THE ROLE OF OLFACTION IN HOST-FINDING FOR TWO SPECIALIST PREDATORS OF HEMLOCK WOOLLY ADELGID (HOMOPTERA: ADELGIDAE).

by

Corey D. Broeckling

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APPROVED:

_____	_____
Scott M. Salom: Chair	Joseph C. Dickens
_____	_____
Richard D. Fell	John R. Seiler

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Dr. Scott Salom, Committee Chair

Department of Entomology

ABSTRACT

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Homoptera: Adelgidae), is forest pest introduced to eastern North America in the early 1950's. Although this pest occurs on both landscape and nursery stock as well as in natural stands of hemlock forest, pesticides are only practical and effective in urban settings. Ecological and economical considerations prevent utilization of chemical treatment in the forest setting, thus biological control is viewed as the most promising option for slowing the spread of HWA. It is essential for researchers to be able to accurately assess the population levels of biocontrol agents after release into the environment. No method currently exists for sampling HWA predators. This project was designed to determine whether two species of predators are able to utilize olfactory cues from eastern hemlock and/or HWA in host-finding. If predators use olfactory cues, we may develop an attractive sythnetic blend of compounds to draw them to a trap, thereby simplifying the sampling and improving its accuracy. To address this question we executed three experiments. The first involved examination of the antennae of the predators for the presence and abundance of olfactory sensilla. The second experiment was designed to detect a behavioral response by the predators following exposure to host volatile compounds. The final experiment involved identifying compounds emitted from eastern hemlock, and the affect of HWA-feeding on volatile emissions.

Laricobius nigrinus Fender (Coleoptera: Derodontidae) antennae are densely populated with sensilla, several of which are potentially olfactory in function. In

addition, we observed a behavioral response to olfactory cues which included altered flight behavior. However, the behavior was not clearly attraction. *Pseudoscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) has few sensilla on a very short antennae and only one type of sensilla possesses wall pores suggestive of an olfactory function. In addition, we did not observe a significant behavioral response to host-volatiles. It seems unlikely that this species uses olfaction in long-range host location. We identified 10 monoterpenes that were consistently expressed in the hemlock volatile profile and were unable to isolate volatile emissions from HWA. There is an increased monoterpene release rate from HWA-infested hemlock foliage as compared to uninfested foliage apparently driven indirectly by HWA through a reduction in new growth at branch tips. In addition there was a slight but statistically significant change in the percent composition of the individual compounds.

We see potential in developing a more efficient sampling procedure for *L. nigrinus* through utilization of olfactory cues. More biological assays must be conducted to determine whether an attractive blend exists and electrophysiological assays are required to isolate physiologically active compounds. However, our data suggest that *P. tsugae* is not likely to be reliant on olfaction in long-range host location.

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CHAPTER 1
Introduction and Literature Review

Life history, impact and control of Hemlock woolly adelgid in the Eastern U.S.

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, has been recognized as a major pest of eastern hemlock, *Tsuga canadensis* Carriere, and Carolina hemlock, *T. caroliniana* Engelm. (McClure *et al*. 2001). It is presumed to have been introduced to the North American continent from Asia (McClure 1987) in 1924, when it was found in Oregon on *T. heterophylla* Raf. (Annand 1924). HWA was discovered in the eastern United States in the early 1950's in Richmond, VA and has spread as far north as Massachusetts and south and west to North Carolina and West Virginia (United States Forest Service, 2001). HWA has killed native stands of eastern hemlock, as well as ornamental, landscape and nursery (Young *et al*. 1995).

Conditions contributing to HWA's success include the ability to produce two parthenogenic generations per year, a lack of natural enemies, the ability of individuals to spread via birds, wind, deer, and human activity (McClure 1990), and an apparent lack of host-plant resistance (McClure, 1995). All stages of the life cycle excluding the first instar crawler stage are spent in the same location at the base of a needle (McClure *et al*. 1996), with feeding stylets removed at the time of each molt. Adelgid species have either a primary and secondary host (holocyclic) or only one host (anholocyclic). The primary host of holocyclic Adelgidae is spruce (*Picea spp.*) and the secondary host a separate conifer (Hain *et al*. 1991). A winged form of HWA, the sexuparae of the spring generation, migrates from hemlock to *Picea spp.* in the spring, but has not been successful in colonization of North American species (McClure 1987, 1989).

