2 = 8.71; \text{df} = 3; P = 0.033$) and S. tsugae ($\chi^2 = 11.23; \text{df} = 3; P = 0.011$).

Affected
The proportions of beetles affected generally increased with dose and time. Affected ranks were significantly different among treatments for both *L. nigrinus* ($\chi^2 = 10.41; \text{df} = 3; P = 0.015$) and *S. tsugae* ($\chi^2 = 13.13; \text{df} = 3; P = 0.004$).

**Location**
A binomial test of significance tested the proportion of observations of beetles residing on the two branchlets against the test proportion of 50% of observations on each branch. *L. nigrinus* beetles were observed on control branchlets 50-61% of the time with no difference from treated branchlets (Table 3.8). *S. tsugae* beetles were observed on control branchlets 37-60% of the time. The only significant difference was found in the 1 ppm treatment group, where beetles were more frequently observed on the 1 ppm branch than on the control branch ($P = 0.013$).

**Fecundity**
A repeated measure ANOVA on the total number of eggs laid over time for *L. nigrinus* revealed no significant differences among treatments ($F = 0.476; \text{df} = 3, 66; P = 0.70$). A Wilcoxon signed rank test compared the numbers of eggs laid by *L. nigrinus* on treated or untreated branchlets (Table 3.9). In the 1 ppm treatment group, *L. nigrinus* laid significantly more eggs on control branchlets than treatment branchlets ($P = 0.021$), but there were no significant differences in the 10, 100 ppm, or control groups. *S. tsugae* eggs were difficult to find as they lay eggs under bud scales and in bark crevices, making observation of eggs very challenging without destructive sampling. Therefore, no egg data for this species are reported.

**Predator topical application**
The 6 d LD$_{50}$ values for *L. nigrinus* and *S. tsugae* were 1.8 and 0.71 ng per beetle, respectively (Table 3.10).

**Flip time**
Both beetle species also required longer flip times after imidacloprid treatment than controls, with higher concentrations resulting in longer times than lower concentrations. (Table 3.11). Flip times were significantly different among treatments for *L. nigrinus* ($F = 18.51; \text{df} = 5, 139; P = 0.001$) and for *S. tsugae* ($F = 37.29; \text{df} = 5, 96; P = 0.001$).
Mortality
Mortality increased with dose. *L. nigrinus* and *S. tsugae* mortality were significantly different among treatments ($F = 10.50; \text{df} = 5, 9; P = 0.002$) and ($F = 7.22; \text{df} = 5, 9; P = 0.039$), respectively.

Intoxicated
Both beetle species displayed tremors and paralysis after treatment, with increasing intensity of poisoning symptoms and mortality over time and with increasing dose concentration. The ratings from the intoxication scale were significantly different for both *L. nigrinus* ($\chi^2 = 304.50; \text{df} = 5; P = 0.001$) and *S. tsugae* ($\chi^2 = 326.90; \text{df} = 5; P = 0.001$).

Affected
Proportions of beetles affected increased with dose. Affected beetle ratings were significantly different *L. nigrinus* ($\chi^2 = 72.88; \text{df} = 5; P = 0.001$) and *S. tsugae* ($\chi^2 = 62.26; \text{df} = 5; P = 0.001$).

Imidacloprid and metabolite recovery in beetles
Imidacloprid was detected in both beetle species in all experiments. No metabolites were detected from the choice or no-choice experiments. In the topical application experiment, olefin and 6-CNA were recovered from the 50 ng treatment group from both *L. nigrinus* and *S. tsugae*. Beetle mortality corresponded with recovered imidacloprid concentrations from the choice and no-choice experiment (Figure 3.4) and the topical application experiment (Figure 3.5).

3.4 Discussion
Our results show that in the laboratory, very low concentrations (< 242 ppb) of imidacloprid in wood tissue killed 50% of HWA within 30 d of treatment. Our results support Cowles et al. (2006) who conducted a similar experiment with imidacloprid and HWA sistentes. They determined the LC$_{50}$ and its 95% CI for imidacloprid after 20 d to be 300 and 150-600 ppb, respectively. Their results with sistentes and this study on progredientes suggest that the two
generations display similar susceptibility to imidacloprid. However, these two studies investigated short-term imidacloprid impacts (< 30 d). In the field, HWA will be exposed to imidacloprid for periods of time much longer than 30 d, and over several generations. Field LC₅₀ values therefore are likely to be lower than 300 ppb. This was shown by Cowles et al. (2006) who estimated the field LC₅₀ for HWA to be to be approximately 120 ppb.

Based on analysis by LC/MS/MS, imidacloprid was effectively taken up and metabolized by cut hemlock branches within 10 d after treatment in the laboratory. Olefin was the primary metabolite recovered from the hemlock wood tissues. It displays biological efficacy against insects, in some cases even higher than its parent compound imidacloprid. For example, olefin was 10 times as biologically efficacious as imidacloprid against whiteflies, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) (Nauen et al. 1999). In the honeybee, *Apis mellifera* L., (Hymenoptera: Apidae) olefin was a product of biotransformation of oral doses of imidacloprid and was associated with the onset of mortality within hours after ingestion, while imidacloprid was the cause of initial neurotoxicity symptoms (Suchail et al. 2003, 2004). Olefin’s efficacy against HWA has not been studied, however, it could display similar increased efficacy against HWA and its predators. Hemlock biotransformation of imidacloprid into the olefin metabolite may provide additional protection from HWA infestation, although this could make imidacloprid application more toxic to predators.

A strong relationship was observed in the 100 ppm treatment group when HWA mortality, predator mortality and imidacloprid concentrations recovered by LC/MS/MS were compared over time (Figure 3.2). Both imidacloprid concentrations and HWA mortality generally increased with time. When mortality and imidacloprid concentrations are compared across treatments, HWA mortality parallels imidacloprid concentrations (Figure 3.3). *L. nigrinus* mortality was similar to HWA mortality and imidacloprid recovery in the no-choice test (Figure 3.3A), but mortality was not as pronounced in the choice test (Figure 3.3B). *S. tsugae* mortality was much lower than *L. nigrinus* in both no-choice and choice tests.

Predators feeding on adelgids surviving on treated trees may be affected directly by insecticide toxicity or indirectly by reduced prey quality and density. Although both predators consumed
more adelgids from untreated than treated branchlets, this may be influenced by the fact that adelgids were dying at a faster rate on the treated branchlets. Besides contact with a systemic insecticide, mortality and fitness seem to be affected also by mortality associated with ingestion of insecticide. Mortality was less pronounced in the choice tests than in the no-choice tests, suggesting that the availability of healthy, untreated HWA may encourage the survival of predators.

Some beetles displayed intoxication symptoms after feeding on treated adelgids, suggesting that imidacloprid may be passed from an adelgid to a predator under certain conditions. Intoxication response was variable; some beetles showed intoxication symptoms within 5 days of feeding on HWA from treated branchlets, others displayed symptoms later, and some never displayed intoxication symptoms. Not all beetles that displayed intoxication symptoms died, suggesting that consumption of low concentrations of imidacloprid are not always lethal as the beetle may be able to metabolize the insecticide to non-biologically efficacious metabolites within a few days. Some beetles that died did not display any intoxication symptoms, indicating natural mortality from reduced prey availability or quality rather than direct insecticidal effects.

Imidacloprid was the main toxicant recovered from beetles post-mortem. The metabolites olefin and 6-CNA were recovered from the highest dose in the topical application experiment, however, no metabolites were recovered from any other beetle cadavers. In general, higher concentrations of imidacloprid were recovered from L. nigrinus than S. tsugae. The recovery of imidacloprid from beetles post-mortem suggests that imidacloprid was passed on to the predators through feeding on contaminated adelgids. If the imidacloprid concentrations that were found in these lab-treated branches are similar in hemlocks from field treatments of imidacloprid, it is possible that systemic imidacloprid could directly affect nontarget predators. L. nigrinus mortality was highest in the 100 ppm treatment group in the no-choice test, however, the highest concentration of imidacloprid was also found in beetles from the 100 ppm treatment group in the choice test.

L. nigrinus was more sensitive to feeding on adelgids from treated branchlets compared with S. tsugae because it was more intimately linked to HWA. For instance, egg laying was within
HWA ovisacs while *S. tsugae* egg laying was primarily on the bark. Predators could prefer to lay eggs on untreated branchlets than treated branchlets in order to utilize healthier, denser, higher quality adelgid populations. Differences in predator behavior may play a role in susceptibility; *L. nigrinus* consumed whole adelgid adults while *S. tsugae* was more often observed feeding on eggs or partially consuming adelgids. Adelgid eggs might not have imidacloprid within them and could be a safer food source for both predators and their larvae, although further experiments are required to test this hypothesis.

Topical applications demonstrated the intolerance of both beetle species to direct exposure to imidacloprid. Both predator species displayed intoxication symptoms at the lowest dose 24 h after treatment. At doses of <10 ppm, some beetles were able to survive for the 6 d experiment with tremors. In some cases, mortality may have resulted from starvation or dehydration as opposed to acute mortality, because beetles were paralyzed and unable to move or feed. These LD$_{50}$ values are similar to the LD$_{50}$ for a honeybee, reported to be 46 ng/bee 96 h after oral dosing (Suchail et al. 2001). In the choice and no-choice tests, mortality and intoxication symptoms were less pronounced for both species than acute mortality. For instance, in the no-choice experiment *L. nigrinus* beetles displayed 47% mortality after 20 d of feeding on 100 ppm branchlets. Similarly, in the topical application experiment, *L. nigrinus* displayed mortality of 46% after only 6 d from a topical application of 5 ng of imidacloprid. Therefore, < 5 ng of imidacloprid may have been passed on to predators through feeding on the adelgids. If any of this mortality was from lower quality food, the amount of imidacloprid exchanged through feeding on adelgids would be even lower. Differences in beetle size and weight could play a role in susceptibility; *L. nigrinus* weighs approximately 740 ± 38 µg each, and *S. tsugae* weighs 390 ± 36 µg each. The LD$_{50}$ value for *L. nigrinus* was more than twice the value for *S. tsugae*, which may reflect that they weigh approximately twice as much. If LD$_{50}$ values are calculated by body weight, the LD$_{50}$ values are 2.4 and 1.8 µg/g for *L. nigrinus* and *S. tsugae*, respectively. Topical applications of imidacloprid are more acutely toxic to *S. tsugae* compared with *L. nigrinus*.

Another factor that could have affected susceptibility is that the test was carried out in June and *L. nigrinus* adults had been alive and feeding for over 9 months and were at the end of their lifecycle. *S. tsugae* adults were less than 3 months old and compared with *L. nigrinus* they were
younger and more robust. Thus, beetle age and health could be factors impacting subsequent survivorship and it would be beneficial if *L. nigrinus* assays were conducted during early winter when they were younger and more robust.

In the field, very low concentrations of imidacloprid are capable of controlling HWA. Imidacloprid concentrations in field-treated hemlocks can be less than 250 ppb (Cowles et al. 2006) or generally less than 1 ppm (Chapter 4). In these experiments imidacloprid concentrations in the branchlets from the 100 ppm treatment group ranged from 4.5-15 ppm, concentrations that are much higher than would be expected in the field. The 1 ppm treatment group had imidacloprid concentrations in the branchlets ranging from 60-200 ppb, which are concentrations that are more likely to be found in field treated hemlocks. At these concentrations, predator mortality and fitness impacts were less pronounced than the 10 and 100 ppm treatments, suggesting that the concentrations found in field-treated hemlocks may not impact predators to a quantifiable degree. Negative effects of imidacloprid on HWA predators would probably be the result of reduced prey quality and density. Although imidacloprid exposure through feeding on adelgids on treated trees is possible, predator preference for healthier food stock could drive them away from treated stands towards denser, healthier adelgid populations.

Different life cycle behaviors by these two predator species could be the basis for their differences in susceptibility to imidacloprid treatments. For instance, *L. nigrinus* larvae drop from hemlock branches into the soil where they pupate and aestivate through the summer as adults. If treatments of imidacloprid are applied to soil at the base of hemlock trees where *L. nigrinus* larvae pupates, larva and adult survivorship, and emergence could also be affected.

Short term acute insecticide effects can be so efficacious against pests that they can reduce a population so that it cannot support a natural enemy population, causing natural enemies to either starve, disperse or find new food sources (Johnson and Tabashnik 1999). Any antifeedant or survivorship impacts that imidacloprid has on HWA will result in lower quality food for predators. This effect is greater on host-specific predators like *L. nigrinus* and on generalists that would prey on HWA during winter or early spring when few other food sources are available. In
high value hemlock stands biological and chemical control could be advantageous when used together; chemicals could eradicate HWA populations on a proportion of hemlocks in a stand and predators could subsequently be released on the remaining trees. Chemical and biological control of HWA are both needed to protect hemlocks in eastern forests. These studies suggest that predator releases in treated stands would benefit when untreated adelgid populations are available as additional food sources, and biological and chemical control could be used together for protection of high value hemlock stands if predator releases are well timed for least adverse insecticidal effects on them.
**Table 3.1** 2007 timeline of hemlock woolly adelgid development in southwestern VA.

<table>
<thead>
<tr>
<th>Generation</th>
<th>Life Stage</th>
<th>2007¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006 Sistentes</td>
<td>Adults</td>
<td>Before April 1</td>
</tr>
<tr>
<td></td>
<td>Eggs</td>
<td>April 21</td>
</tr>
<tr>
<td></td>
<td>Crawlers</td>
<td>May 5</td>
</tr>
<tr>
<td></td>
<td>2nd instar</td>
<td>May 12</td>
</tr>
<tr>
<td></td>
<td>3rd instar</td>
<td>May 16</td>
</tr>
<tr>
<td></td>
<td>4th instar</td>
<td>May 20</td>
</tr>
<tr>
<td></td>
<td>Adults</td>
<td>May 25</td>
</tr>
<tr>
<td>2007 Sistentes</td>
<td>Eggs</td>
<td>May 30</td>
</tr>
<tr>
<td></td>
<td>Crawlers</td>
<td>June 5</td>
</tr>
</tbody>
</table>

¹Date in 2007 HWA life stage first observed.

**Table 3.2** Ordinal intoxication scale used to quantify the degree of intoxication observed for beetles. Each beetle was given an intoxication ranking from 0-5 at each observation period.

<table>
<thead>
<tr>
<th>Rating</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beetle behavior</td>
<td>Alive, healthy</td>
<td>Mobile but twitching</td>
<td>Mobile but with severe tremors</td>
<td>Immobile with severe tremors</td>
<td>Paralyzed, unable to right themselves</td>
<td>Dead</td>
</tr>
</tbody>
</table>

**Table 3.3** Mean (± se) percent mortality of hemlock woolly adelgid sampled at 10, 20, and 30 days after branchlets were placed in imidacloprid solutions.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>10</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>45 ± 4 a</td>
<td>25 ± 5 a</td>
<td>29 ± 11 a</td>
</tr>
<tr>
<td>1</td>
<td>53 ± 5 ab</td>
<td>40 ± 8 a</td>
<td>70 ± 16 b</td>
</tr>
<tr>
<td>10</td>
<td>55 ± 4 ab</td>
<td>70 ± 5 b</td>
<td>84 ± 6 b</td>
</tr>
<tr>
<td>100</td>
<td>65 ± 3 b</td>
<td>78 ± 6 b</td>
<td>96 ± 2 b</td>
</tr>
</tbody>
</table>

Within each column, means followed by different letters are significantly different (Tukey’s HSD test, P = 0.05).
Table 3.4 Mean (± se) concentrations (ppb) of imidacloprid and four of its most prevalent metabolites recovered from eastern hemlock wood tissue. Percentage of total concentration each compound comprises for each dose/time combination in parenthesis.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>DAT(^1)</th>
<th>Imidacloprid</th>
<th>Olefin</th>
<th>Des-Nitro</th>
<th>Urea</th>
<th>6-CNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>68 ± 21 (64%)</td>
<td>30 ± 9.1 (27%)</td>
<td>2 ± 0.4 (2%)</td>
<td>0 (0%)</td>
<td>0 ± 0 (0%)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>216 ± 206 (87%)</td>
<td>21 ± 7.9 (8%)</td>
<td>3 ± 2 (1%)</td>
<td>0 (0%)</td>
<td>1 ± 1 (1%)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>200 ± 97 (90%)</td>
<td>16 ± 8.9 (7%)</td>
<td>2 ± 1 (1%)</td>
<td>2 ± 1 (1%)</td>
<td>2 ± 2 (1%)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>89 ± 56 (78%)</td>
<td>17 ± 4 (15%)</td>
<td>3 ± 1 (2%)</td>
<td>0 (0%)</td>
<td>1 ± 0.4 (1%)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>470 ± 247 (93%)</td>
<td>23 ± 11 (5%)</td>
<td>4 ± 3 (1%)</td>
<td>6 ± 4 (1%)</td>
<td>3 ± 1 (1%)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1254 ± 638 (94%)</td>
<td>35 ± 18 (3%)</td>
<td>9 ± 6 (1%)</td>
<td>19 ± 12 (1%)</td>
<td>7 ± 3 (1%)</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>4488 ± 2497 (97%)</td>
<td>93 ± 31 (2%)</td>
<td>38 ± 17 (1%)</td>
<td>13 ± 11 (0%)</td>
<td>8 ± 4 (0%)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>7799 ± 4454 (96%)</td>
<td>172 ± 86 (2%)</td>
<td>56 ± 35 (1%)</td>
<td>88 ± 77 (1%)</td>
<td>29 ± 16 (0%)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>15282 ± 4002 (93%)</td>
<td>442 ± 155 (3%)</td>
<td>110 ± 53 (1%)</td>
<td>294 ± 187 (2%)</td>
<td>84 ± 24 (1%)</td>
</tr>
</tbody>
</table>

\(^1\)Days after treatment.

Table 3.5 Impacts on two predators resulting from feeding on hemlock woolly adelgid on eastern hemlock branchlets treated with imidacloprid in a no-choice experiment.

<table>
<thead>
<tr>
<th>Spp(^1)</th>
<th>Dose (ppm)</th>
<th>HWA Eaten per day(^2)</th>
<th>Flip time(^3) (s)</th>
<th>Mortality(^3) (%)</th>
<th>Intoxicated(^4) (%)</th>
<th>Affected(^5) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ln</td>
<td>0</td>
<td>2.2 ± 0.14 a</td>
<td>19.9 ± 2.7 a</td>
<td>0 a</td>
<td>0 a</td>
<td>27 a</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.6 ± 0.17 b</td>
<td>28.4 ± 3.6 ab</td>
<td>15 ab</td>
<td>13 b</td>
<td>52 b</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.5 ± 0.15 b</td>
<td>34.4 ± 3.4 b</td>
<td>41 b</td>
<td>20 b</td>
<td>77 b</td>
</tr>
<tr>
<td>St</td>
<td>0</td>
<td>3.6 ± 0.26 A</td>
<td>11.9 ± 1.6 A</td>
<td>0 A</td>
<td>0 A</td>
<td>0 A</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.4 ± 0.18 A</td>
<td>13.4 ± 2.1 A</td>
<td>3 A</td>
<td>7 A</td>
<td>10 AB</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.4 ± 0.28 A</td>
<td>18.7 ± 2.6 A</td>
<td>7 A</td>
<td>13 A</td>
<td>20 B</td>
</tr>
</tbody>
</table>

\(^1\)Ln = *Laricobius nigrinus*, St = *Sasaicynnus tsugae*
\(^2\)Mean ± standard error. For each species, means within each column followed by the same letter are not significantly different (Tukey’s HSD test, \(P = 0.05\)).
\(^3\)Displayed means are corrected for control mortality. For each species, means within each column followed by the same letter are not significantly different (Tukey’s HSD test, \(P = 0.05\)).
\(^4\)Percent of beetles observed intoxicated at least once during course of the experiment. Differences among treatment ranks were separated with a pairwise comparison using the Wilcoxon signed rank test (\(P = 0.05\)).
\(^5\)Percent affected (observed intoxicated or dead) during the course of the experiment. Differences among treatment ranks were separated with a pairwise comparison using the Wilcoxon signed rank test (\(P = 0.05\)), ranks not shown.
Table 3.6 Impacts on two predators resulting from feeding on hemlock woolly adelgid on eastern hemlock branchlets treated with imidacloprid in a choice experiment.

<table>
<thead>
<tr>
<th>Spp</th>
<th>Dose (ppm)</th>
<th>Flip time (s)</th>
<th>Mortality (%)</th>
<th>Intoxicated (%)</th>
<th>Affected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ln</td>
<td>0</td>
<td>16.4 ± 3.6 a</td>
<td>0 a</td>
<td>0 a</td>
<td>27 a</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>24.4 ± 3.4 a</td>
<td>11 a</td>
<td>27 b</td>
<td>57 bc</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>23.6 ± 3.8 a</td>
<td>11 a</td>
<td>23 b</td>
<td>48 b</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>28.8 ± 3.6 a</td>
<td>26 a</td>
<td>23 b</td>
<td>60 c</td>
</tr>
<tr>
<td>St</td>
<td>0</td>
<td>12.5 ± 1.3 A</td>
<td>0 A</td>
<td>7 A</td>
<td>7 A</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>13.9 ± 1.4 A</td>
<td>0 A</td>
<td>0 A</td>
<td>0 A</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>19.5 ± 2.4 AB</td>
<td>3 A</td>
<td>13 B</td>
<td>13 B</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>23.2 ± 2.6 B</td>
<td>3 A</td>
<td>10 B</td>
<td>10 B</td>
</tr>
</tbody>
</table>

1 Ln = *Laricobius nigrinus*, St = *Sasajiscymnus tsugae*

2 Mean ± standard error. For each species, means within each column followed by the same letter are not significantly different (Tukey’s HSD test, *P* = 0.05).

3 Displayed means are corrected for control mortality. For each species, means within each column followed by the same letter are not significantly different (Tukey’s HSD test, *P* = 0.05).

4 Percent of beetles observed intoxicated at least once during course of the experiment. Differences among treatment ranks were separated with a pairwise comparison using the Wilcoxon signed rank test (*P* = 0.05), ranks not shown.

5 Percent affected (observed intoxicated or dead) during the course of the experiment. Differences among treatment ranks were separated with a pairwise comparison using the Wilcoxon signed rank test (*P* = 0.05), ranks not shown.
Table 3.7 Mean number (± se) of ravaged hemlock woolly adelgid ovisacs on control and treated eastern hemlock branchlets in paired choice tests.

<table>
<thead>
<tr>
<th>Spp</th>
<th>Dose (ppm)</th>
<th>Control</th>
<th>Treated</th>
<th>Difference ± se</th>
<th>t - statistic</th>
<th>df</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7.3 ± 0.95</td>
<td>6.2 ± 0.63</td>
<td>1.1 ± 0.66</td>
<td>1.71</td>
<td>29</td>
<td>0.098</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8.8 ± 1.1</td>
<td>7.3 ± 0.75</td>
<td>1.5 ± 1.1</td>
<td>-1.44</td>
<td>29</td>
<td>0.162</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.1 ± 0.97</td>
<td>6.4 ± 0.70</td>
<td>1.7 ± 0.98</td>
<td>-1.77</td>
<td>29</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.8 ± 0.84</td>
<td>5.3 ± 0.59</td>
<td>2.5 ± 0.84</td>
<td>-2.96</td>
<td>28</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.6 ± 0.54</td>
<td>14.6 ± 0.80</td>
<td>0.0 ± 0.69</td>
<td>0.00</td>
<td>28</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>14.8 ± 0.75</td>
<td>14.6 ± 0.92</td>
<td>0.21 ± 1.1</td>
<td>-0.19</td>
<td>28</td>
<td>0.853</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>14.6 ± 0.75</td>
<td>13.9 ± 0.62</td>
<td>0.59 ± 0.99</td>
<td>-0.59</td>
<td>28</td>
<td>0.562</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>14.6 ± 0.67</td>
<td>13.4 ± 0.69</td>
<td>1.2 ± 0.71</td>
<td>-1.74</td>
<td>29</td>
<td>0.093</td>
</tr>
</tbody>
</table>

1 Ln = Laricobius nigrinus, St = Salsicynmus tsugae
2 Mean ± standard error
3 For the 0 dose group “treated” branchlets are untreated.

Table 3.8 Results from a binomial test of significance comparing the proportion of observations of beetles on control vs. treated branchlets with a hypothetical proportion of 50% of total observations on each branchlet.

<table>
<thead>
<tr>
<th>Spp</th>
<th>Dose (ppm)</th>
<th>Control</th>
<th>Treated</th>
<th>P-value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 (%)</td>
<td>50 (%)</td>
<td>1</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>50 (%)</td>
<td>50 (%)</td>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td>Ln</td>
<td>10</td>
<td>58 (%)</td>
<td>42 (%)</td>
<td>0.26</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>61 (%)</td>
<td>39 (%)</td>
<td>0.11</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 (%)</td>
<td>60 (%)</td>
<td>0.062</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>37 (%)</td>
<td>63 (%)</td>
<td>0.013</td>
<td>102</td>
</tr>
<tr>
<td>St</td>
<td>10</td>
<td>51 (%)</td>
<td>49 (%)</td>
<td>0.917</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>60 (%)</td>
<td>40 (%)</td>
<td>0.076</td>
<td>92</td>
</tr>
</tbody>
</table>

1 Ln = Laricobius nigrinus, St = Salsicynmus tsugae
2 Percent of total observations where beetle was located on control branchlet.
3 Percent of total observations where beetle was located on treated branchlet. For the 0 dose group “treated” branchlets are untreated.
Table 3.9 Wilcoxon signed rank test results comparing average number of eggs laid by *Laricobius nigrinus* females on control vs. treated branchlets in a choice test.

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Control(^1)</th>
<th>Treated(^1)</th>
<th>Difference</th>
<th>Z statistic</th>
<th>n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.88 ± 0.43</td>
<td>1.1 ± 0.68</td>
<td>-0.19 ± 0.31</td>
<td>-0.302</td>
<td>16</td>
<td>0.763</td>
</tr>
<tr>
<td>1</td>
<td>2.8 ± 1.09</td>
<td>1.2 ± 0.53</td>
<td>1.6 ± 0.72</td>
<td>-2.316</td>
<td>20</td>
<td>0.021</td>
</tr>
<tr>
<td>10</td>
<td>1.4 ± 0.54</td>
<td>0.94 ± 0.34</td>
<td>0.5 ± 0.55</td>
<td>-0.718</td>
<td>16</td>
<td>0.472</td>
</tr>
<tr>
<td>100</td>
<td>1.3 ± 0.46</td>
<td>1.6 ± 4.08</td>
<td>-0.28 ± 0.84</td>
<td>-0.309</td>
<td>18</td>
<td>0.758</td>
</tr>
</tbody>
</table>

\(^1\)Mean ± se. For the 0 dose group “treated” branchlets are untreated.

Table 3.10  LD\(_{50}\) and LD\(_{90}\) values for *Laricobius nigrinus* and *Sasajiscymnus tsugae* 6 d after topical application of imidacloprid to the ventral abdomen.

<table>
<thead>
<tr>
<th>Spp(^1)</th>
<th>n</th>
<th>LD(_{50}) (95% CI)</th>
<th>LD(_{90}) (95% CI)</th>
<th>(\chi^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ln</td>
<td>145</td>
<td>1.8 (1.7-133.7)</td>
<td>5.8 (3.2-47.8)</td>
<td>11.10</td>
</tr>
<tr>
<td>St</td>
<td>102</td>
<td>0.71 (0.5-1.7)</td>
<td>1.3 (0.88-3.6)</td>
<td>0.076</td>
</tr>
</tbody>
</table>

\(^1\)Ln = *Laricobius nigrinus*, St = *Sasajiscymnus tsugae*

CI = confidence interval; LD\(_{50}\) and LD\(_{90}\) values are ng imidacloprid/beetle
Table 3.11 Effects of topical application of imidacloprid on *Laricobius nigrinus* and *Sasajiscymnus tsugae* biology 6 d after treatment.

<table>
<thead>
<tr>
<th>Spp</th>
<th>Dose (ng/beetle)</th>
<th>Flip time (s)</th>
<th>Mortality (%)</th>
<th>Intoxicated (%)</th>
<th>Affected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ln</em></td>
<td>0</td>
<td>15.9 ± 3.7 a</td>
<td>0 a</td>
<td>0 a</td>
<td>7 a</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>30.5 ± 4.3 ab</td>
<td>19.4 ab</td>
<td>70 b</td>
<td>70 b</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>32.8 ± 3.5 ab</td>
<td>31.9 abc</td>
<td>60 b</td>
<td>70 b</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>43.6 ± 3.5 bc</td>
<td>46.3 bc</td>
<td>83 c</td>
<td>87 bc</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>59.4 ± 3.5 c</td>
<td>82.1 bc</td>
<td>77 d</td>
<td>100 c</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>60.0 c</td>
<td>100 c</td>
<td>63 cd</td>
<td>100 c</td>
</tr>
<tr>
<td><em>St</em></td>
<td>0</td>
<td>15.1 ± 2.8 A</td>
<td>0 A</td>
<td>0 A</td>
<td>5 A</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>32.4 ± 2.8 B</td>
<td>5.3 AB</td>
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<td>31.6 B</td>
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<td>70 E</td>
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<td></td>
<td>50</td>
<td>60.0 C</td>
<td>100 C</td>
<td>70 E</td>
<td>100 C</td>
</tr>
</tbody>
</table>

1 *Ln = Laricobius nigrinus, St = Sasajiscymnus tsugae*

2 Mean ± standard error. For each species, means within each column followed by the same letter are not significantly different (Tukey’s HSD test, *P* = 0.05).

3 Displayed means are corrected for control mortality. For each species, means within each column followed by the same letter are not significantly different (Tukey’s HSD test, *P* = 0.05).

4 Percent of beetles observed intoxicated at least once during course of the experiment. Differences among treatment ranks were separated with a pairwise comparison using the Wilcoxon signed rank test (*P* = 0.05), ranks not shown.

5 Percent affected (observed intoxicated or dead) during the course of the experiment. Differences among treatment ranks were separated with a pairwise comparison using the Wilcoxon signed rank test (*P* = 0.05), ranks not shown.
Figure 3.1 Hemlock woolly adelgid percent mortality (mean ± se) 30 d after treatment versus mean imidacloprid concentration (ppm) recovered from hemlock branchlets by LC/MS/MS. Concentrations are means from branchlets treated with 1, 10, and 100 ppm imidacloprid. Mortality adjusted for control mortality using Abbott’s formula.
**Figure 3.2** No-choice test comparing mortality and imidacloprid concentrations in hemlock wood tissue from 1 ppm (A) and 100 ppm (B) treatments. Mortality adjusted for control mortality using Abbott’s formula. Imi = imidacloprid; HWA = hemlock woolly adelgid; Ln = *Laricobius nigrinus*; St = *Sasajiscymnus tsugae.*
Figure 3.3 No-choice (A) and choice (B) tests comparing hemlock woolly adelgid and beetle mortality to imidacloprid concentrations recovered from hemlock wood tissue 30 d after treatment. Mortality adjusted for control mortality using Abbott’s formula. Imi = imidacloprid; HWA = hemlock woolly adelgid; Ln = *Laricobius nigrinus*; St = *Sasajiscymnus tsugae*.
Figure 3.4  No-choice (A) and choice (B) test results comparing beetle mortality with imidacloprid concentrations recovered from beetles. Imi = imidacloprid, Ln = *Laricobius nigrinus*, St = *Sasajiscymnus tsugae.*
Figure 3.5 Comparing beetle mortality (lines) with imidacloprid concentrations recovered from beetles (bars) 6 d after topical application of imidacloprid to the ventral abdomen. Imi = imidacloprid, Ln = *Laricobius nigrinus*, St = *Sasajiscymnus tsugae*.
Chapter 4

Impacts of field applications of imidacloprid on hemlock woolly adelgid (Adelges tsugae) and two of its predators, Laricobius nigrinus and Sasajiscymnus tsugae

Abstract
Eastern hemlocks (Tsuga canadensis) at two sites in southwestern Virginia were treated between 2004 and 2006 by trunk and soil injections of imidacloprid to determine the insecticide’s impact on the hemlock woolly adelgid (HWA), Adelges tsugae, and its predators. Treatments were 0 (control), 0.25, 0.5 and 1.0 of the highest labeled dosage rates. Three to four years after treatment the half and full rates had significantly reduced HWA populations, which was accompanied by improved tree vigor with greater production of new shoot growth and higher scores on a visual rating scale. Imidacloprid and metabolite concentrations in wood tissue from treated trees were determined by liquid chromatography-tandem mass spectrometry. HWA populations decreased as imidacloprid concentrations increased in wood tissue. HWA was absent from all trees with imidacloprid concentrations higher than 413 ppb. Olefin, di-hydroxy, and 6-cna were the imidacloprid metabolites recovered in the highest proportions. In 2005, two Coleopteran biological control agents, Laricobius nigrinus and Sasajiscymnus tsugae were significantly affected by intoxication and death after feeding on HWA from hemlocks trunk injected with the quarter rate of imidacloprid one year earlier. In 2006, flip times differed among beetles feeding on HWA-infested trees treated with imidacloprid compared with untreated trees, although intoxication and mortality were not significantly different. Predators feeding on HWA from surviving trees displayed impaired mobility and limited signs of intoxication; however, mortality was not consistent among treatments groups. HWA was very susceptible to imidacloprid, and concentrations in wood tissues from treated trees with no HWA populations were in the lower parts per billion range. In field situations it is more likely that predators will be faced with indirect impacts from treatments rather than direct toxicity.

Keywords: Tsuga canadensis; biological control, nontarget impacts.
4.1 Introduction

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an invasive pest of eastern hemlock (*Tsuga Canadensis* Carriere) and Carolina hemlock (*T. caroliniana* Engelm.) in the eastern U.S. Hemlocks are important components of forest health and have unique roles in the ecology of eastern forests by providing habitat for many important aquatic, terrestrial and avian species (Evans et al. 1995, Yamasaki et al. 1999, McClure et al. 2001).

HWA is spreading throughout the native range of the eastern hemlock. It kills trees by feeding on fluids and nutrients from parenchyma cells at the base of needles (McClure 1987a, 1991b, Shields et al. 1995, Young et al. 1995). Infestation causes needle desiccation and discoloration and can inhibit production of new growth and can kill trees less than 10 years after infestation (McClure 1987a, McClure et al. 2001). HWA has a polymorphic life cycle and completes two generations each year on eastern hemlock (McClure 1987a, 1995). The sistentes, or the overwintering generation, hatch in early summer and undergo aestival diapause, resuming development in the fall and feed throughout the winter (McClure 1987a, McClure et al. 2001, Salom et al. 2001). Sistentes eggs hatch in April-May as one of two types of progeny, either as sexual sexuparae or asexual progregentes. The sexuparae are ecologically insignificant in the eastern U.S. Progregentes feed throughout late spring, their eggs hatch as sistentes in late June (McClure 1989).