The apparent lack of resistance by *T. canadensis* to HWA allows quick establishment of local populations. An uncontrolled population may kill both individual hemlocks as well as whole stands within a period as brief as 4 years (McClure 1991). Orwig and Foster (1998) found up to 95% *T. canadensis* mortality in stands in southern Connecticut. With saplings being particularly vulnerable to HWA, they concluded that

advance regeneration and seedbanks will not be important mechanisms in hemlock reestablishment. Young *et al*. (1995) noted that unlike other adelgids, which feed on cortical parenchyma cells, HWA appears to feed only on parenchyma storage cells that make up the xylem ray. HWA is thought to be spreading northward at a rate of 10-30 km a year from its established eastern range (McClure *et al*. 2001, Souto *et al*. 1996), and is adapted to high elevations and winter temperatures commonly below -35°C in its native range in Japan. Parker *et al*. (1999) experimentally deduced the low temperature survival threshold for North American populations to occur at -30°C. This finding implied that the extreme temperatures of the New England winters may limit the northward spread of HWA, but the authors acknowledged that HWA may adapt to colder temperatures and continue spreading northward.

Hemlock is an ecologically important late succession species, with about 800,000 ha of forest characterized as *T. canadensis* or *T. canadensis-Pinus strobus* L. (white pine) (Orwig and Foster 1998). It plays a unique role in natural landscapes, as it is extremely long lived, shade tolerant, and present in either pure stands or in combination with white pine and deciduous hardwoods. Undisturbed stands are characterized by a cool damp microclimate, low light levels, sparse understory vegetation, and a relatively stable forest composition due to hemlock casting deep shade and depositing acidic litter. Only 1% of sunlight that reaches the top of the canopy penetrates through the foliage to the understory (Hadley 2000).

Hemlock stands are found on exposed slopes as well as more protected sites such as ravines and stream bottoms. Hemlock creates structural diversity and provides cover for a number of terrestrial wildlife species (Godman and Lancaster 1990). Additionally, Jenkins *et al*. (1999) found that the nitrogen cycle in stands destroyed by HWA is altered such that an increase in nitrogen leaching will likely result. They also concluded that increased light levels in the understory are likely to increase the air and soil temperature as compared to pre-HWA levels. Thus significant impact on associated aquatic habitats are to be expected.

Adelgids damage hemlock by feeding on ray parenchyma cells via long stylets, causing needle loss, bud mortality, and branch and tree mortality (Young *et al*. 1995). During heavy infestation, new growth is drastically reduced and sistens eggs hatch shortly after bud-break, making recovery virtually impossible without control. Curiously, western hemlock, *T. heterophylla*, and the Asian *Tsuga spp*. are generally not fatally injured (Furniss and Carolin 1977, McClure 1995). Exposing the mechanism of this resistance may prove valuable to understanding the apparent lack of defense in *T. canadensis* and *T. caroliniana*.

Biological control of HWA

Although chemical controls can be easily and successfully applied in ornamental and landscape settings, such methods are not feasible in natural settings due to environmental impacts of pesticide use, the prohibitive cost of such applications, and geographical and logistical limitations (McClure 1991,1992). For these reasons, biological control efforts are currently being pursued.

Two definitions of biological control were offered by DeBach (1964): (1) "The action of parasites, predators, or pathogens in maintaining another organism's population density at a lower average than would occur in their absence," and (2) "Utilization of parasites, predators, and pathogens for the regulation of host population densities." The first is a natural process and the second requires human intervention. The native predator complex on the introduced HWA is minimal in the eastern United States, and predators introduced in other biological control programs appear to offer little hope of slowing the spread of HWA (Montgomery and Lyon 1996, Wallace and Hain 2000). Therefore the premeditated and deliberate manipulation or movement of native and introduced enemies is being examined (Cheah and McClure 1998, Salom *et al.* 2001*a*).