A complex of predators associated with HWA in the western U.S., Japan, and China are under evaluation as components of a classical biological control program. *Sasajiscymnuss tsugae* Sasaji and McClure, (Coleoptera: Coccinellidae), is a predator associated with HWA in Japan (Cheah and McClure 1995, Sasaji and McClure 1997) that is amenable to mass rearing in the laboratory (Cheah and McClure 1998). *S. tsugae* displays seasonal synchrony with HWA and was able to overwinter in Connecticut hemlock stands (Cheah and McClure 2000, 2002, Cheah et al. 2005). Over 3,000,000 have been released in eastern forests since 1995 (Cheah and McClure 2002, Cheah et al. 2004, Mausel 2007). *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) is native to the Pacific Northwest of North America, where both adults and larvae have been found in association with HWA on western hemlock species. (McClure 2001, Zilahi-Balogh et al. 2003a). The beetle shows good synchrony with HWA’s lifecycle, and strong host specificity
(Zilahi-Balogh et al. 2002, 2003b). Adults are univoltine and feed on sistentes throughout winter before the females oviposit into adelgid ovisacs in spring. In Virginia, HWA densities on branches where *L. nigrinus* adults were released in cages were lower than HWA densities on control branches, and F2 beetle progeny was recovered in the field one year after release (Lamb et al. 2006). F3 generation progeny was recovered from 13 of 22 sites in eight states in the eastern U.S. (Mausel 2007). Releases through 2008 have brought the total to over 50,000 individuals released at over 80 sites.

While biological control of HWA is a long-term objective, temporary control on individual trees can be realized with chemical applications of foliar sprays and systemic insecticides (McClure 1987, 1991, Steward and Horner 1994, Cowles et al. 2006). Chemical controls are primarily used in ornamental and landscape settings and on high-value hemlock stands in National Forests and State Parks (Rhea 1995, Johnson et al. 2005). Systemic injections of imidacloprid have become the most prevalent treatment method for HWA. Imidacloprid is a neonicotinoid insecticide that has physical characteristics that make it effective as a foliar, seed, or soil treatment against a wide variety of insect pests (Pflüger and Schmuck 1991, Placke and Weber 1993, Silcox 2002) and is especially effective against sucking insects (Elbert et al. 1991, Mizell and Sconyers 1992, Lind et al. 1999).

The Merit® formulation of imidacloprid can be injected into hemlock root systems using a Kioritz® soil injector (Steward et al. 1998, Cowles et al. 2006). Imidacloprid can also be trunk injected using a variety of application systems, including the Mauget® system (J. J. Mauget Company, Arcadia, California), which uses self-pressurized capsules containing a 10 % formulation called Imicide. These products have been used with success against HWA, and both soil and trunk injections have proven efficacious as systemic treatments and have minimal impacts against non-target beneficials. Predators released as biological control agents could be affected directly or indirectly by systemic treatments. Indirect effects would be from changes in HWA density, distribution, and availability as a food source for sustaining predator populations. Direct effects would include exposure to systemic imidacloprid within treated hemlock trees by contact through feeding on affected adelgids. In this study, soil and trunk injections of
imidacloprid using the Mauget and Kioritz systems were evaluated for control of HWA and their impacts on hemlock health and nontarget predators.

4.2 Materials and methods

4.2.1 Imidacloprid impacts on HWA and hemlock health

A. Abingdon, VA

Hemlock Treatments

Eastern hemlocks were selected in the yard of a private homeowner in Abingdon, VA. At the time of treatment all trees appeared to be healthy but infested and had not been treated with any insecticides for HWA or any other pests. Trunk injections consisted of Imicide using the Mauget system (J. J. Mauget Co., Arcadia California). Imicide is a 10% imidacloprid insecticide that is injected from self-pressurized capsules into the xylem at the root flare of each tree. A portable electric drill with a 0.4 cm bit drilled a hole approximately 0.6 – 1.3 cm deep into the xylem. One capsule was placed into each hole. Holes were drilled into control trees, but no capsules were applied. Capsules were left in each tree until empty or until 24 hours had elapsed.

Control, quarter, half and full (highest labeled) rates were applied. For the full rate, one 3 mL capsule was used for each 2.5 cm of dbh. For the half rate, one capsule was applied every 5 cm dbh, and the quarter rate required a capsule for every 10 cm dbh, resulting in the total amount of imidacloprid applied equal to 0.15, 0.075, and 0.038 g for each 2.5 cm of trunk dbh (Table 4.1). When the study began trees were approximately 50-60 years old. Tree dbh ranged from 23 – 64 cm with a mean of 41 cm. Trees were treated with Mauget trunk injections on April 24, 2004. 24 trees were treated at one of four rates resulting in 6 trees per treatment rate. A completely randomized design was employed.

Proportion of shoots infested with HWA

To quantify HWA infestations, the proportion of shoots infested (PSI) with the sistentes generation was measured before treatment and subsequently one year post-treatment in March
2005. PSI is based on a binomial sequential sampling plan (Fidgen et. al. 2006). Fifty shoots of the most recent growth from the distal ends of each of four branches were examined for the presence or absence of HWA, resulting in 200 shoots examined per tree. The PSI was calculated for each tree as the number of infested shoots divided by the total number of shoots examined. Higher proportions correspond to higher adelgid infestation. The four branches were randomly selected from the lower to mid-crown in each of the four cardinal directions. Branches were not included in the analysis if no HWA was present because of an absence of new growth due to branch-tip dieback.

**B. Marion, VA**

*Hemlock Treatments*

Eastern hemlock trees of approximately equal size, health, age, and level of infestation were selected from forested stands in Hungry Mother State Park in Marion, VA in 2004. These trees had never been treated with imidaclorpid or any other insecticides. Of the 32 trees selected, 8 were controls, 12 were soil-injected and 12 were trunk-injected. Study trees were given one of four treatment levels: control, quarter, half or the full (highest) labeled rate.

Soil injections were applied on September 7, 2004. A Kioritz applicator (Kioritz Corp., Tokyo, Japan) was used to inject Merit 75 WP and water into the soil around the base of each hemlock tree. Injection holes spaced evenly less than 30 cm from the base of the tree were made less than 20 cm below the soil surface into the shallow root system for passive uptake. At least four injection holes into the soil were used for each tree. Rates were 1.4, 0.7, and 0.35 g active ingredient (a.i., imidaclorpid) applied for every 2.5 cm of tree diameter at breast height (dbh, 1.4 m) (Table 4.1). The number of injection sites was the same for all treatment rates. On the day of treatment the temperature was 21 °C, and approximately 2.0 cm of rain fell on the site.

Mauget trunk injections were applied on May 11, 2005 using the methodology described previously. On the day of treatment it was sunny and the air temperature was 26 °C. Uptake was generally efficient; 90% of the capsules were empty when removed. At the time of treatment the hemlock tree mean dbh was 32 cm and ranged from 13 to 63 cm.
Proportion of shoots infested with HWA

To quantify HWA infestations, the PSI based on the 2004/2005 sistentes generation was measured before treatment and subsequently each spring post-treatment from 2006-2008.

HWA density

To estimate HWA density before treatment, on May 5, 2007 four branches were cut from the lower crown of each tree at Hungry Mother State Park in Marion, VA. Only branches exhibiting HWA populations were cut. Branches were taken to Virginia Tech in Blacksburg, VA and examined under a microscope. The amount of new growth (cm) on the distal 30 cm section of each branch was recorded, and each adelgid residing on new growth was counted and probed to determine if it was alive or dead. An adelgid that could be observed moving when disturbed or displaying bright and aqueous hemolymph when punctured was noted as alive. Adelgids that were dry or exuded thick black hemolymph when punctured were recorded as dead. The number of live HWA per cm of new growth was calculated for 4 branches from each tree.

New shoot length

In late summer of 2005 following treatment, the length (cm) of new shoots was measured on the distal 30 cm of four randomly selected branches in the lower to mid-crown of each tree. The distal 30 cm section was measured beginning from the distal terminal point of old growth on the central leader, and measured proximally along the main stem. The length of new growth shoots on all branches in the 30 cm section was measured to the nearest cm. The total length of new shoots on the four branches was summed and divided by 4 to give a mean amount of new shoot length for each tree. Measurements were rounded to the nearest cm.

Note: At the time writing, 2008 growth is not yet present in the field, therefore, new growth measurements are through the 2007 season.

Hemlock health ratings

Each spring, trees were visually rated according to a scoring system called hemlock health rating. Five parameters were used to score each tree: percent live branches, percent tips alive, percent new foliage, color, and an overall appearance grade (Table 4.2). Each parameter was worth a maximum of 100 points. The sum of the five parameter scores was divided by 5 to give each
tree a final score on a 0-100 point scale. This rating system is similar to the hemlock vigor index (Mausel 2007).

4.2.2 Imidacloprid wood tissue concentrations and HWA infestation level

Treated trees were used from both field sites in experiments designed to correlate the PSI with imidacloprid concentrations recovered in wood tissue.

At Hungry Mother State Park in Marion, four branches were cut at random from the lower crown of each tree in spring 2007 and 2008, or two and three years post-treatment, respectively. Branches were prepared for liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis.

At the Abingdon field site, trees were re-treated if HWA populations were still prevalent 1 year after the 2004 injections; so 12 trees were re-treated in May 2005 with a subsequent Mauget trunk injection of either quarter or full rate of imidacloprid. In May 2006, 10 trees with remaining HWA populations were treated with a soil injection of Merit 2f at the full rate. In March 2008, four branches were cut from the lower crown of each tree in preparation for LC/MS/MS analysis. Treatment timing, methods, rates and imidacloprid volume applied are listed in Table 4.3.

To extract imidacloprid for LC/MS/MS analysis, hemlock branches were dried in an oven overnight at 40 °C or until needles could easily be removed. Branches were mechanically pulverized with a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) until tissues were able to pass through a 1x1 mm screen. 150 mg of the dried, ground wood tissue were added to a 1.5 mL micro-centrifuge tube. Samples were combined with 1.5 mL of acetonitrile, shaken overnight, and centrifuged at 13,000 x g for 13 min the following day. Supernatant was removed with a 1 mL syringe and filtered through a 0.45 μm PVDF filter before being added to a 1.8 mL autosampler vial to be analyzed by LC/MS/MS by Dr. A. Lagalante at Villanova University in Villanova, PA. The procedure quantifies imidacloprid, and its metabolites olefin, urea, 6-chloronicotinic-acid (6-cna), des-nitro, des-nitro-olefin and dihydroxy metabolites.
4.2.3 Imidacloprid impacts on predators feeding on HWA from treated trees

Experiments were conducted to evaluate any impacts of HWA from field-treated hemlocks on the biology and survivorship of *L. nigrinus* and *S. tsugae* in 2005 and 2006. All beetles used in these experiments came from the following sources. *L. nigrinus* adults were F1 progeny reared from field collected beetles at the Virginia Tech insectary in Blacksburg, VA using the techniques described in Lamb et al. (2005b). *S. tsugae* was reared from adults obtained from Clemson University, South Carolina and the University of Tennessee in Knoxville, TN. Experiments with *L. nigrinus* were kept at 12:9 °C (D:N), 14:10 (L:D) and *S. tsugae* were kept at 20:18 °C (D:N), and 14:10 (L:D).

As the gender of adult *L. nigrinus* beetles cannot be determined by external physical characteristics, *L. nigrinus* was sexed by observation of oviposition after 72 h as reported in Lamb et al. (2005a). *L. nigrinus* beetles determined to be “male” are non-ovipositing individuals. *S. tsugae* adults were observed under a microscope with sexes identified according to distinguishing morphological characteristics as discussed by Cheah and McClure (1998).

A. 2005 Predator Experiments

*L. nigrinus* feeding comparison

Beginning March 14, 2005, 24 branches, 20 cm long, were cut from an untreated hemlock and adelgids were removed by hand until 24 live adelgids remained. Branches were placed in floral foam, and one beetle was placed on each branch. Feeding was compared between two periods. Fifty-six *L. nigrinus* beetles were starved for 12 h and then allowed to feed for 48 h on the untreated foliage (period 1). Branches and beetles were then separated. Fresh branches with surviving HWA were cut from Abingdon trees that had been trunk injected in April 2004 at the quarter rate (Table 4.4). The number of HWA on each branch was standardized at 24. The same individual beetles were then starved again for 12 h. Half fed on HWA from treated branches while the other half fed on HWA from control trees (period 2). Period 2 was 48 h in duration. After each period branches were examined under a microscope for predator eggs. For each
treatment, the difference in the number of HWA consumed and eggs laid between the two periods was compared.

*L. nigrinus and S. tsugae feeding and survivorship*

In 2005, HWA population densities from full and half rate trees trunk injected in 2004 at the Abingdon field site were less than 40 adelgids per 30 cm branch tip. This density was not sufficient for predator experiments. As a result, only quarter rate and control trees from the 2004 treatments were used in these experiments. Three trees were selected from each treatment group and four infested branches from each tree were cut. Branches with moderate to heavy HWA populations were chosen, and adelgids were removed by hand until 24 live adelgids remained. Adelgids were scored as alive if honeydew and healthy white ovisac were present, or if they responded when lightly probed. They were scored as dead if they were dried out, black or did not respond when probed. Dead adelgids were removed by hand. One predator was placed on each branch of 24 adelgids, with four beetles of each species and sex per tree. Beetles were observed every 3 d and rated as healthy, intoxicated, or dead. Beetles were considered “affected” if they died or displayed intoxication symptoms at any point during the experiment. Mortality and affected data were recorded as a binomial response. The experiment concluded after 18 d. Beetles were scored as dead if no response was elicited after probing. Dead beetles were removed and frozen at -60 °C for imidacloprid residue analysis. Intoxicated beetles included individuals with tremors, spasms, or paralysis. Each branch was observed microscopically, and the number of eaten adelgids counted. Adelgids were recorded as eaten if the ovisac appeared at least 50% ravaged and/or if the adelgid was ruptured or desiccated. Predator eggs were counted at each observation.

The experiment was done twice. For *L. nigrinus*, one trial began March 26 and the second beginning April 29, 2005. For *S. tsugae*, the first trial began on April 2 and the second on May 6, 2005. The first and second trials occurred during two generations of the adelgid life cycle, and are referred to as “sistentes” and “progredientes,” respectively.

**B. 2006 Predator Experiments**
**Treatments**

In spring 2006 all treated trees at both field sites in Marion and Abingdon were examined for HWA populations. Most trees did not have many infested branches or HWA populations were not dense enough for predator experiments. As a result, trees in Marion and Abingdon that were treated with imidacloprid in 2005 and 2006 in a similar but unrelated research project were examined for HWA. Two treated trees in Marion and three treated trees in Abingdon had sufficient HWA density and shoots infested for predator experiments and were chosen for incorporation into this study. All five trees had been treated with Merit 2f, a suspension containing a 21.4% concentration of imidacloprid as the active ingredient. Merit 2f and water were injected with the Kioritz soil injector using the basal system technique described previously. 30 ml of Merit 2f was injected for every 2.5 cm of trunk dbh. The high and low labeled rates were used: 0.64 (half rate) or 1.27 (full rate) g of imidacloprid injected for every 2.5 cm of DBH for the two rates, respectively (Table 4.1). The Marion trees were treated on April 6, 2005, and one received half rate and the other full rate. Abingdon trees were treated on October 14, 2005, and all three received the full rate. From all trees treated with these varying rates and methods, five were chosen from the Abingdon field site and six from Marion (Table 4.4). These were the only trees available in 2006 with sufficient HWA densities to use in predator trials.

*L. nigrinus and S. tsugae feeding, mobility and survivorship*

From these trees, the distal 30 cm of 4-10 infested branches from the lower crown of each tree were cut in April, 2006 and brought to Virginia Tech. The infested branches were cut into 10 cm sections, and cut ends were inserted into water-soaked floral foam blocks wrapped in parafilm. *L. nigrinus* experiments began on April 4 and *S. tsugae* on April 11. For each predator species, 20 total beetles were added to branches from each tree. Ten beetles were added to 5 branches and stored in 950 ml polyethylene plastic containers (Trx-Tech Corp., Kent OH). The containers had three ventilation holes (2.5 cm diameter) and a lid with fine mesh (0.14 mm², Sefar America Inc, KC, MO) coverings. This was done in duplicate. Beetles were allowed to feed for 20 d, and each individual was removed every 5 d and examined under a light microscope to measure the following parameters:
Flip time
To measure loss of coordination or mobility due to imidacloprid a flip test based on Bai et al. (2006) was used. An individual beetle was placed in the center of a Petri dish with a 9 cm filter paper and was flipped onto its dorsum with a small paintbrush. The span of time it took each beetle to flip over was measured with a stopwatch to the nearest 0.01 s. Timing ceased at 60 s for beetles that did not right themselves. The amount of time taken by each beetle to right itself was recorded as flip time. Flip time has been used as an indicator to quantify beetle health and mobility (Bai et al. 2006, Smith and Krischik 1999, Lundgren and Wiedenmann 2002, 2005). Each beetle’s flip time was recorded before placement in the experiment and subsequently at each observation event every 5 d. Observations were terminated 20 d after beetles were placed on branches.

Intoxication
Evidence of intoxication from imidacloprid was recorded at each observation and percent affected calculated. Imidacloprid intoxication symptoms include twitching, spasms, or paralysis. An ordinal scoring system rated each beetle according to the degree of intoxication symptoms observed (Table 4.5). Ratings increased in value as intoxication symptoms became more severe.

Mortality
Beetles were labeled as dead when no response could be elicited after probing with a small paintbrush. Percent mortality was calculated for each treatment. Dead beetles were removed and frozen at -60 °C until imidacloprid residue analysis.

4.2.4 Imidacloprid concentrations recovered from beetles

Beetles from each species were pooled by treatment group, and all beetles from each group were weighed and placed in a 1.5 mL plastic micro-centrifuge tube. For extraction, they were frozen in liquid nitrogen, transferred to a glass tube and crushed with a pestle. 0.5 mL of acetonitrile was then added to each sample, and samples were transferred to 1.8 mL auto-sampler vials to be analyzed by the LC/MS/MS procedure described previously.
**Statistical analysis**

Data were analyzed using SPSS® for Macintosh OS X version 11.0.4. When necessary, control mortality was corrected using Abbott’s formula (Abbott, 1925). Proportional data were arcsine square-root transformed before analysis (Zar 1998). Flip times were converted to a proportion by dividing each value by 60, then each datum point was arcsin square-root transformed before analysis (Zar 1998). All reported means are untransformed. For all tests, a significance level of $P = 0.05$ was used to separate means.

For imidacloprid impacts on HWA and hemlock health at each sampling date, the proportion of shoots infested, HWA density, cm of new growth on each tree, and hemlock health ratings were analyzed by application method and treatment rate using two-way analysis of variance (ANOVA). When application method was not significant, data were pooled and analyzed by treatment rate. Means were separated using Tukey’s HSD test. Mean differences were calculated as indicated in the text and analyzed by treatment rate with a one-way ANOVA and mean separation by Tukey’s HSD test. Imidacloprid concentration LC/MS/MS data were corrected by subtracting the control mean concentration from the treatment group mean.

For the 2005 feeding comparison test, the difference in the number of HWA consumed during periods 1 and 2 were compared for each treatment using paired t-test.

2005 predator fitness and survivorship data were analyzed by predator species and HWA generation. The number of HWA eaten and eggs laid by *L. nigrinus* were normally distributed (Levene’s test confirmed the equality of variances), and treatment means were compared by Student’s t-test. Mortality and affected data were binomial so treatment ranks were compared with a Mann-Whitney U test.

For the 2006 predator experiments, the two field sites were analyzed separately, as were the two predator species. For each site-species combination, data were blocked by tree. Differences in flip time between treatments were compared using ANOVA with pair-wise comparisons of
means performed with Tukey’s HSD test. Mortality and intoxication data were binomial, (0 = alive or not intoxicated, 1 = dead or intoxicated) and analyzed using logistic regression.

4.3 Results

4.3.1 Imidacloprid impacts on HWA and hemlock health

A. Abingdon, VA

Proportion of shoots infested with HWA
At the time of treatment in April, 2004 the proportion of shoots infested were not significantly different between treatments ($F = 0.445; \text{df} = 3, 21; P = 0.72$). In March 2005, there was a significant difference between treatments ($F = 3.37; \text{df} = 3, 19; P = 0.0446$) (Figure 4.1).

B. Marion, VA

Proportion of shoots infested with HWA
Application method was not significant in 2005 ($F = 0.188; \text{df} = 3, 29; P = 0.669$), 2006 ($F = 1.67; \text{df} = 1, 32; P = 0.208$), 2007 ($F = 0.919; \text{df} = 1, 28; P = 0.349$), or 2008 ($F = 1.81; \text{df} = 1, 28; P = 0.192$). Data were pooled by treatment method and analyzed by treatment rates. PSI varied among years but treatment effects were significant 2-3 years post-treatment (Figure 4.2). The PSI of the sistentes generation on the trees at the time of treatment in Fall 2004 and Spring 2005 showed a significant difference among treatments ($F = 4.85; \text{df} = 3, 29; P = 0.01$). In the subsequent 2006 generation, however, the HWA population in Hungry Mother State Park saw a substantial reduction and the PSI was low on all trees (mean < 11%), with no significant differences among treatments ($F = 1.16; \text{df} = 3, 32; P = 0.342$). In 2007, the HWA population was still relatively low (mean < 12%) but with significant difference between treatments ($F = 9.13; \text{df} = 3, 28; P < 0.001$). In 2008, HWA populations had increased on all trees, but proportions were higher on control (mean: 78%) trees than quarter rate (38%), half (28%), or full rate trees (4%). Treatments were significantly different ($F = 13.45; \text{df} = 3, 28; P < 0.001$).
HWA density
Two years after treatments, HWA density decreased as treatment rate increased (Figure 4.3). Application methods were not significantly different ($F = 1.85; \text{df} = 1, 25; P = 0.074$). There was a significant difference between treatments ($F = 4.85; \text{df} = 3, 22; P < 0.01$).

New shoot length
Application method did not significantly affect shoot length in 2005 ($F = 0.031; \text{df} = 1, 29; P = 0.86$), 2006 ($F = 13.37; \text{df} = 1, 28; P = 0.10$), and 2007 ($F = 0.028; \text{df} = 1, 29; P = 0.868$) and data were pooled by treatment method and analyzed by treatment rates. New growth length means in all treatments increased each year; however, a treatment effect was not observed until 2007 (Figure 4.4). In 2005, mean new growth from all trees was 10 cm, and in 2006 it had tripled to 29 cm, and increased to 100 cm in 2007. Corresponding new growth of control trees increased from 10.8 cm in 2005 to 22.2 cm in both 2006 and 2007. Full rate trees increased from 7.8 cm in 2005 to 160.5 cm in 2007. No significant differences were observed for treatments in 2005 ($F = 0.133; \text{df} = 3, 29; P = 0.939$) or 2006 ($F = 1.14; \text{df} = 3, 28; P = 0.354$), but treatments were significantly different ($F = 5.94; \text{df} = 3, 29; P = 0.004$) in 2007.

Hemlock health rating
Application method was not significant in any year [2005 ($F = 0.005; \text{df} = 1, 27; P = 0.946$), 2006 ($F = 2.63; \text{df} = 1, 26; P = 0.121$), 2007 ($F = 1.045; \text{df} = 1, 28; P = 0.319$), or 2008 ($F = 0.433; \text{df} = 1, 29; P = 0.517$)], therefore, data were pooled by treatment method and analyzed by treatment rates. Hemlock health ratings were not different across treatments each year until 2008 (Figure 4.5). In 2005, the mean hemlock health rating scores were equal among treatments ($F = 1.5; \text{df} = 3, 27; P = 0.245$). In 2006, ratings were lower than 2005 with no significant treatment differences ($F = 1.259; \text{df} = 3, 26; P = 0.317$). In 2007, mean ratings for half and full rate trees increased while control and quarter rate trees decreased; yet treatment differences were not significant ($F = 1.425; \text{df} = 3, 28; P = 0.265$). In 2008, the mean control rating fell to 61.8, the lowest score observed among all treatments while the full rate trees increased to 88.3, the highest score of all treatment groups during the 4 years. In 2008, dosage treatments were significant ($F$
= 3.646; df = 3, 29; \( P = 0.028 \)). Although full and half rate scores were statistically equal, only the full rate was significantly different from control and quarter rate trees.

### 4.3.2 Imidacloprid wood tissue concentrations and HWA infestation level

At Hungry Mother State Park in Marion, VA, mean concentrations of imidacloprid recovered from each treatment group had a negative correlation with corresponding PSI means in both 2007 (-0.938, Figure 4.6A) and 2008 (-0.811, Figure 4.6B). Imidacloprid concentrations recovered by LC/MS/MS were not significant among treatments in 2007, \( (F = 1.248; \text{df} = 3, 22; \ P = 0.325) \) but were significant in 2008 \( (F = 4.36; \text{df} = 3, 27; \ P = 0.016) \). Application method was not significant for 2007 \( (F = 4.479; \text{df} = 1, 22; \ P = 0.052) \) or 2008 \( (F = 0.821; \text{df} = 1, 27; \ P = 0.376) \).

When imidacloprid concentrations and PSI were pooled over field sites, application methods, rates, and timing, the PSI generally declined as imidacloprid concentrations increased in wood tissues (Figure 4.7). The mean concentration for branches with 0% PSI was 211 ppb and the mean for trees with PSI ≥1% was 41 ppb. Proportions greater than 30% of shoots infested inhibit hemlock growth and represents a physiological injury threshold (Fidgen et al. 2006). Only 3 out of 31 trees with wood tissue concentrations >27 ppb imidacloprid had PSI >30%. The mean imidacloprid concentration from trees with < 30% PSI was 187 ppb.

When comparing all treated tree samples, imidacloprid was the most prevalent of the detected compounds in hemlock wood tissue (85%), followed by the olefin (7%), dihydroxy (5%), and 6-cna (3%) metabolites, respectively. Des-nitro and des-nitro-olefin were also detected but were < 1% of the total metabolites detected. The urea metabolite was not of consequence. Imidacloprid concentrations were generally in the ppb range (50-800 ppb), however, three samples were in the ppm range (4.8, 5.6, and 33 ppm). The median concentration of positive samples was 24 ppb, and the mean (± se) concentration was 360 ± 190 ppb. The maximum metabolite concentrations detected from all tree tissue samples were 912 ppb (olefin), 104 ppb (6-cna) and 36 ppb (dihydroxy).
4.3.3 Imidacloprid impacts on predators feeding on HWA from treated trees

A. 2005 Predator experiments

*L. nigrinus feeding comparison*

*L. nigrinus* fed on HWA adults in paired feeding periods. Paired t-tests indicated no differences in adelgids eaten on control branches between the two periods. The number of HWA consumed increased significantly in the treated (quarter rate) branches during the second feeding period (Table 4.6). There was no mortality during this trial.

*L. nigrinus and S. tsugae feeding and survivorship*

*L. nigrinus* consumed more HWA on quarter branches than on control branches, but differences were not significant (Table 4.7). *L. nigrinus* oviposition varied, but differences were not significant. More eggs were laid during the sistentes generation than the progredientes generation. *S. tsugae* consumed significantly more adelgids on control branches during the sistentes generation, but not for the progredientes generation (Table 4.7).

No predators died while feeding on HWA sistentes from control branches (Table 4.8). *L. nigrinus* exhibited mortality on all quarter rate branches. A significantly greater number of *L. nigrinus* were affected on the quarter rate branches compared with the control branches during the sistentes generation. *S. tsugae* mortality on quarter rate branches was significantly higher than control branches during the sistentes generation. Affected *S. tsugae* were significantly higher on the quarter rate than control branches, and all died subsequently. The high mortality of *S. tsugae* feeding on control progredientes negated any treatment effects.

B. 2006 Predator experiments

Pre-trial flip tests of both species represented are shown in Figure 4.8. All beetles took less than 30 s to right themselves. The mean (± standard error) number of seconds it took each beetle to right itself was 6.6 ± 0.36 and 4.5 ± 0.29 s for *S. tsugae* and *L. nigrinus*, respectively. Beetle flip times, percent observed intoxicated, and percent mortality were compared by grams
imidacloprid applied per 2.5 cm dbh for each species/field site combination (Figures 4.9 and 4.10). These same dependant variables were also compared by each tree for all species/field site combinations (Tables 4.9 and 4.10).

**Flip Time**

There were no significant differences in flip time means on day 0 for beetles added to each treatment (S. tsugae Marion: $F = 1.18; \text{df} = 4, 112; P = 0.320$, S. tsugae Abingdon: $F = 2.09; \text{df} = 4, 94; P = 0.088$, L. nigrinus Marion: $F = 2.075; \text{df} = 5, 114; P = 0.0736$, L. nigrinus Abingdon: $F = 0.679; \text{df} = 4, 95; P = 0.608$).

After 5 d, a two-way ANOVA (time, treatment) determined that there were no significant effects of time on beetle flip times (S. tsugae Marion: $F = 0.91; \text{df} = 3, 370; P = 0.437$, S. tsugae Abingdon: $F = 0.53; \text{df} = 3, 249; P = 0.660$, L. nigrinus Marion: $F = 0.98; \text{df} = 3, 367; P = 0.400$, L. nigrinus Abingdon: $F = 5.12; \text{df} = 3, 299; P = 0.198$), so data from each day were analyzed together.

One-way ANOVA for each site-species combination revealed some significant differences between treatments (S. tsugae Abingdon: $F = 5.22; \text{df} = 4, 309; P = 0.004$, L. nigrinus Marion: $F = 6.28; \text{df} = 5, 386; P < 0.001$, L. nigrinus Abingdon: $F = 3.33; \text{df} = 4, 263; P = 0.0111$). However, no significant differences were observed for S. tsugae on branches from Marion ($F = 2.17; \text{df} = 5, 379; P = 0.0568$). For S. tsugae beetles on branches from Abingdon, flip time means from only one treatment was significantly different from controls. For L. nigrinus beetles, flip time means from three treatments at Marion and only one treatment at Abington were significantly different from controls.

**Intoxication**

Intoxication was not significantly different among treatments (S. tsugae Marion: Wald $\chi^2 = 1.26; \text{df} = 1; P = 0.261$, S. tsugae Abingdon: Wald $\chi^2 = 0.277; \text{df} = 1; P = 0.598$, L. nigrinus Marion: Wald $\chi^2 = 0.324; \text{df} = 1; P = 0.569$, L. nigrinus Abingdon: Wald $\chi^2 = 0.286; \text{df} = 1; P = 0.593$). S. tsugae intoxication was relatively low, with 6% intoxication being the highest. L. nigrinus
intoxication was higher when feeding on treated branches, however the difference was not significant (Figures 4.9 and 4.10).

Mortality
Survival was not significantly different among treatments (S. tsugae Marion: Wald $\chi^2 = 0.027$; df = 1; $P = 0.871$; S. tsugae Abingdon: Wald $\chi^2 = 0.535$; df = 1; $P = 0.464$; L. nigrinus Marion: Wald $\chi^2 = 0.176$; df = 1; $P = 0.675$; L. nigrinus Abingdon: Wald $\chi^2 = 1.39$; df = 1; $P = 0.238$). S. tsugae mortality was relatively low in all treatments; highest mortality was from beetles feeding on a control tree (19%). L. nigrinus mortality was higher than S. tsugae, but mortality did not have a strong relationship with treatment (Figures 4.9 and 4.10).

4.3.4 Imidacloprid concentrations recovered from beetles

Imidacloprid was detected in L. nigrinus cadavers from 3 treatments. Concentrations ranged from 6.7 - 151 ppb. Imidacloprid was detected in three S. tsugae groups, with concentrations ranging from 6.6 ppb - 9.5 ppm. No metabolites were detected in any beetles.

4.4 Discussion

Trunk and soil injections of imidacloprid were efficient in controlling HWA in the field in southwestern VA, although treatment effects were not significant until two or three years after application. The half rate did not reduce HWA populations as efficiently as the full rate, but PSI levels were below the 30% threshold. The quarter rate reduced HWA in some cases, but results were much more variable than the half or full rate. Observations of quarter rate trees indicated that large sections of the tree were not-infested, but some branches scattered through the canopy had dense populations. Incomplete distribution of imidacloprid could explain this patchiness, as some sections of the crown may have received imidacloprid while other sections did not. HWA surviving on treated trees may have survived because of the low incidence of imidacloprid, as the majority of trees exhibiting a PSI > 30% had less than 27 ppb in their wood tissues.
Mauget trunk injections worked as well as soil injections, in contrast to a previously published report in Connecticut that June and October trunk applications were less efficient in HWA control than soil treatments (Cowles et al. 2006). In this study applications were made in April during warm, sunny, breezy days bracketed by rainfall. The efficacy of trunk injections may be increased when they are performed on days when trees are transpiring high volumes of water. Successful uptake using trunk injection depends upon the volume of material injected, plant species, environmental conditions, tree health and size, water movement and availability, and the molecular properties of the product injected (Sánchez-Zamora and Fernández-Escobar 2000, 2004). Success is more likely on healthy trees transpiring large volumes of water during treatment, which would promote uptake and distribution of the insecticide.

Stem injections work more quickly and require less pesticide volume than soil injection because they are injected directly into the vascular system. For instance, Tattar et al. (1998) injected imidacloroprid in June using both trunk injections with the Mauget® system and soil injections with a hydraulic sprayer. They found that soil-injected imidacloroprid took 8-12 weeks to reach a concentration of approximately 0.15 ppm in eastern hemlock foliage, while trunk-injected imidacloroprid only took 4 weeks to reach the same concentrations. The capsules were left on the trees up to 24 h which is a longer amount of time than is often feasible in many treatment environments or situations because of labor limitations or concerns over possible non-target impacts on humans or pets in a landscape environment. One drawback to stem injection is damage to the tree by drilling into or puncturing the bark or vascular bundle, which can lead to wounding and girdling (Doccola et al. 2003, Smith and Lewis 2005). Each time a tree is trunk injected a small hole must be drilled into the trunk. This stresses the trees, although more research is needed to clarify hemlock wound response.

Soil injection is efficient but slower than trunk injections. Soil treatment success is dependant upon soil type, moisture levels, and tree root system efficiency and health. Imidacloroprid residues in soils after treatment harms non-target organisms such as earthworms, Eisenia fetida Savigny, (Kreutzweiser et al. 2008) or could potentially affect soil aestivation of beneficials such as L. nigrinus.
Systemic imidacloprid can be metabolized by plants, resulting in metabolites that can provide complementary control and residual activity alongside the parent compound (Nauen et al. 1998, 1999). In cotton plants, metabolites have shown insecticidal properties against aphids, sometimes with properties more toxic than imidacloprid itself and protecting the plant even after parent compound concentrations declined (Nauen et al. 1998b). Oral ingestion of the olefin metabolite was ten times more active than imidacloprid against a susceptible strain of cotton whiteflies, *Bemisia tabaci* Gennadius (Nauen et al. 1999). More research is needed to elucidate the metabolic pathways of imidacloprid in eastern hemlocks. Since olefin was the primary metabolite recovered, one possible metabolic pathway eastern hemlocks may utilize is the hydroxylation of the imidazolidine component of the imidacloprid molecule into the 5-hydroxyl metabolite which, after elimination of water, is converted to olefin. This is a common pathway of metabolization in a variety of plant species (Sur and Stork 2003). If olefin is a primary byproduct of imidacloprid metabolism in hemlocks, this could provide additional protection as metabolism leads to bioactivation of the parent into a more toxic compound.