Early successes of biological control encouraged others to attempt the importation of natural enemies (see DeBach 1964 for history), however a relatively small percentage of importations have been deemed successful. Problems may occur from either too fast or too slow a dispersal rate of biological control agents, local climatic conditions, a lack

of genetic diversity, lack of knowledge about the control agent, or other poorly understood factors. Still, predictions can be made based on deductions from previous understanding of both the predator and prey species as well as ecological relationships and the environment of interaction (Hodek and Honek 1996). In some instances a single predator or parasite is sufficient and even preferable for control, a position supported by Myers *et al*. (1989). On the other hand predator-predator interactions can have a synergistic effect such that the predation rate of the pair of predators is nearly double the sum of the individual predation rates, as demonstrated by Losey and Denno (1998). In still other cases, native or introduced generalist predators can interfere with biological control programs (Snyder 2001). Most predator species found associated with HWA in North Carolina were generalists (Wallace and Hain 2000), while all predators imported for HWA control are specialists (Salom *et al.* 2001*a*).

There is a long tradition of successful biological control programs for homopteran pests. The first successful organized biological control program in the U.S. utilized the Australian vedalia beetle to control an introduced homopteran pest of citrus, cottony-cushion scale (van den Bosch *et al*.1982). More recently, the endoparasitoid *Apoanagyrus lopezi* De Santis (Hymenoptera: Encyrtidae) was introduced for use against the cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero in Africa (Souissi *et al*. 1998). The citrus mealybug, *Planacoccus citri* Risso (Homoptera: Pseudococcidae), has been successfully controlled in citrus orchards and in greenhouses by the parasitoids *Leptomastix dactylopii* Howard and *Leptomastidea abnormis* Girault (Hymenoptera: Encyrtidae) (Cadee and van Alphen 1997). The introduced parasitoids *Coccobius fulvus* Compere et Annecke and *Aphytis yanonensis* DeBach et Rosen (Hymernoptera: Aphelinidae) successfully depressed populations of arrowhead scale, *Unaspis yanonensis* Kuwana (Hemiptera: Diaspididae), to 1/60 of the original density over an eight year period (Itioka *et al*. 1997). *Leucopis obscura* Haliday (Diptera: Chamaemyiidae) has effectively controlled the adelgids (Homoptera: Adelgidae) *Pineus boernerion* on Monterey pine*, Pinus radiata* D. Don in New Zealand (Rawlings 1958), and *Pineus pini* Macquart in Hawaii (Culliney *et al*. 1988). The latter studies suggest that biological control of adelgids can be successful.

Several organisms are currently found at varying levels of association with HWA in North America and have potential to reduce populations. Some are native to their respective regions while others have been introduced previously for control of other pest species. Gouli *et al* (1997) found entomopathogenic fungi representing 13 genera associated with approximately 37% of field collected HWA. Three of those genera contain species known to be major entomopathogens, *Verticillium, Beauveria,* and *Paecilomyces*. *Alternaria* sp. was isolated from one third of all adelgids collected.

Midges (Diptera: Cecidomyiidae) (McClure 1987), flower flies (Diptera: Syrphidae) and lacewings (Neuroptera: Chyrsopidae) (McClure 1987; Montgomery and Lyon 1996) have been found associated with HWA in New England, but at densities too low to affect HWA populations. *Scymnus suturalis* Thunberg (Copeoptera: Coccinellidae), a predator introduced into North America from Europe for control of the aphids, was found feeding on HWA on hemlock growing near eastern white pine and Scotch pine (Lyon and Montgomery 1995; Montgomery and Lyon 1996). *S. suturalis* larvae and adults feed on all stages of HWA, consuming on average 16 eggs per day as an adult and 6 eggs per day as an immature. *Laricobius rubidus* LeConte (Coleoptera: Derodontidae), a native of North America, was found on the same trees species as *S. suturalis*. In addition, the life cycles of the two beetles are complementary, with *L. rubidus* active several weeks before *S. suturalis* in early spring. The complimentary nature of life cycles of the two species allows peak feeding to affect subsequent generations of the adelgid (Montgomery and Lyon 1996). The coccinellid, *Aphidecta obliterata* L., was introduced for control of the balsam woolly adelgid and the most frequently collected predator in a study by Humble (1994). Amman (1966) reported this species to be established in North Carolina. Montgomery and Lyon (1996) suggested that an introduction and evaluation of *A. obliterata* against HWA has potential to contribute to HWA control. However, preliminary results obtained by Salom (pers. com. 1999) indicate that this beetle is not likely to have a significant impact on HWA populations.

A number of host specific predators from the native range of HWA have been identified and are under investigation as biological control candidates of HWA. *Pseudoscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) was introduced to the United States in the mid-1990's and has since been mass released (Cheah and McClure 1998, Salom *et al.* 2001*a*). Of some 50 coccinellid species collected from hemlock in China, half of which are new to science, three promising *Scymnus spp.* have been imported for further evaluation (Montgomery *et al*. 2000, Lu and Montgomery 2001). *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) is associated with HWA on western hemlock and is currently being evaluated for biological control potential at Virginia Tech (Zilahi Balogh 2001).

The coccinellid *P. tsugae* (Coleoptera: Coccinellidae) was observed feeding on HWA in Japan. This predator is moderately host-specific, and has a life cycle highly synchronized with that of HWA (Cheah and McClure1998). The predatory success of *P. tsugae* in its natural habitat is promising, killing 86-99% of its prey in certain situations (Sasaji and McClure 1997). A population has been imported to the United States, and a breeding colony established (McClure 1995, Cheah and McClure 1998). Several releases have occurred throughout the eastern distribution of HWA and both overwintering capabilities and successful establishment offer promise that this species may contribute to successful biological control of HWA.

Scymnus spp. and *Pseudoscymnus spp.* are members of the tribe Scymnini (Coccinellidae). Scymnini is one of the largest groups within the family Coccinellidae (Hodek and Honek 1996), but due to their small size (almost exclusively less than 4 mm) (Sasaji 1971) and difficult identification, little is known of their biology and ecology (Pang and Gordon 1986). Nevertheless, several attempts have been made to introduce scymnids to North America for biological control with varying success (Gordon 1977). The genus *Pseudoscymnus* was created by Chapin in 1962 for some of the Japanese *Scymnus* species formerly treated as members of the subgenus *Scymnus* (Sasaji 1971). Separation from the subgenus *Scymnus* and inclusion in the genus *Pseudoscymnus*

requires a very short nine-segmented antennae and three segmented tarsi (Sasaji 1971, Pang and Gordon 1986, Gordon 1977).

Although only recently described (Sasaji and McClure, 1997), *P. tsugae* has been studied intensively due to its potential for control of HWA. Cheah and McClure (1998) found that both adults and larvae of *P. tsugae* fed on all stages of HWA. Life stages of the predator include egg, 4 larval stages, prepupa, pupa, and adult. This species requires 29.8 days at 20°C and 17.9 days at 25°C to complete development. At 20°C, males matured at 30 days, and females at 31.8, while at 25°C males matured at 18.8 days and females at 22.4 days. Mean longevity was 162.9 days for males and 125.6 days for females, although individuals of both genders lived beyond 300 days. Lifetime fecundity averaged 280 eggs, with a maximum of 513 over a period of 14 weeks. The species enters facultative reproductive diapause when prey is scarce, but can survive on diapausing HWA nymphs and other adelgid species. The life cycle of the beetle is well synchronized with that of HWA.