All branches used in these trials were cut from the lower crown of each tree and it is unclear whether treatment effects would be more or less pronounced on predators feeding on adelgids at different crown levels within each tree. The branches selected were chosen because they had high densities of surviving adelgids and were easily accessible. Because adelgids are extremely sensitive to imidacloprid (field LC$_{50}$ of 120-600 ppb, Cowles et al. 2006), the fact that adelgids are surviving on these branches could suggest that very low or no concentrations of imidacloprid were present in the microenvironment where the adelgids were feeding.

Mortality patterns differed between the two beetle species. In 2005, mortality during the sistentes trial had no mortality in controls and higher mortality in treated branches. This pattern continued in the progradientes generation for *L. nigrinus*, but not for *S. tsugae* where control mortality dramatically increased. In 2006, *L. nigrinus* exhibited 6-26% mortality after 20 d among all treatment groups, while *S. tsugae* mortality was only observed in the two controls and in one of the Kioritz treatments at Abingdon. The higher mortality in *L. nigrinus* could be attributed to the fact that *L. nigrinus* beetles had emerged in Fall 2005 and were older than the *S. tsugae* beetles which had pupated only a few weeks before these trials began. If mortality is due to contact with
imidacloprid residues, differences in beetle behavior could affect survivorship. For instance, *L. nigrinus* females lay their eggs within HWA ovisacs, while *S. tsugae* females lay their eggs on branches and twigs. Increased contact with adelgids and egg masses could expose *L. nigrinus* adults to insecticide residues that *S. tsugae* adults would not necessarily be exposed to. Control mortality in both species could result from increased pressure of starvation, as adelgids inhabiting imidacloprid free branches would be eaten in greater quantity. In treated branches, the presence of imidacloprid could result in lower quality adelgids, and less feeding by the predators, leading to starvation.

More beetles of both species were observed with intoxication symptoms on branches from treated trees although a high incidence of intoxication symptoms did not always result in higher mortality. Longer beetle flip times were more prevalent in treated trees, but longer flip times also did not appear linked with mortality.

Imidacloprid was only recovered from beetle cadavers from three treatment groups from both predator species. The lack of imidacloprid and metabolite detection from beetle cadavers suggests that either concentrations were below the detection limit of the LC/MS/MS procedure, or no insecticide was present in the cadavers. If imidacloprid did not directly affect the predators then beetle mortality would likely be indirect due to poor nutritional HWA quality or from natural mortality.

In conclusion, HWA infestations were efficiently controlled with the labeled rate of both trunk and soil applications of imidacloprid. The scarcity of HWA infesting trees treated at one half the labeled rate suggests that HWA is unlikely to infest treated trees in the field at least 1-3 years after treatment. Concentrations > 27 ppb imidacloprid in wood tissue can inhibit HWA infestations below an injury threshold of 30% infestation, and reduce adelgid density on infested branches. Based on this and the lack of consistent mortality of the two predator species, it seems unlikely that imidacloprid poses a threat to non-target predators in the field. These data suggest that if impacts on predators exist, it is more likely from a lack of quality food caused by reduced HWA populations.
**Table 4.1** Mass (g) of imidacloprid applied for every 2.5 cm of tree diameter at breast height for each combination of treatment rate/method/formulation.

<table>
<thead>
<tr>
<th>Treatment Rate</th>
<th>Mauget trunk injection</th>
<th>Kioritz soil injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full</td>
<td>0.15</td>
<td>1.05</td>
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<tr>
<td>Half</td>
<td>0.075</td>
<td>0.45</td>
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<tr>
<td>Quarter</td>
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<td>0.23</td>
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<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 4.2** Scoring system for hemlock health ratings. Each parameter category is worth up to 100 points. Parameter scores are summed and divided by 5 to give each tree a score ranging from 0-100 points. Higher scores correspond with healthier trees.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Scoring system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live branches (%)</td>
<td>0-100 points</td>
</tr>
<tr>
<td>Tips alive (%)</td>
<td>0-100 points</td>
</tr>
<tr>
<td>New foliage (%)</td>
<td>0-100 points</td>
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<tr>
<td>Color</td>
<td>Dark green = 100 points</td>
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<tr>
<td></td>
<td>Yellow = 50 points</td>
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<tr>
<td></td>
<td>Grey = 0 points</td>
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<tr>
<td>Overall appearance</td>
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<tr>
<td></td>
<td>Fair = 66 points</td>
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<td></td>
<td>Poor = 33 points</td>
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<td></td>
<td>Dead = 0 points</td>
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Table 4.3 Timeline for 2004-2006 eastern hemlock treatments in Abingdon, VA and imidacloprid recovery by LC/MS/MS in 2008.

<table>
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<tr>
<th>Tree&lt;sup&gt;a&lt;/sup&gt;</th>
<th>April 2004</th>
<th>Rate</th>
<th>Method</th>
<th>May 2005</th>
<th>Rate</th>
<th>Method</th>
<th>May 2006</th>
<th>Rate</th>
<th>Method</th>
<th>Imi. applied&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>F</td>
<td>K</td>
<td>1.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F = full rate, H = half rate, Q = quarter rate, C = control.
M = Mauget trunk injection, K = Kioritz soil injection.

<sup>a</sup> Tree number is arbitrary and is provided as a reference.

<sup>b</sup> Total grams of imidacloprid applied over three years per 2.5 cm of tree diameter at breast height.
Table 4.4 Treatment timing and methodology for eastern hemlock trees in Abingdon and Marion, VA. Surviving hemlock woolly adelgid from these trees were used in 2005 and 2006 predator feeding trials.

<table>
<thead>
<tr>
<th>Predator experiment year&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Field site</th>
<th>Application method</th>
<th>Treatment rate</th>
<th>Imidacloprid per 2.5 cm&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Treatment date</th>
<th>Years after treatment&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005 Abingdon</td>
<td>Control</td>
<td>Mauget Quarter</td>
<td>0.038</td>
<td>4/24/2004</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2006 Abingdon</td>
<td>Control</td>
<td>Mauget Full</td>
<td>0.15</td>
<td>4/24/2004</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2006 Abingdon</td>
<td>Control</td>
<td>Kioritz Full</td>
<td>1.27</td>
<td>10/14/2005</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>2006 Marion</td>
<td>Control</td>
<td>Kioritz Full</td>
<td>1.27</td>
<td>10/14/2005</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>2006 Marion</td>
<td>Control</td>
<td>Mauget Quarter</td>
<td>0.038</td>
<td>5/11/2005</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2006 Marion</td>
<td>Control</td>
<td>Mauget Half</td>
<td>0.075</td>
<td>5/11/2005</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2006 Marion</td>
<td>Control</td>
<td>Mauget Full</td>
<td>0.15</td>
<td>5/11/2005</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2006 Marion</td>
<td>Control</td>
<td>Kioritz Half</td>
<td>0.64</td>
<td>4/6/2005</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2006 Marion</td>
<td>Control</td>
<td>Kioritz Full</td>
<td>1.27</td>
<td>4/6/2005</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Calendar year the predator experiment was carried out.

<sup>b</sup> Total grams of imidacloprid applied per 2.5 cm of tree diameter at breast height.

<sup>c</sup> Years after treatment that surviving HWA from tree were fed to predators. Rounded to the nearest year or half year.

Table 4.5 Fitness scale for predator beetles feeding on hemlock woolly adelgid-infested branches treated with imidacloprid.

<table>
<thead>
<tr>
<th>Rating</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beetle behavior</td>
<td>Alive, healthy Mobile but twitching Mobile with severe tremors Immobile with severe tremors Paralyzed, unable to right themselves Dead</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6  Hemlock woolly adelgid adults consumed by *Laricobius nigrinus* in 2005 feeding comparison test. In each period, individual beetles fed for 48 h on a hemlock branch infested with 24 adelgids. Trees treated in 2004 by Mauget trunk injection. Adelgids are from untreated trees in period 1 and untreated or quarter rate trees in period 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HWA eaten (mean ± se)</th>
<th>Difference (mean ± se)</th>
<th>t statistic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.7 ± 0.4</td>
<td>3.2 ± 0.4</td>
<td>0.42 ± 0.5</td>
<td>0.87</td>
</tr>
<tr>
<td>Quarter</td>
<td>2.7 ± 0.3</td>
<td>3.8 ± 0.4</td>
<td>1.1 ± 0.5</td>
<td>2.31</td>
</tr>
</tbody>
</table>

*All branches during period 1 were not treated with imidacloprid. A paired t-test compared the total HWA eaten between the two feeding periods.*
Table 4.7 Comparison of the number of *Laricobius nigrinus* and *Sasajiscymnus tsugae* feeding and oviposition on HWA infested branches from control or quarter-rate dosages of imidacloprid treatments on eastern hemlocks in 2005. Trees were treated in 2004 by Mauget trunk injection.

<table>
<thead>
<tr>
<th>Predator species</th>
<th>HWA generation</th>
<th>Dependant variable</th>
<th>Mean ± se</th>
<th>t</th>
<th>P</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. nigrinus</em></td>
<td>Sistentes</td>
<td>HWA eaten</td>
<td>9.7 ± 0.5</td>
<td>-1.77</td>
<td>0.083</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Progredientes</td>
<td></td>
<td>5.3 ± 0.3</td>
<td>-0.171</td>
<td>0.865</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Sistentes</td>
<td>Eggs laid</td>
<td>8.7 ± 0.6</td>
<td>0.961</td>
<td>0.342</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Progredientes</td>
<td></td>
<td>2 ± 1.5</td>
<td>-2.03</td>
<td>0.064</td>
<td>13</td>
</tr>
<tr>
<td><em>S. tsugae</em></td>
<td>Sistentes</td>
<td>HWA eaten</td>
<td>12.3 ± 0.7</td>
<td>2.03</td>
<td>0.048</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Progredientes</td>
<td></td>
<td>12.3 ± 1.2</td>
<td>0.75</td>
<td>0.457</td>
<td>24</td>
</tr>
</tbody>
</table>

HWA = hemlock woolly adelgid, *Adelges tsugae*; Quarter rate = 0.038 g imidacloprid per 2.5 cm of tree diameter at breast height.

A Student’s t-test compared differences between control and quarter rates.

Table 4.8 Comparison of proportion survivorship of *Laricobius nigrinus* and *Sasajiscymnus tsugae* on branches from control or quarter-rate dosages of imidacloprid treatments on eastern hemlocks in 2005. Trees were treated in 2004 by Mauget trunk injection.

<table>
<thead>
<tr>
<th>Predator species</th>
<th>HWA generation</th>
<th>Dependant variable</th>
<th>Percent mean ± se</th>
<th>Z</th>
<th>P</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. nigrinus</em></td>
<td>Sistentes</td>
<td>Mortality</td>
<td>0</td>
<td>7 ± 5</td>
<td>-1.38</td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>Progredientes</td>
<td></td>
<td>4 ± 4</td>
<td>13 ± 7</td>
<td>-1.03</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Sistentes</td>
<td>Affected</td>
<td>0</td>
<td>21 ± 8</td>
<td>-2.48</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>Progredientes</td>
<td></td>
<td>8 ± 6</td>
<td>25 ± 9</td>
<td>-1.53</td>
<td>0.13</td>
</tr>
<tr>
<td><em>S. tsugae</em></td>
<td>Sistentes</td>
<td>Mortality</td>
<td>0</td>
<td>15 ± 7</td>
<td>-2.06</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>Progredientes</td>
<td></td>
<td>17 ± 8</td>
<td>13 ± 7</td>
<td>-0.35</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>Sistentes</td>
<td>Affected</td>
<td>0</td>
<td>15 ± 7</td>
<td>-2.06</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td>Progredientes</td>
<td></td>
<td>29 ± 10</td>
<td>22 ± 9</td>
<td>-0.58</td>
<td>0.56</td>
</tr>
</tbody>
</table>

HWA = hemlock woolly adelgid, *Adelges tsugae*; Quarter rate = 0.038 g imidacloprid per 2.5 cm of tree diameter at breast height; Affected beetles were dead or displayed intoxication symptoms during trial.

A Mann-Whitney U test compared proportions between control and quarter rates.
Table 4.9 2006 *Laricobius nigrinus* survivorship and health after feeding on hemlock woolly adelgid infested branches from imidacloprid-treated eastern hemlocks at two field sites in southwestern VA.

<table>
<thead>
<tr>
<th>Field site</th>
<th>Treatment method</th>
<th>Treatment rate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mortality&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>Intoxicated&lt;sup&gt;c&lt;/sup&gt; (%)</th>
<th>Flip time&lt;sup&gt;d&lt;/sup&gt; (s)</th>
<th>Flip time n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abingdon</td>
<td>Control</td>
<td>-</td>
<td>23</td>
<td>0</td>
<td>5.9 ± 1.9 a</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Mauget</td>
<td>0.15</td>
<td>17</td>
<td>11</td>
<td>12.7 ± 4.7 b</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>1.27</td>
<td>25</td>
<td>0</td>
<td>5.4 ± 2.4 ab</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>1.27</td>
<td>20</td>
<td>0</td>
<td>7.0 ± 3.3 ab</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>1.27</td>
<td>16</td>
<td>11</td>
<td>7.0 ± 2.3 a</td>
<td>65</td>
</tr>
<tr>
<td>Marion</td>
<td>Control</td>
<td>-</td>
<td>17</td>
<td>0</td>
<td>7.3 ± 2.4 a</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Mauget</td>
<td>0.038</td>
<td>26</td>
<td>32</td>
<td>23.1 ± 6.2 b</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Mauget</td>
<td>0.075</td>
<td>26</td>
<td>37</td>
<td>23.6 ± 6.2 b</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Mauget</td>
<td>0.15</td>
<td>21</td>
<td>16</td>
<td>17.3 ± 5.1 ab</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>0.64</td>
<td>16</td>
<td>10</td>
<td>19.0 ± 5.1 b</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>1.27</td>
<td>6</td>
<td>8</td>
<td>13.8 ± 3.6 ab</td>
<td>70</td>
</tr>
</tbody>
</table>

<sup>a</sup> Grams imidacloprid applied per 2.5 cm dbh.

<sup>b</sup> Mortality after 20 days of feeding.

<sup>c</sup> Total number of beetles observed with intoxication symptoms (1-4 rating) throughout the 20 day trial.

<sup>d</sup> Means ± standard error. Means with same letter are not significantly different (Tukey’s HSD test, P = 0.05).
Table 4.10 *Sasajiscymnus tsugae* survivorship and health after feeding on hemlock woolly adelgid infested branches from imidacloprid-treated eastern hemlocks at two field sites in southwestern VA.

<table>
<thead>
<tr>
<th>Field site</th>
<th>Treatment method</th>
<th>Treatment rate$^a$</th>
<th>Mortality$^b$ (%)</th>
<th>Intoxicated$^c$ (%)</th>
<th>Flip time$^d$ (s)</th>
<th>Flip time n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abingdon</td>
<td>Control</td>
<td>-</td>
<td>7</td>
<td>0</td>
<td>6.5 ± 1.3 ab</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Mauget</td>
<td>0.15</td>
<td>0</td>
<td>4</td>
<td>10.6 ± 2.9 abc</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>1.27</td>
<td>0</td>
<td>3</td>
<td>10.5 ± 2.9 ab</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>1.27</td>
<td>0</td>
<td>0</td>
<td>9.3 ± 1.8 ab</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>1.27</td>
<td>13</td>
<td>6</td>
<td>16.2 ± 4.6 c</td>
<td>64</td>
</tr>
<tr>
<td>Marion</td>
<td>Control</td>
<td>-</td>
<td>19</td>
<td>2</td>
<td>8.5 ± 2.7 a</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Mauget</td>
<td>0.038</td>
<td>0</td>
<td>3</td>
<td>12.9 ± 3.7 a</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Mauget</td>
<td>0.075</td>
<td>0</td>
<td>0</td>
<td>8.3 ± 2.3 a</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Mauget</td>
<td>0.15</td>
<td>0</td>
<td>5</td>
<td>14.4 ± 3.9 a</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>0.64</td>
<td>0</td>
<td>2</td>
<td>9.6 ± 2.7 a</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Kioritz</td>
<td>1.27</td>
<td>0</td>
<td>1</td>
<td>9.6 ± 2.4 a</td>
<td>69</td>
</tr>
</tbody>
</table>

$^a$ Grams imidacloprid applied per 2.5 cm dbh.

$^b$ Mortality after 20 days of feeding.

$^c$ Total number of beetles observed with intoxication symptoms (1-4 rating) throughout the 20 day trial.