L. nigrinus is a member of the coleopteran family Derodontidae. This family is comprised nearly exclusively of fungus feeding species (Lawrence 1991, Lawrence and Hlavac 1979). The exception to mycophagous habit of the derodontids is the genus *Laricobius*. Members of this genus have shifted from feeding on sooty molds associated with Homoptera to feeding on the insects themselves (Leschen 2000). In particular, members of the genus *Laricobius* are tightly associated with members of the Adelgidae (Homoptera). Generally, very little is known about this family, as its members are rarely encountered and are generally not of economic importance. However, the predatory nature of *Laricobius spp*. have resulted in sporadic interest from researchers in the field of biological control of adelgid species. *L. erichsonii* Rosnh. was examined as a biological control agent of *Adelges piceae* Ratz. (Homoptera: Adelgidae), the balsam woolly adelgid, in the 1950's and 60's. This interest resulted in the most thorough examination of any derodontid species (Balch *et al.* 1958, Brown and Clark 1962, Buffam 1962, Clark and Brown 1958, Franz 1958) previous to the current interest in *L. nigrinus*.

L. nigrinus was found associated with HWA on western hemlock, *T. heterophylla*, in British Columbia, Canada (Zilahi Balogh 2001). With the knowledge that members of this genus are predatory on adelgids and the history of *L. erichsonii* as a biological control agent, *L. nigrinus* was imported to Virginia Tech where its biology and control potential for HWA have been and continue to be studied (Zilahi Balogh 2001, Salom *et al.* 2001*a*). *L. nigrinus* is highly host specific, completing development only on HWA. It passes through one generation per year and displays an aestival period coinciding with that of HWA. Females lay approximately 100 eggs over a 13 week period and both adults and larvae feed on all stages of the adelgid, although the egg stage is preferred (Zilahi Balogh 2001). In addition, caged field trials suggest that this predator is capable of significantly reducing adelgid populations (A. Lamb pers. com. 2001).

Rearing of biological control agents is costly and time-consuming, but efficiency continues to improve. Two of the coccinellid species are commercially reared (Rich Pais, Per. Comm. Ecoscientific Solutions, Scranton, PA) and *L. nigrinus* is near the point that it may be mass-released. Salom *et al.* (2001b) have devised a way to prevent HWA from entering its aestival period, which has the potential to allow for continuous rearing of adelgid predators (Salom 2002).

Insect Olfaction

The field of chemical ecology has made important contributions to the knowledge and understanding of biological control. Studies within the field attempt to uncover the nature, function, and use of semiochemicals, primarily by arthropods. Ridgway *et al*. (1990) refered to the discovery of *Bombyx mori* pheromone in 1959 as "the beginning of a new era." In the 40 years that followed, at least 1000 sex pheromones have been isolated from moths, and hundreds of other pheromones of various functions identified from other orders of insects. Pheromones have been identified from Coleoptera of the families Scarabaeidae (Hasegawa *et al*. 1993, Leal *et al*. 1994, Zhang, *et al*. 1994), Curculionidae (Rochat *et al*. 1991, Eller *et al* 1994), Nitidulidae (Bartelt and James 1994), Dermestidae (Finnegan and Chambers 1993), Scolytidae (Byers *et al*. 1990), as

well as others. Pheromones have been successful attractants for trapping male scarabs (Hasegawa *et al*. 1993). Often pheromones and kairomones synergize each other (Byers *et al* 1990, Landolt 1997). Although pheromones (chemicals used in intraspecific communication) are the most common and understood of the semiochemicals, interspecific compounds of communication including kairomones, allomones, and synomones play important roles in host finding for both herbivores and predators (Karg and Suckling 1999). A kairomone is an interspecific chemical signal which benefits the receiver of the signal, an allomone benefits the sender of the chemical cue, and a synomone benefits both species involved (Shorey 1977).

Predators and parasitoids may use semiochemicals in locating prey or host species. Neuenschwander and Ajuonu (1995) released 34,500 exotic insects of five predacious or parasitic species in the center of a circle of cassava plants infested with various levels of mealybug, *P. manihoti*, and found high levels of correlation between mealybug density and number of observed predators and parasitoids at each site. Soiussi and Le Ru (1999) found that host and host plant odors stimulated locomotory behavior in the parasitoid *Apoanagyrus lopezi* (Hymenoptera: Encyrtidae). Morgan and Hare (1998) noted that a parasitic wasp's ability to orient to host volatile compounds was not innate but behavior learned through association. Coccinellids species are commonly employed in biological control programs. For decades, it was thought that generalist coccinellids searched primarily randomly, although visual, tactile, and gustatory cues were implicated for some species (Hodek and Honek 1996). It is becoming evident that olfaction is a more commonly employed modality by both generalist and specialist Coccinellidae than previously supposed (Obata 1986, van den Meiracker *et al*. 1990, Heidari and Copland 1992, Jourdan *et al*. 1995, Hamilton *et al*. 1999, Ponsonby and Copland 1995).