$^d$ Means ± standard error. Means with same letter are not significantly different (Tukey’s HSD test, $P = 0.05$).
Figure 4.1  Proportion of hemlock shoots infested with HWA in April 2004 and May 2005 in Abingdon, Virginia. Trees were treated by trunk injection in April 2004. For each year, means (± se) with same letter are not significantly different (Tukey’s HSD test, $P = 0.05$).
Figure 4.2 Proportions of hemlock shoots infested with hemlock woolly adelgid in Marion, Virginia. Trees were treated with imidacloprid using Mauget trunk injections of Imicide or Kioritz soil injections of Merit 75 WP. Soil and trunk injections were in fall 2004 and spring 2005, respectively. Means with same letter are not significantly different (Tukey’s HSD test, $P = 0.05$).
Figure 4.3 Mean (± se) number of HWA density (# alive per cm new growth) on eastern hemlocks 2 years after treatment in Marion, Virginia. Means with same letter are not significantly different (Tukey’s HSD test, $P = 0.05$).
Figure 4.4 Mean (± se) length (cm) of new shoots per eastern hemlock branch in Marion, Virginia. Trees were treated with imidacloprid using Mauget trunk injections of Imicide or Kioritz soil injections of Merit 75 WP. Soil and trunk injections were made in fall 2004 and spring 2005, respectively. For each year, means with same letter are not significantly different (Tukey’s HSD test, \( P = 0.05 \)).
Figure 4.5 Mean (± se) hemlock health ratings in Marion, Virginia. Trees were treated with imidacloprid using Mauget trunk injections of Imicide or Kioritz soil injections of Merit 75 WP. Soil and trunk injections were in fall 2004 and spring 2005, respectively. For each year, Means with same letter are not significantly different (Tukey’s HSD test, $P = 0.05$).
Figure 4.6 Mean (± se) concentration of imidacloprid recovered from hemlock wood tissue in Marion, Virginia in 2007 (A) and 2008 (B) compared with mean proportion of shoots infested with hemlock woolly adelgid.
Figure 4.7 Comparison of the proportion of hemlock shoots infested with hemlock woolly adelgid with imidacloprid concentrations recovered in 2008 wood tissue from the same tree. Each point on x axis is a hemlock tree. Trees were treated with varying rates, methods, and timing. Field sites, rates, and application methods were pooled in order to show the relationship between imidacloprid concentrations in wood tissue with corresponding hemlock woolly adelgid populations.
Figure 4.8 Histograms of *L. nigrinus* and *S. tsugae* flip times before placement in no-choice and choice experiments in 2006.
Figure 4.9 *L. nigrinus* response after feeding on hemlock woolly adelgid surviving on treated eastern hemlocks from Abingdon (A) and Marion (B) field sites. Each datum point is the mean from its x axis category.
Figure 4.10 *S. tsugae* response after feeding on hemlock woolly adelgid surviving on treated eastern hemlocks from Abingdon (A) and Marion (B) field sites. Each datum point is the mean from its x axis category.
Chapter 5

Application of high performance thin-layer chromatography (HPTLC) for imidacloprid detection in eastern hemlock (Tsuga canadensis) tissues

Abstract
High performance thin-layer chromatography (HPTLC) is a relatively simple and rapid tool for imidacloprid detection and quantification in eastern hemlock wood tissues. This method is accurate and repeatable, and can be advantageous compared with other methods such as ELISA because hemlock wood tissue extract does not interfere with analysis. The limit of detection of imidacloprid in hemlock wood tissue is 2.1 ng/µl. Although the technique is efficient, it may not be sensitive enough to quantify imidacloprid from field-treated eastern hemlock trees.

Keywords: Hemlock woolly adelgid, Adelges tsugae, chemical control.
5.1 Introduction

Imidacloprid is the primary insecticide applied to protect eastern hemlock, *Tsuga canadensis* (L.) Carriere trees from the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae) trees in forest and urban landscapes in the eastern United States. Trunk and soil injections of imidacloprid are the most widely used chemical control methods for HWA infestation (Cowles et al. 2006).

High performance thin-layer chromatography (HPTLC) is a technique that is inexpensive, rapid (< 1 hour), and requires a relatively small amount of solvent to perform. HPTLC has been used to quantify imidacloprid from liver and crop samples from birds (Berny et al. 1999) and Chinese cabbage, *Brassica rapa* (Cao et al. 2005).

Thin-layer chromatography (TLC) was used for purification of avocado tree tissue samples before ELISA analysis (Byrne et al. 2007) and for qualitative analysis of imidacloprid residues in date palm, *Phoenix dactylifera*, before HPLC analysis (Kaakeh 2006). TLC has also been used to quantify radiolabeled (14C) imidacloprid in sugar beets, *Beta vulgaris* L. (Westwood et al. 1998), whiteflies, *Bemisi tabaci* Gennadius (Homoptera: Aleyrodidae) (Rauch and Nauen 2003), and honey bees, *Apis mellifera* L. (Hymenoptera: Apidae) (Suchail et al. 2004). Currently, no standardized method of extracting, qualifying, or quantifying imidacloprid from hemlock tree tissues exists. Enzyme linked immunoassays (ELISA) have been used to quantify imidacloprid from treated hemlock trees, however, inherent properties of hemlock extracts cause non-specific binding that can interfere with ELISA kit properties (Cowles et al. 2006). A flow injection analysis has been used to quantify imidacloprid from hemlock xylem fluid but not wood tissues (Lagalante and Greenbacker 2007). To date no method has been reported describing HPTLC analysis of imidacloprid in eastern hemlock tissues. The purpose of this study was to validate a simple analytical technique using HPTLC for imidacloprid in hemlock tissues.

5.2 Materials and methods

Reagents and standards
A stock solution of imidacloprid (1 mg/ml) was prepared with imidacloprid (Bayer Crop Science, Kansas City, MO 99.2% purity) in methanol (HPLC grade, Fischer Scientific). Stock was diluted with methanol for standard solutions.

**Instrumentation**

HPTLC 10 cm x 10 cm 60 F_{254} silica gel plates (Merck Darmstadt, Germany) were washed with chloroform-acetone-methanol (23:1:1, v/v/v) solvent. Plates were then dried at 120 °C for 60 min. Standards and samples were spotted 1.2 cm from the bottom of each plate and 10 mm apart in 1 µl aliquots using a Camag Nanomat II (Camag, Switzerland). Nine spots were applied per plate. After drying at 120 °C for 5 min, plates were placed in twin-trough elution chambers (Camag). 7 mL of the chloroform-acetone-methanol solvent were applied to one trough in the chamber and the plate was placed in the empty trough to allow vapor saturation before elution. After 30 min, the plate was moved to the trough containing solvent for the mobile phase. Elution required less than 40 min, and migration distance was approximately 8-8.5 cm. After elution, plates were removed and dried for 10 minutes at 120 °C. Spot absorbance was measured with a Camag II densitometer with a deuterium lamp at 275 nm. Densitograms were analyzed with a Camag SP 4270 integrator. The absorbance area for each spot was plotted against its imidacloprid concentration (ng/spot) to yield a standard curve. Concentrations were determined by their x axis intercept based on the regression line slope.

**Standard curve**

From the stock solution, concentrations of 1-1000 ng/spot were spotted to establish a standard curve. Inter-day precision was calculated by independently prepared standards spotted in duplicate on individual plates over three days. Applications were 1 µl of 100 µg/g concentration and resulted in 100 ng imidacloprid applied per spot.

**Spikeovers with hemlock extract**

Eastern hemlock branches were cut from trees in Montgomery County, Virginia, in spring 2005 and 2006. All trees were in a forested environment and had never been treated with imidacloprid. Branches were dried in an oven at 40 °C for 24 h or until needles could easily be removed. Needles were separated from wood tissue and discarded. Dried wood was ground
using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA), which mechanically pulverizes the tissue until it is able to pass through a 1x1 mm screen. 100 mg of dried wood tissue was added to 1.5 mL micro-centrifuge tubes.

Imidacloprid stock solution was diluted with methanol in 10-fold increments, resulting in 100, 10, and 1 µg/ml imidacloprid. 1 ml of each dilution was added to each 100 mg wood sample which was equal to 10, 1 and 0.1 µg imidacloprid per gram of wood tissue, respectively. Samples were sonicated for 10 min and shaken overnight at room temperature. The following day samples were sonicated for 10 min and centrifuged at 10,000 x g for 10 min. Supernatant was removed in 1 µl increments and spotted.

*Spiked eastern hemlock branch samples*

Eastern hemlock branches infested with HWA were cut from trees in Montgomery County, Virginia, beginning in March, 2005. All trees were in a forested environment and had never been treated with imidacloprid. Branches were taken to Virginia Tech and cut into 30-40 cm sections for the experiment. The cut ends of these sections were placed in different concentrations of imidacloprid for 10-14 days. Concentrations used were 0.125%, 0.25%, 0.5%, 1%, and 10% imidacloprid using Imicide (J. J. Mauget Company, Arcadia, CA) and water solutions. Ten ml solutions were poured into 4 x 10 cm plastic vials and the tops sealed with parafilm. Cut ends of the branches were pushed through the parafilm and submerged into the solution. Needles and twigs were clipped from the lower 10 cm of these branches so that the bare stem could be pushed through the parafilm to be in contact with the insecticide, but the infested sections of the branch were above the parafilm barrier. Branches were kept at 14:12 °C (D:N) with approximately 30-40% relative humidity for 10-14 d or until most of the solution was taken up. Water was added as needed. Branches were then carefully removed and the bottom 10 cm sections of the branch that were below the parafilm were clipped and discarded. The remaining branch portions were used in predator feeding experiments and stored at either 4:4, 12:9 or 20:18 °C (D:N) and 14:10 (L:D) photoperiod for 20 d. The experiment was repeated weekly from March 4 through April 15, 2005 to create 7 blocks. For extraction, branches were analyzed separately according to block (time) within each temperature/predator combination. Two 30-cm branches were spiked per treatment dose within each block.
Before extraction branches were dried and ground as previously mentioned, 100 mg of dried wood tissue were added to 1.5 ml micro-centrifuge tubes. 1 ml methanol was added to each sample. Samples were sonicated for 10 min and allowed to sit overnight at room temperature. The following day samples were again sonicated for 10 min and centrifuged at 10,000 x g for 10 min. Supernatant was removed in 1 µl increments and spotted in duplicate on a plate.

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism for Macintosh, v4.0c (GraphPad Software Inc.). Linear regression compared standard curves. Spiked eastern hemlock branch samples data were pooled by treatment dose within each block.

**5.3 Results**

**Standard curve**

Spot area displayed a sigmoidal relationship with spot concentration. Linearity was achieved using standards of 5, 10, 50 and 100 ng imidacloprid per spot.

**Spikeovers with hemlock extract**

Curves of imidacloprid in both methanol and hemlock extract exhibited strong linearity, with coefficient of determination ($r^2$) of 0.999 and 0.993, respectively (Figure 5.1). A standard curve of imidacloprid prepared in methanol (slope = 2.29x+7.48; $r^2 = 0.999$) was compared with hemlock tissue extract (slope = 2.24x+7.56; $r^2 = 0.993$). Slopes ($F = 0.010$; df = 1, 43; $P = 0.919$) and intercepts ($F = 0.023$; df = 1, 44; $P = 0.881$) were equal. Percent recovery, detection and quantification limits of imidacloprid in methanol and hemlock tissue extract were comparable (Table 5.1).

**Spiked eastern hemlock branch samples**

Imidacloprid concentrations in the hemlock branches ranged from 7-874 ppm. Imidacloprid recovery had a strong correlation with the initial treatment dose (slope = 42.38x + 22.96; $r^2 = 0.997$, Figure 5.2).
5.4 Discussion

HPTLC can be used to quantify imidacloprid from hemlock wood tissue extract. This method is accurate and repeatable, and clean-up or dilution steps are not necessary. HPTLC has the advantage of no matrix interference, so hemlock tissue sample analysis is not complicated by false positives or elevated concentration readings as occur in ELISA. The HPTLC working range of 5-100 ppm is higher than ELISA’s working range of 0.2-6 ppb. However, the need to dilute ELISA samples 100-1000 fold (Chapter 2) brings its upper working range to 0.6 - 6 ppm. Although HPTLC is not as sensitive as ELISA, the dilution steps necessary to absolve matrix effects from tissue extract in ELISA decreases the sensitivity to a level that is comparable with HPTLC.

Analysis of field-treated eastern hemlocks using liquid chromatography-tandem mass spectrometry suggests that imidacloprid concentrations in wood tissue can be in the ppm range, however, the majority of samples were in the 50 – 800 ppb range (Chapter 3). If imidacloprid-treated hemlocks have concentrations primarily in the ppb range, the HPTLC technique is not currently sensitive enough for field use. The technique is advantageous in certain situations, such as analysis of branches spiked in the lab (Appendix A).

Imidacloprid is used to combat tree pests such as the exotic Asian longhorned beetle, Anoplophora glabripennis Motschulsky (Coleoptera: Cerambycidae) and Emerald ash borer, Agrilus planipennis Fairmaire (Coleoptera: Buprestidae). In pin oak, Quercus palustris Muenchh, up to 2 ppm imidacloprid was detected in foliage 1-12 wk after soil injections (Tattar et al. 1998). Populus nigra L. twigs 3 months after trunk injection with imicide had < 7 ppm at the highest dose (Wang et al. 2005b). If imidacloprid concentrations from tree tissues treated for those pests reach the ppm range, this technique may be a practical alternative to ELISA and other extraction methods.
Table 5.1 Validation parameters for imidacloprid in methanol and hemlock tissue extract.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Imidacloprid in methanol</th>
<th>Imidacloprid in hemlock extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Curve</td>
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<tr>
<td>Slope (area in thousands)</td>
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<td>2240x + 7.6</td>
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<tr>
<td>Linearity ($r^2$)</td>
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<td>Linearity range (ng/spot)</td>
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</tr>
<tr>
<td>Quantification limit (ng/spot)</td>
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<td>7.0</td>
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<tr>
<td>Percent Recovery</td>
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<td>Mean</td>
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<td>Range</td>
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<td>68-108 %</td>
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<tr>
<td>Inter-day mean*</td>
<td>98.3 %</td>
<td>98.8 %</td>
</tr>
</tbody>
</table>

* 100 ng/spot, based on three plates over three days.
Figure 5.1 Comparison of standard curves of imidacloprid prepared in methanol compared with imidacloprid prepared in hemlock wood tissue extract. Curve data points are 5, 10, 50, and 100 ng imidacloprid/spot.
Figure 5.2 Imidacloprid concentrations in hemlock wood tissue extract recovered by high performance thin-layer chromatography from eastern hemlock branches placed in 0-10% imidacloprid solutions.
Chapter 6

Summary

The hemlock woolly adelgid (HWA) is an exotic invasive pest from Japan that infests and kills eastern hemlock trees throughout much of their native range in the eastern United States. A classical biological control program is underway, and several predators are being mass-reared and released. *Laricobius nigrinus* and *Sasajiscymnus tsugae* are two biological control agents that have been released in the eastern United States to control HWA and imidacloprid is the primary insecticide used against HWA. Trunk and soil injections of imidacloprid are the primary methods of control in forest and urban landscapes and can provide protection against infestation for several years after application. There continues to be applications of imidacloprid in public and private forests and parks, often geographically close to releases of adelgid predators in a coordinated biological control program. The purpose of this study was to investigate potential nontarget impacts of imidacloprid on HWA predators.

Chapter 2

An enzyme linked immunoassay (ELISA) technique has been used for quantification of imidacloprid within treated hemlock tissues. The kits are commercially available, easy to use, highly sensitive and selective, and are relatively rapid and inexpensive. ELISA is susceptible to matrix effects associated with hemlock wood and needle tissues, which can result in false positives and overestimated imidacloprid concentrations. Matrix effects can be avoided if samples are sufficiently diluted at least 100-1000 fold with water. The recommended minimum total dilution factor of 1000 fold effectively raises the lower limit of detection of the kit from 0.2 ppb to 200 ppb. Imidacloprid can be extracted for analysis by soaking samples overnight in water at room temperature. Wood, and needle tissue can be analyzed by this method. This investigation provides a standardized method to account for matrix effects associated with ELISA.

Chapter 3

Eastern hemlock branches infested with HWA were placed in 1-100 ppm imidacloprid solutions and used in a HWA bioassay and predator no-choice and choice tests. HWA mortality increased
with time and dose and was correlated with the amount of imidacloprid recovered from the branches. HWA’s 30 d LC50 from imidacloprid in wood tissue was 242 ppb. These results suggest that HWA is highly susceptible to imidacloprid, and that over time, concentrations in the low ppb range are efficient in causing substantial mortality.

In the no-choice experiment with *L. nigrinus* and *S. tsugae*, mortality was generally higher on treated branches than controls. *S. tsugae* consumed the same number of adelgids on treated branches as in controls, while *L. nigrinus* beetles consumed significantly fewer adelgids from the 100 ppm branches than the number of adelgids consumed on controls. When given no choice in prey, both beetle species will feed on HWA residing on treated branches.

In predator choice experiments, beetles consumed significantly fewer adelgids on the 100 ppm branches than those on the untreated branches, probably because on the 100 ppm branches over 90% of the adelgids were dead by the end of the trial and the beetles prefer to feed on live adelgids over dead ones. Beetle mortality generally increased in the higher dose treatments, however, means were not significantly different from control mortality. It is unclear if beetles died from natural causes associated with feeding on poor quality adelgids or from ingesting imidacloprid in the adelgids. Beetles were observed feeding more on control branches than treated branches, suggesting feeding preference on healthier, untreated adelgids.

The recovery of imidacloprid from beetle cadavers suggests that systemic imidacloprid can be passed to predators feeding on adelgids. This route of exposure impacted predators in a laboratory setting, but imidacloprid concentrations in hemlock tissue were higher than concentrations recovered from field treated hemlocks (Chapter 4). HWA was very sensitive to imidacloprid, and in the field it is more likely that predators will be impacted indirectly by reduced prey quality, density, and distribution.

**Chapter 4**
Over 50 eastern hemlocks were treated with trunk or soil injections of imidacloprid in Abingdon or Marion VA in 2004-2006. Treatment rates were 100%, 50%, or 25% of the recommended rate. At the Marion site, treatment effects were most evident three years after treatment. For both
soil and trunk injected trees, LC/MS/MS analysis of branches cut 2 and 3 years after treatment recovered the des nitro, des nitro olefin, 5-hydroxy, di-hydroxy, olefin, and 6-e-n-a metabolites.

When adelgids surviving on branches from field-treated hemlocks were fed to the two predator species, both displayed reduced mobility and fitness. Branches from quarter-rate trees were cut 1 year after treatment and surviving adelgids fed to the two predators. In 2005, beetle mortality was higher when beetles fed on adelgids from treated trees, but the total amount of adelgids consumed was not different on treated branches compared with the untreated. In 2006, mortality varied among treatments but was not significantly higher for predators feeding on treated adelgids than the control. Beetle fitness was reduced for predators feeding on treated adelgids, as they took more time to right themselves in flip tests compared with beetles feeding on untreated adelgids. HWA surviving on imidacloprid trees in the field has the potential to impact predator survivorship and health. However, for most trees HWA populations were low or not-dense, suggesting that predator exposure may be limited because of the high efficacy of imidacloprid against HWA; populations on treated trees will be insufficient for predation.

Chapter 5
High performance thin-layer chromatography (HPTLC) was evaluated as a potential technique for quantification of imidacloprid in hemlock wood tissue. The technique was accurate, rapid, inexpensive, and repeatable. Hemlock wood tissue extract did not cause interference of quantification. The working range of the technique is 5-100 ppm. In Chapter 4, the majority of hemlock wood samples from field-treated trees had imidacloprid concentrations < 1 ppm, therefore, in its current form this technique is not sensitive enough for field application.

Conclusions
Imidacloprid displays biological efficacy against HWA at very low concentrations. The two predator species displayed sensitivity to imidacloprid from topical applications in the ng range. Prey-mediated effects of systemic imidacloprid on Laricobius nigrinus and Sasajiscynnum tsugae are possible, however, in some cases mortality and fitness seem to be affected as a result of reduced prey quality and density rather than direct mortality associated with ingesting the insecticide. Some individuals did display poisoning symptoms after feeding on treated adelgids,
suggesting that imidacloprid could potentially be passed from an adelgid to a predator under certain conditions. Imidacloprid exposure through feeding on adelgids on treated trees is possible but predator preference for healthier and denser food stock could drive them away from treated stands towards denser, healthier adelgid populations. HWA populations and densities on treated trees were very low or absent 2-3 years after treatment, and this lack of food may lead predators to feed on untreated trees with healthy, dense HWA populations. Beetles should not be released in treated stands until HWA populations begin to recover after imidacloprid treatment, which in some cases may be 4+ years after treatment. Both chemical and biological control of HWA are important components in the effort to save hemlocks in eastern forests, and both methods should be employed; however, predator releases in or near treated stands would benefit from untreated adelgid populations as additional food sources.

**Future work**

Soil and trunk injections of imidacloprid at 50% of the recommended field rate in some cases were as efficient in controlling HWA as the recommended rate. Reduced application rates would be economically and environmentally advantageous, however, more research should be done to elucidate when lower rates may be appropriate, their effectiveness, and the length of residual protection. Chapter 4 compared the proportion of hemlock shoots infested with imidacloprid concentrations recovered from a sub-sample of wood tissue from those same branches. A more specific relationship could be investigated by comparing HWA densities on individual branches with imidacloprid concentrations recovered from that same branch. Additionally, the recovery of imidacloprid and its metabolites from adelgids would give insight on feeding, susceptibility and metabolism. Relatively little is known about metabolic pathways in eastern hemlock trees. Since the olefin metabolite’s biological efficacy is 10-times as acutely toxic as imidacloprid, future studies should investigate bioactivation pathways for olefin and its distribution and persistence in hemlock tissues.

Since *L. nigrinus* aestivates in the soil, it would be interesting to determine its aestivation success in treated soils. As predator spread and establishment is monitored, it would be beneficial to use GIS to track the success and/or failure of predator establishment and impact on HWA populations and hemlock health. Using imidacloprid treatments as a variable could help
determine if such treatments have sustained impacts on hemlock, HWA and predator populations on a large scale.

This study has focused on nontarget impacts of arthropod predators, but in the Great Smoky Mountains, due to the sheer number of trees that have been treated with imidacloprid it is important to make sure that the treatments are not having ecological impacts. Many treated hemlocks are near streams located in important headwaters. A study sampling streams and watersheds for imidacloprid and its metabolites would be beneficial to ensure that nontarget ecological impacts are limited. Likewise, as imidacloprid is placed the soil at the base of hemlocks, these trees are often located in areas of dense Rhododendron or other vegetation. It would be interesting to sample these plants and see if nontarget plants could be taking up imidacloprid from treated soils.
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Yu, Y.-S., S. Xue, J.-C. Wu, F. Wang, J.-L. Liu, and H. Gu. 2007. Distribution of


Appendix A

Impacts of imidacloprid on Laricobius nigrinus and Sasajiscymnus tsugae feeding on hemlock woolly adelgid from eastern hemlock branches

A.1 Introduction

Hemlock woolly adelgid (HWA), Adelges tsugae Annand (Hemiptera: Adelgidae) is an introduced pest of eastern hemlock, Tsuga canadensis (L.) Carriere, and Carolina hemlock, T. caroliniana Engelmann, two hemlock species that are native to the eastern U.S. Laricobius nigrinus Fender, (Coleoptera: Derodontidae), and Sasajiscymnus tsugae Sasaji and McClure, (Coleoptera: Coccinellidae), are two HWA predators being reared and released in a classical biological control program. The neonicotinoid imidacloprid is the primary insecticide used to control HWA infestations in forested and urban landscapes and can be applied by foliar sprays or administered systemically through trunk or soil injections (McClure et al. 2001, Doccola et al. 2003). Imidacloprid poisoning symptoms mimic nicotine poisoning with loss of coordination, twitching, tremors, and paralysis. As predators are released into the environment, it is important to investigate what factors could impact their spread, survival and establishment (Mausel 2007). The fitness and survivorship of predators could be exposed to systemic imidacloprid by feeding on HWA from treated hemlocks. This study investigated prey-mediated effects of systemic imidacloprid on two predators of HWA.

A.2 Materials and methods

In February and May, 2005 a large branch was cut from an HWA infested eastern hemlock trees in Montgomery County, Virginia. The tree was in a forest landscape and of approximately 30 cm dbh. Trees at this site had been infested for at least two years, and adelgid densities were >1 adelgid/cm new growth. Branches were taken to Virginia Tech in Blacksburg, VA and 30-40 cm branchlets were cut for the experiments. The cut ends of these branchlets were placed in different concentrations of imidacloprid for 10-14 d. Concentrations used were 0.125%, 0.25%, 0.5%, 1%, and 10% imidacloprid using Iocide (J. J. Mauget Company, Arcadia, CA) and water solutions. 10 ml solutions were poured into 4 x 10 cm plastic vials and the tops were sealed with
parafilm. Cut ends of the branches were pushed through the parafilm and submerged into the solution. Needles and twigs were clipped from the lower 10 cm of these branches so that the bare stem could be pushed through the parafilm to be in contact with the insecticide but the adelgid-infested sections did not have contact with the insecticide. Branches were kept in a growth chamber at 14:12 °C (D:N) and 14:10 h (L:D) and approximately 30-40 % relative humidity for 10-14 d or until most of the solution was taken up. Branches were then carefully removed and the bottom 10 cm sections of the branch that were below the parafilm were clipped and thrown away. The upper portions were used for the experiment and were inserted into wetted floral foam blocks wrapped in parafilm and placed in 950 ml polyethylene plastic containers (Trx-Tech Corp., Kent OH). The plastic containers had three ventilation holes (2.5 cm diameter) with fine mesh (0.14 mm², Sefar America Inc, KC, MO) coverings. Branchlets were watered by hand as needed.

Five beetles, either *L. nigrinus* or *S. tsugae*, were randomly selected and added to the branch in each container. *L. nigrinus* beetles were from the colony at the Virginia Tech insectary, and *S. tsugae* beetles were from a colony comprised of individuals from both the Clemson insectary and the Beneficial Insects Lab at the University of Tennessee. Beetles were examined every day for 20 d and noted as alive, dead, or intoxicated. Individuals were labeled as intoxicated if they exhibited hyperactivity, tremors, un-coordinated movement, spasms, or paralysis. Dead individuals were removed and frozen at -70 °C. Containers were maintained in growth chambers at two temperature regimes for each species: *L. nigrinus* was stored at 5:3 and 12:9 °C (D:N). *S. tsugae* was stored at 12:9 and 20:18 °C (D:N). Chambers were maintained with a photoperiod of 14:10 h (L:D) and approximately 40-50 % relative humidity. After the experiment concluded branches were frozen for pesticide residue analysis.

Branches were prepared for imidacloprid extraction and analysis using high performance thin layer chromatography (HPTLC) according to the protocol outlined in Chapter 5.

*Statistical analysis*
Mortality was adjusted for control mortality using Abbott’s formula (Abbott 1925). Two-way ANOVA for each species using the factors temperature and dose. Linear regression compared beetle mortality with percent imidacloprid.

A.3 Results

No *L. nigrinus* mortality was observed in controls (Figure A.1A). Mortality was higher at 12 °C than 5 °C. Mortality and observations of intoxication generally increased with time and dose (Figure A.2). When the highest rate (10%) was treated as an outlier and removed from analysis, the linear regression of *L. nigrinus* percent mortality means with dose of imidacloprid the slopes for 12 °C were \(y = 185.88 + 217.84, r^2 = 0.484\) and 5 °C \((y = 99.173x + 99.77, r^2 = 0.788)\). Imidacloprid concentrations recovered from hemlock wood tissue by HPTLC showed good correlation with *L. nigrinus* mortality (Figure A.4), although the relationship was more evident at 5 °C.

No *S. tsugae* mortality was observed in controls at 12 °C but in the 20° experiment there was 25% control mortality. Mortality was higher at 20 °C than 12 °C (Figure A.1B). Mortality and observations of intoxication generally increased with time and dose (Figure A.3). When the highest rate (10%) was removed from analysis to achieve linearity, the linear regression of *S. tsugae* percent mortality means with dose of imidacloprid the curves for 12 °C \((y = 81.952x + 101.71, r^2 = 0.307)\) and 20° \((y = 181.64x + 215.93, r^2 = 0.876)\). Imidacloprid concentrations recovered from hemlock wood tissue by HPTLC showed good correlation with *S. tsugae* mortality (Figure A.5).

A.4 Discussion

Increasing temperatures and imidacloprid concentrations affected predator survivorship. Mortality increased in both species at warmer temperatures and at increasing concentrations of imidacloprid. Warmer temperatures probably increase beetle feeding, resulting in more ingestion of sublethal doses, although the number of adelgids eaten at each temperature was not
monitored. Warmer temperatures could also increase adelgid feeding, increasing the imidacloprid concentrations in the adelgids, or the amount of uptake of imidacloprid by the hemlock branches through higher transpiration and metabolic rates within the tissues. This would lead to more insecticide being stored within the parenchyma cells. Imidacloprid has a positive temperature coefficient of toxicity (Elbert et al. 1991), a factor observed in the different observed mortality rates.

There was some control mortality in the 20 °C S. tsugae trial, and in all four trials some beetles feeding on control branches exhibited some level of poisoning, especially during the last 10 days of the trial (Figures A.2 and A.3). S. tsugae colony beetles showed evidence of poisoning, as did L. nigrinus colony beetles kept in feeding containers before the experiments. Cleaning the containers and growth chambers did not stop the effects on controls and colony beetles. With the exception of the 20° S. tsugae trials, no control mortality occurred, suggesting that any intoxication in control beetles was not enough to cause mortality. One possible reason for intoxicated controls is that beetles were labeled “intoxicated” if it displayed any signs of poisoning, from hyper-excited to complete paralysis. This experiment would have benefited if intoxication symptoms were divided into several categories, such as un-coordinated movement, tremors, spasms, and paralysis, which would help quantify the degree of intoxication rather than simply recording intoxication symptoms as present/absent. Since some beetles with relatively trivial symptoms such as hyper-excitation were labeled intoxicated, the resulting proportions of intoxicated beetles may exaggerate the actual signs of relevant imidacloprid intoxication symptoms. The beetles were examined daily at the same lab station. Some cross-contamination from using the same paintbrush to handle all beetles during the daily handling could have been passed to the controls, as evidenced by their signs of poisoning. Intoxication symptoms often began as trembling and progressed to spasms and paralysis and, eventually death. This generally increased with time and dosage. Death occurred 1-3 days after paralysis, possibly as a result of starvation or dehydration as paralyzed beetles were unable to feed or drink water. Paralyzed beetles were usually found at the bottom of the feeding containers. Intense intoxication symptoms prohibited individuals from residing on hemlock branches and caused them to fall from the branches. In the field, paralysis could cause predators to fall from hemlock branches.
and land on the surface of the ground, where they will be at higher risk of predation and more exposed to elements and temperature fluctuations.

This study would have benefited from a quantification of adelgid mortality over time at each imidacloprid dose. HWA has an LD$_{50}$ of 242 ppb (Chapter 3). HPTLC analysis confirmed that concentrations in the hemlock wood tissue were in the ppm range. HWA mortality was high during the course of the trial, as beetles may have been exposed to imidacloprid through another route of exposure. For instance, imidacloprid concentrations were so high that beetles may have been exposed through concentrations expressed externally in hemlock sap, bark or needles. Analysis of branches from field-treated hemlocks in Chapter 4 suggests that imidacloprid concentrations in wood tissue are more likely to be in the ppb range. The concentrations in this experiment were several orders of magnitude higher than are likely to be observed in the field. While these results suggest that predators can be exposed to systemic imidacloprid through feeding on affected adelgids, the high concentrations do not accurately represent concentrations in a realistic field situation. However, because of imidacloprid’s positive coefficient of toxicity warmer temperatures could increase imidacloprid’s efficacy against both adelgids and predators. Warmer temperatures could increase uptake and distribution by hemlocks and increase feeding rates by both adelgids and predators. This suggests that systemic imidacloprid could negatively affect fitness and survivorship of nontarget predators through prey-mediated exposure.
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Figure A1. Percent mortality of Laricobius nigrinus (A) and Sasajiscymnus tsugae (B) at different temperatures after feeding for 20 d on HWA from imidacloprid-spiked hemlock branches. Branches were placed in 0.125 – 10 % imidacloprid solutions. The 10% dose is not shown in this figure for clarity. Mortality corrected using Abbott’s formula.
**L. nigrinus**

Figure A2. Percentages of *Laricobius nigrinus* intoxicated or dead per day while feeding on hemlock woolly adelgids infesting eastern hemlock branches spiked with imidacloprid. Experiments were carried out at 5 °C and 12 °C for 20 d.
Figure A3. Percentages of *Sasajiscymnus tsugae* intoxicated or dead per day while feeding on hemlock woolly adelgids infesting eastern hemlock branches spiked with imidacloprid. Experiments were carried out at 12 °C and 20 °C for 20 d.
Figure A4. Laricobius nigrinus mortality and imidacloprid concentrations recovered by HPTLC from experiments at 12 °C (A) and 5 °C (B).
Figure A5. *Sasajiscynnus tsugae* mortality and imidacloprid concentrations recovered by HPTLC from experiments at 12 °C (A) and 20 °C (B). Mortality corrected using Abbott’s formula.
Appendix B

Oral toxicity of imidacloprid to *Hippodamia convergens* and *Harmonia axyridis*

B.1 Introduction

The multicolored Asian ladybird beetle *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) is a generalist predator widely distributed in the U.S. and was originally imported from China for biological control of homopteran pests (Koch 2003). Another generalist coccinellid, *Hippodamia convergens* Guérin-Méneville, is commercially available for biological control releases in gardens and interiortscapes (Flint and Dreistade 2005).

Imidacloprid, a neonicotinoid insecticide, is widely used as a seed treatment, foliar spray, or soil drench against many agricultural pests including Lepidopterans, Coleopterans, Hemipterans (aphids, whiteflies, planthoppers) (Nauen and Breitschneider 2002), Isoptera (Ramakrishnan et al. 2000, Thorne and Breisch 2001), Blattaria (Appel and Tanley 2000), Diptera (Stelinski and Liburd 2001, Barry et al. 2004, Liburd et al. 2004, Paul et al. 2006), and Siphonaptera (Jacobs et al. 2000, Rust et al. 2002). It binds to and blocks nicotinic acetylcholine receptors in the central nervous system (Lind et al. 1999, Silcox 2002, Tomizawa and Casida 2003) causing twitching, spasms, paralysis, and death. The relatively high water solubility of imidacloprid makes it an effective systemic that provides protection throughout the entire plant.