Insect olfaction has been subject to the fascination of researchers for centuries, but it wasn't until behavioral experiments of von Frisch in 1921 that the antennae were recognized as the primary organs of olfaction (Keil 1999). A variety of methods exist for testing behavioral responses of insects to olfactory cues. Generally, an individual or group of individuals is offered a choice between one to many different odors or blends in

the form of walking or flying bioassays. Behavioral responses are recorded, quantified, and analyzed to deduce the behavioral role of the odor. Further testing is then carried out with field tests of the active volatiles.

In addition to behavioral examinations of olfaction, morphological clues can provide information on the importance of olfaction to a given species. The sensillum is the smallest functional unit for stimulus reception on insect antennae (Keil 1999). Several types of sensilla have been identified based on external and internal morphology. By recording physiological responses from these sensilla during exposure to various stimuli, researchers have been able to attribute certain morphological characters to specific function (Altner 1977, Zacharuk 1985). We are now able to utilize this correlation of morphology to function to determine whether structures are present to allow for detection of olfactory cues.

Olfactory cues may be used in IPM programs for control of a pest species. If a successful trapping technique is established, it can be applied to monitor the presence or dispersion of an insect population, resistance to pesticides, to predict an upcoming infestation, or directly control a population (Karg and Suckling 1999). One of the most prominent and large-scale uses of this technology is the use of the sex pheromone, disparlure, for mating disruption of gypsy moth in eastern hardwood forests (Leonhardt *et al*. 1996). In addition to their use as control agents, olfactory attractants can be used for sampling of pest and predator populations and predicting future trends (Borden 1995), as with the southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae), and its principal predator, *Thanasimus dubius* F. (Coleoptera: Cleridae) (Billings *et al.* 1995).

To utilize an attractive odor, it is essential to identify the compounds that comprise the odor so that it can be synthesized. When interested in predator behavior, it is of interest to examine the prey species, the host plant on which the prey feeds, and the bitrophic complex which may include induced responses from the plant due to exposure to herbivore feeding (Dickens 1999, Geervleit *et al*. 1994). Thus for predators of HWA, we are interested in identification and use of volatile emissions from both HWA and

eastern hemlock, as well as any changes in hemlock odor induced by HWA feeding. The most frequently utilized technology is the coupling to gas chromatography (a separation tool) to mass spectrometry (an identification tool). Solvent extracts of the foliage are performed, which reveal a breadth of metabolites that may or may not be emitted at biologically significant levels (see examples below). Other studies utilize techniques designed to capture only those compounds that are released from the foliage.

Foliar and twig chemistry studies have been conducted to reveal non-volatile and volatile metabolites from several conifer species. Muzika *et al.* (1990) summarized and compared various methods of such extractions. They found major constituents of blue spruce, *Picea pungens* Engelm., to include camphor, limonene, α-pinene, camphene, myrcene, β-pinene, and bornyl acetate. In grand fir, *Abies grandis* Dougl., the major compounds were β-pinene, camphene, β-phellandrene, and bornyl acetate. McClure and Hare (1984) analyzed foliar terpenoids of *T. canadensis* and *T. sieboldii* Carr. and identified and quantified 15 compounds through steam distillation methods. From subsequent analyses, they concluded that there was no significant wound response to infestation by two scale species (Homoptera: Diaspididae). Von Rudloff (1975) summarized his work on the chemosystematics of conifers, including analysis of *T. canadensis*, *T. heterophylla* Sarg. and *T. mertensiana* Carr. His studies have resulted in the most comprehensive analysis of hemlock terpenoids to date.

A sampling method for volatile release was utilized by Wibe *et al*. (1998) to determine the enantiomeric composition of monoterpenes in seedlings of *Pinus sylvestris* L., branches of *Juniperus communis* L., and seedlings of *Picea abies* L. (Karst.). Volatile compounds identified included enantiomers of α-pinene, *B*-pinene, camphene, sabinene, 3-carene, limonene, and *B*-phellandrene. Both (+) and (-) enantiomers of all compounds were found in all three species except for (-)-3-carene, which was found in none of the samples. Shu *et al*. (1997) examined the volatile release of white pine, *P. strobus* (L.), and found the most abundant monoterpenes to include α-pinene, *B*-pinene, myrcene, and limonene. Smaller amounts of camphene, *B*-phellandrene, and α-terpinolene as well as

traces of sabinene, borneol and bornyl acetate were also present. To my knowledge, *Tsuga spp.* volatile profiles have not been characterized.

Currently, no effective sampling method exists for predators at release sites. Without an effective sampling procedure, researchers and biological control practitioners are unable to monitor for establishment of predators species at release sites. In addition, without the ability to accurately monitor the population dynamics of the predator species, we are unable to attribute fluctuations in pest population levels to predator impact. It is therefore our objective to understand the role of olfaction in *L. nigrinus* and *P. tsugae* host-finding behavior. As with pest species, predator species which use olfactory cues in host-finding may be drawn to a trapping device baited with attractive olfactory cues. If we are able to demonstrate behavioral attraction, we may utilize this information to develop a more effective and efficient sampling method to accurately monitor establishment at release sites, as well as population dynamics of predator species over time. With this ultimate goal in mind, we formulated three objectives:

1. To examine the antennal morphology of the two predators and use this information as an indicator of the importance of olfaction.
2. To conduct behavioral assays to detect responses to host odors.
3. To identify volatiles emitted from the HWA/hemlock complex and determine whether the volatile profiles for infested and uninfested hemlock differ.

CHAPTER 2

Antennal morphology of two specialist predators of hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae)

Introduction

Since its introduction to the eastern United States in 1952 (Souto *et al* 1996), the hemlock woolly adelgid (HWA) has spread through approximately one third of the range of naturally occurring eastern hemlock, *Tsuga canadensis* Car. (USDA Forest Service 2001). HWA population levels grow rapidly at both the tree and stand level, apparently unchecked by either host plant defense or natural enemies. Although in the landscape setting HWA is successfully controlled using chemical means, this option is unavailable in the forest setting due to economic constraints, ecological sensitivity of hemlock habitat, and patchy distribution of both hemlock trees and HWA within trees. To reduce the rate of spread of HWA in the natural setting, several research groups in the eastern U.S. are actively pursuing biological control agents (see Salom *et al* 2001*a*, McManus *et al* 2000).

Commonly, hymenopteran and dipteran parasitoids are the preferred agents for biological control . However, no parasitoids for *Adelges piceae* (Homoptera: Adelgidae), were found after an exhaustive search as part of a massive biological control program (Dahlsten and Mills 1999). In addition, in review of the sparse Adelgidae literature, there are no known parasitoids of any adelgid species (Zilahi-Balogh 2001). Researchers have identified and imported several specialist coleopteran predators for control of HWA. Included in this list are *Pseudoscymnus tsugae* Sasaji and McClure, *Scymnus spp*. (Coleoptera: Coccinellidae: Scymnini), and *Laricobius nigrinus* Annand (Coleoptera: Derodontidae) (Salom *et al* 2001).

The practice of biological control in the forest setting offers several obstacles not encountered in agriculture, including poor accessibility, ecological complexity, and multiple use (Dahlsten and Mills 1999). As a result of these and other constraints, sampling for predators after release in complicated. At this time, *P. tsugae* is the only

predator of HWA to have been mass-released (McClure 2001, Salom *et al* 2001). Current sampling methodology for this predator employs beating lower branches of the release tree and surrounding trees and counting the number of predators that fall off onto a 1.0 m^2 collection sheet. This method has failed to account even for the presence of predators at Virginia release sites. Without accurate information on the population levels of predators, it is impossible to attribute HWA population fluctuations to predator impact. It is our objective to elucidate the innate host-finding mechanisms of *P. tsugae* and *L. nigrinus* as a means of improving the accuracy of sampling methods. In support of this objective, we undertook a project to examine the antennal morphology of the predators to gauge the potential importance of olfactory cues in host location.