Susceptibility to insecticides can vary greatly among different coccinellid species (Obrycki and Kring 1998), although predaceous coccinellids are often more resistant to insecticides than target prey species (Croft 1990). Mode of contact often determines the impact of toxicants. Imidacloprid is highly toxic to both *H. axyridis* and *H. convergens* from topical treatments (Kaakeh et al. 1996, Youn et al. 2003). After topical application the 48 h, LD₅₀ for *H. axyridis* adults was 364 µg/g (Youn et al. 2003) and the 24 h LD₅₀ value for *H. convergens* adults was 0.68 µg/g or 0.01 µg/insect (Stark et al. 1995). *H. convergens* adults exposed to imidacloprid residues on Petri dishes exhibited 78% mortality at 127.4 ppm after 72 h. (Mizell and Sconyers 1992). *H. axyridis* is more tolerant of imidacloprid compared with *H. convergens.*
Predators can be directly or indirectly exposed to pesticides (De Cock et al. 1996, Johnson and Tabashnik 1999). Indirect effects are consequences that impact natural enemies by affecting their prey density, distribution and abundance. (Waage 1989). Direct effects result from contact with the pesticides or their residues and are often exhibited by acute symptoms within the first 24 h after application to their environment. They occur more frequently with foliar sprays, where natural enemies and/or their habitats are directly sprayed. For systemic insecticide applications, predators can be directly exposed by ingesting affected pests (Ahmed et al. 1954, Roach and Moore 1988, Johnson and Tabashnik 1999, Grafton-Cardwell and Gu 2003). Natural enemies who supplement their diet with plant products can be exposed through contaminated nectar or pollen (Smith and Krischik 1999, Stapel et al. 2000). Predators can be exposed to systemic imidacloprid through ingestion of affected aphids (Cole and Horne 2006). Susceptibility differences between predator species can be influenced by predator size and metabolism. As H. axyridis and H. convergens ingest aphids surviving on systemically treated plants, they could be exposed to oral doses of the insecticide that could negatively impact their fitness and survivorship. This study was conducted to investigate the oral toxicity of imidacloprid to two common generalist predators and their subsequent metabolism of imidacloprid.

B.2 Materials and methods:

Wild adult H. axyridis were collected by hand in an agricultural setting in Blackstone, VA and transported to Blacksburg, VA in July 2007. H. convergens were purchased online (Planet Natural, Bozeman Montana) and were shipped to Blacksburg in June 2007. Beetles were maintained in colony at 22 °C, 14:10 h (L:D) and were fed daily with green peach aphids, Myzus persicae Sulzer (Hemiptera: Aphididae) naturally infesting organic turnips in a greenhouse. Ladybeetle diets were supplemented with artificial food consisting of 1 part ladybird artificial diet (Planet Natural, Bozeman Montana), 1 part honey, and 2 parts water. Beetle colonies were stored in 950 ml polyethylene plastic containers (Trx-Tech Corp., Kent OH). The containers had three ventilation holes (2.5 cm diameter) and a lid with fine mesh (0.14 mm², Sefar America Inc, KC, MO) coverings. 2-3 organic turnip leaves with approximately 100-200 aphids each were wrapped in wetted paper towels and placed in each container every 4-7 d. Approximately 100
beetles were stored in each container. Artificial diet was placed on the mesh screen lid of each container every two d. Beetles could feed on the artificial diet through the screen, which protected them from drowning in the mixture.

Imidacloprid (99.2% purity) was obtained from Bayer. Serial dilutions of imidacloprid were created with water, resulting in concentrations of 0, 0.2, 2, 20, and 200 ppm. Adult beetles were provided with artificial food consisting of approximately 200 mg of ladybird artificial diet, 500 mg honey, and 0.8 mL of imidacloprid dilutions. Imidacloprid solutions and diet were freshly prepared before each test. Food was placed in a plastic “feeding tray” (a cap from an 8 x 3 cm plastic vial), and one feeding tray was placed in a 10 cm Petri dish. Five healthy beetles of each species were added to each Petri dish for each treatment level and the assay was carried out three times from June 5-19, 2007. Beetles were fed *M. persicae* for at least two weeks before the experiment began. They were randomly removed from the colony containers andstarved for five hours before testing.

Mortality and signs of neuro-toxicity were measured at 18, 24, 48, 72, 96, and 120 h. To quantify neuro-toxicity symptoms, individual beetles were rated on a 7 point “fitness scale” at each observation period (Table B.1). Beetles were considered dead when they did not move in response to probing with a small paintbrush. They were frozen when dead, and live beetles were frozen at the conclusion of the experiment, and analyzed by liquid chromatography dual-mass spectrometry (LC/MS/MS) by Dr. A. Lagalante at Villanova University in Villanova, PA. The procedure quantifies imidacloprid, and the olefin, urea, 6-chloro-nicotinic-acid (6-cna), des-nitro, N-nitroso, des-nitro-olefin, 5-hydroxy, and di-hydroxy metabolites.

Daily consumption of artificial diet was measured by weighing the remaining food to the nearest mg. One feeding tray was not exposed to beetles to account for weight changes due to evaporation. Weights were corrected for evaporation, and the amount of imidacloprid consumed daily by each beetle was estimated to the nearest mg.

*Statistical Analysis*
Statistical analyses were performed with SPSS for Macintosh, v11.0.4. Mortality was adjusted for control mortality using Abbott’s formula (Abbott 1925). Percent mortality and weights of food consumed were analyzed among treatments by ANOVA. Means were separated using Tukey’s HSD test. Probit analysis determined the LC<sub>50</sub> values. Fitness scale ratings were averaged over the 5 observation periods and means were analyzed by ANOVA. Beetles that survived the experiment were analyzed by LC/MS/MS separately from beetles that perished during the experiment. The recovered imidacloprid and metabolite concentrations were combined for live and dead beetles and were pooled according to dose for analysis. Beetles that died in the feeding tray were not included in the analysis. The significance level for all tests was $P = 0.05$.

**B.3 Results**

Mean fitness ratings (Table B.2) and mortality (Figure B.1) increased with imidacloprid dosage for both beetle species. Increased fitness ratings correspond with decreased beetle health. Mortality generally increased with dosage. After 120 h of feeding, *H. axyridis* fitness ratings ($F = 3.41; \text{df} = 4, 10; P < 0.008$) and mortality ($F = 29.29; \text{df} = 4, 10; P < 0.001$) were significantly different among treatments. For *H. convergens*, fitness ratings ($F = 2.92; \text{df} = 4, 10; P < 0.021$) and mortality ($F=4.93; \text{df} = 4, 10; P < 0.022$), were significantly different among treatments. The amount of food consumed was not different for *H. axyridis* ($F = 1.12; \text{df} = 4, 10; P < 0.399$) or *H. convergens* ($F = 0.104, \text{df} = 4, 10; P < 0.978$). Intoxication symptoms appeared 18 h after feeding commenced, and generally increased in intensity as time progressed, although some beetles showed poisoning symptoms but were able to recover 24-72 h later. Other beetles did not recover and subsequently died.

Imidacloprid and metabolite concentrations recovered by LC/MS/MS generally increased with dosage (Table B.3). Imidacloprid and olefin concentrations corresponded with beetle mortality (Figure B.1). Imidacloprid and olefin were the most prevalent compounds, followed by the di-hydroxy, 5-hydroxy and des-nitro metabolites, respectively. There were small amounts of des-olefin, urea and 6-cna metabolites. When data were pooled across all dosages, imidacloprid was
the primary compound recovered from *H. axyridis* (69% of all compounds recovered) and olefin was the primary metabolite (25%) (Figure B.2). For *H. convergens*, olefin was the primary compound detected (51%) followed by imidacloprid (45%).

After probit analysis, the *H. axyridis* LC$_{50}$ value based on imidacloprid concentrations in their diet was 93.08 μg/g, ($\chi^2 = 34.46$) after 5 d. Confidence intervals could not be calculated because of the lack of mortality in the lower treatment groups. The *H. convergens* LC$_{50}$ value and its 95% confidence intervals were 141.42 μg/g and 88.85 - 277.56 μg/g, respectively ($\chi^2 = 3.33$) after 5 d.

**B.4 Discussion**

One of the major concerns of using pesticides is the impact on nontarget organisms. In integrated pest management programs, nontarget exposure to systemic imidacloprid may occur as a result of predators ingesting affected prey. In this study, we focused on the oral toxicity and subsequent metabolism of imidacloprid by two common generalist predators.

Both predators consumed food regardless of imidacloprid concentration. The amount of food consumed did not vary significantly among treatments, suggesting that there were no antifeedant effects associated with imidacloprid-spiked diet. Poisoned beetles were rarely observed feeding, as tremors and paralysis restricted beetle mobility. Death was generally observed 24-72 h after intoxication symptoms began, suggesting that starvation and dehydration may influence mortality in conjunction with the toxicant. Some beetles did recover from intoxication, as they were able to metabolize the toxicant and their behavior returned to normal. Mortality was observed at all imidacloprid concentrations for *H. convergens*, while *H. axyridis* only displayed mortality in the highest two concentrations.

The honey used in the artificial diet mixture was purchased at a local supermarket. Imidacloprid can be found in honey because systemic imidacloprid can be expressed in plant nectar and pollen, although the lack of significant mortality in the controls suggests that any imidacloprid was inconsequential in terms of mortality. If imidacloprid was present in the honey, it could
explain why < 2 ppb imidacloprid was recovered from *H. axyridis* control beetles. Imidacloprid and olefin concentrations recovered from beetle cadavers generally increased with the spiked imidacloprid concentrations in their food. Beetle mortality and intoxications symptoms could be from imidacloprid, olefin, or both. Oral ingestion of the olefin metabolite is ten times more active than imidacloprid against a susceptible strain of cotton whiteflies, *B. tabaci* (Nauen et al. 1999). Suchail et al. (2004) hypothesized that in bees imidacloprid causes the immediate symptoms of neuro-toxicity, whereas the eventual mortality is from the 5-hydroxyimidacloprid and olefin metabolites.

LC$_{50}$ values were based on imidacloprid concentrations in the artificial diet. Additional LD$_{50}$ values were calculated based on imidacloprid and olefin concentrations recovered in beetle cadavers. After probit analysis, the *H axyridis* LD$_{50}$ value based on recovered imidacloprid concentrations was 289.24 ng/g ($X^2 = 25.63$). Confidence intervals could not be calculated for these values because of the lack of mortality in the lower treatment groups. The olefin LD$_{50}$ value was 218.8 ng/g, with 95% confidence intervals of 82.62-395.69 ng/g ($X^2 = 5.53$). For *H. convergens*, the LD$_{50}$ and its 95% confidence interval based on recovered imidacloprid concentrations was 204.69 ng/g, 129.75-396.28 ng/g, respectively ($X^2 = 204.69$). The olefin LD$_{50}$ value and its 95% confidence interval was 197.56 ng/g and 136.71-331.74, respectively ($X^2 = 0.66$). This analysis correlates mortality with imidacloprid and olefin as independent compounds, although in actuality mortality is most likely a function of the two compounds acting in concert with one another.

Since olefin was the primary metabolite recovered, one possible metabolic pathway *H. axyridis* and *H. convergens* may utilize is the hydroxylation of the imidazolidine component of the imidacloprid molecule into the 5-hydroxyl metabolite which, after elimination of water, is converted to olefin. This is a common pathway of metabolism in a variety of plant species (Sur and Stork 2003). If olefin is a primary byproduct of imidacloprid metabolism in coccinellids, metabolism could bioactivate the parent compound into a more lethal compound and thereby increase the toxicity of imidacloprid.
Both predators displayed similar susceptibilities to oral doses of imidacloprid. Subsequent metabolism of imidacloprid into olefin may increase predator susceptibility. In the field, predators feeding on prey surviving on systemically treated host plants may ingest sublethal or lethal doses of imidacloprid. Imidacloprid did not display detectable antifeedant effects on the two predators. LC/MS/MS was useful for determining imidacloprid and its metabolites in beetle cadavers. Further study is required in order to determine if predators can be affected through prey-mediated doses of systemic imidacloprid.
References cited


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Table B.1. Fitness scale for beetles feeding on artificial diet treated with imidacloprid.

<table>
<thead>
<tr>
<th>Rating</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beetle behavior</td>
<td>Alive, healthy</td>
<td>Mobile with slight twitching</td>
<td>Mobile with heavy tremors</td>
<td>Immobile with heavy tremors</td>
<td>Paralyzed, unable to right themselves</td>
<td>Paralyzed, only move when prodded</td>
<td>Dead</td>
</tr>
</tbody>
</table>

Table B.2. *Hippodamia convergens* and *Harmonia axyridis* means ± standard deviation. For each species, means within each column followed by same letter are not significantly different (Tukey’s HSD test, $P = 0.05$).

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose (ppm)</th>
<th>Mean fitness rating</th>
<th>Mortality (%)</th>
<th>Food consumed (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. axyridis</em></td>
<td>0</td>
<td>0.13 ± 0.52 (A)</td>
<td>6.7 (A)</td>
<td>0.79 ± 0.13 (A)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.076 ± 0.25 (A)</td>
<td>0 (A)</td>
<td>0.79 ± 0.082 (A)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.51 ± 0.80 (A)</td>
<td>0 (A)</td>
<td>0.84 ± 0.20 (A)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.8 ± 1.7 (B)</td>
<td>80 (B)</td>
<td>0.75 ± 0.032 (A)</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>5.0 ± 1.3 (B)</td>
<td>80 (B)</td>
<td>0.66 ± 0.026 (A)</td>
</tr>
<tr>
<td><em>H. convergens</em></td>
<td>0</td>
<td>0.43 ± 1.6 (a)</td>
<td>7.1 (a)</td>
<td>0.60 ± 0.051 (a)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.72 ± 2.0 (a)</td>
<td>11.1 (a)</td>
<td>0.61 ± 0.093 (a)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.4 ± 2.1 (a)</td>
<td>14.3 (ab)</td>
<td>0.61 ± 0.10 (a)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.8 ± 2.1 (a)</td>
<td>35.7 (ab)</td>
<td>0.58 ± 0.074 (a)</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>4.93 ± 1.16 (a)</td>
<td>64.3 (b)</td>
<td>0.60 ± 0.014 (a)</td>
</tr>
</tbody>
</table>
Table B.3 Imidacloprid and major metabolite concentrations (ppb) recovered from *Harmonia axyridis* and *Hippodamia convergens*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose (ppm)</th>
<th>Imidacloprid</th>
<th>Olefin</th>
<th>Di-hydroxy</th>
<th>5- hydroxy</th>
<th>Des- nitro</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. axyridis</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>9.5</td>
<td>2.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.5</td>
<td>61.6</td>
<td>10.5</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1172.5</td>
<td>255.6</td>
<td>74.3</td>
<td>3.0</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>624.1</td>
<td>364.9</td>
<td>87.9</td>
<td>8.5</td>
<td>1.9</td>
</tr>
<tr>
<td><em>H. convergens</em></td>
<td>0</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.5</td>
<td>29.3</td>
<td>0</td>
<td>2.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>35.3</td>
<td>105.0</td>
<td>0</td>
<td>2.8</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>290.7</td>
<td>270.5</td>
<td>7.4</td>
<td>15.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*The des olefin, urea and 6-cna metabolites were not of consequence.*
Figure B.1. Percent mortality of *Harmonia axyridis* (A) and *Hippodamia convergens* (B) in relation to imidacloprid and olefin recovery from beetle cadavers by LC/MS/MS. Beetles fed on artificial diet and honey spiked with imidacloprid for 5 d. Mortality corrected using Abbott’s formula.
Figure B.2. Proportions of imidacloprid and its major metabolites recovered from *H. axyridis* and *H. convergens* cadavers after oral exposure to imidacloprid-spiked artificial diet. Data pooled for all treatment dosages.
**Appendix C.** Comparison of eastern hemlock, hemlock woolly adelgid and *Laricobius nigrinus* biological processes throughout the year.

<table>
<thead>
<tr>
<th>Species</th>
<th>Fall</th>
<th>Winter</th>
<th>Early Spring (March-April)</th>
<th>Late Spring (May-June)</th>
<th>Summer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. canadensis</em></td>
<td>Increase carbon exchange</td>
<td>Advantageous carbon storage</td>
<td>High carbon exchange</td>
<td>Bud break, new growth, highest carbon exchange</td>
<td>Reduced carbon storage when hot or dry</td>
</tr>
<tr>
<td><em>A. tsugae</em></td>
<td>Sistentes nymphs break aestivation, begin feeding</td>
<td>Sistentes nymphs feeding, developing</td>
<td>Sistentes mature, lay eggs</td>
<td>Progredientes hatch, mature, lay eggs. Sistentes hatch and move to new growth</td>
<td>Aestivation</td>
</tr>
<tr>
<td><em>L. nigrinus</em></td>
<td>Adults break diapause, begin feeding</td>
<td>Adults feeding</td>
<td>Adults feeding, begin egg laying</td>
<td>Eggs hatch, larval feeding and development.</td>
<td>4th instar larvae enter soil for pupation, begin diapause</td>
</tr>
</tbody>
</table>
Appendix D. Differences (mean ±se) of first and final years of the proportion of shoots infested with hemlock woolly adelgid (A), amount (cm) of new growth (B), and hemlock health rating (C). Since 2004 proportions of shoots infested with HWA were significantly different, changes were calculated by subtraction of 2006 means from 2008 means (A). Differences in B and C were calculated by subtracting the mean of each treatment group at the beginning of the study from the mean at the end of the study. Means with same letter are not significantly different (Tukey’s HSD test, $P = 0.05$).
Appendix E. Non-infested hemlock shoots and imidacloprid concentration recovered from wood tissue. Each point is the mean of grams of imidacloprid applied per 2.5 cm dbh. Data pooled over 3 years from both application methods and both field sites.
Appendix F. Imidacloprid concentrations in hemlock wood tissue and corresponding proportion of shoots infested with hemlock woolly adelgid (A) and mean new growth shoot length (B). Data pooled over 3 years from both application methods and both field sites.