Coccinellids are commonly employed as biological control agents and have been relatively well studied (Hodek and Honek 1996). Several recent studies have suggested that olfaction is a more commonly employed modality than previously supposed. *Adalia bipunctata* L. was shown to be attracted to the combined odors of aphid prey and the host plant (Raymond *et al* 2000). Hamilton *et al* (1999) conducted a behavioral and morphological examination of *Hippodamia convergens* (Gguerin-Meneville), concluding that this species can perceive olfactory cues from their prey and its host plant. *Semadalia undecimnotata* Schn. was found to possess wall pore sensilla suggestive of olfactory function on the terminal two segments of the antennae and a marked sexual dimorphism (Jourdan *et al* 1995). *Chilocorus nigritus* (F.) show a strong directed behavioral response to odors of its prey (Ponsonby and Copland 1995). *C. nigritus* is of particular interest, as it is of the same tribe, Scymnini, as *P. tsugae* and *Scymnus spp.*

The family Derodontidae is poorly studied, being a relatively small family and rarely of economic importance. All members of the family are exclusively mycophagous, except the genus *Laricobius*, which has shifted to a tight predatory association with the Adelgidae (Lawrence 1991, Lawrence and Hlavac1979). *Laricobius erichsonii* Rosnh. was imported from Europe as a potential biological control agent of *A. piceae*, which resulted in the most comprehensive studies of any derodontid species (Balch *et al* 1958, Brown and Clark 1962, Buffam 1962, Clark and Brown 1958, Franz 1958) previous to

our recent focus on *L. nigrinus*. No studies have rigorously examined olfaction in this family. We report the functional antennal morphology of *P. tsugae* and *L. nigrinus* in support of biological control efforts and as the first study on the antennal morphology of a species from the family Derodontidae or the tribe Scymnini.

Materials and methods

Insects

A colony of *P. tsugae* was established from individuals obtained from the New Jersey Department of Agriculture, Bureau of Biological Pest Control. *P. tsugae* were reared on field collected HWA-infested hemlock in moistened floral foam contained within one gallon plastic oviposition jars and kept in a room maintained at 25°C, 65% RH, and a photoperiod of 16L:8D (Cheah and McClure 1998). A honey/Wheast® nutritional supplement was provided (Planet Natural, Bozeman, MT, Prod # 176). Twigs and gauze (for monitoring oviposition) were removed and replaced weekly. Material from oviposition jars was then placed in a rearing chamber constructed of Plexiglas (60 x 45 x 45 cm). Each week a new rearing chamber was used. Fresh infested hemlock foliage was provided each week in moist floral foam and newly eclosed adults were collected five weeks after the eggs and foliage were first placed in the chamber.

In December 2000, *Laricobius nigrinus* individuals were collected from infested western hemlock in British Columbia, Canada and transported to Virginia Tech for colony establishment. During the months of January to March, adults were held at 12L:12D photoperiod and 8°C day/4°C night temperature regime. In March, the photoperiod was extended to 14L:10D and temperature was held at 10°C. *L. nigrinus* adults were sexed through manual extrusion of the genitalia (Zilahi Balgh 2001) while *P. tsugae* adults were sexed through examination of the penultimate abdominal segment (Sasaji and McClure 1997).

Scanning and Transmission Electron Microscopy:

For scanning electron microscopy (SEM), three adult insects of each species and gender were fixed in Karnovsky's fixative, post fixed in OsO_4 and critical point dried

(Baker 2001) before mounting on a stub using silver based adhesive. Samples were then sputter coated with gold and studied using a Phillips 505 SEM at 15-30eV. For transmission electron microscopy (TEM), antennae were excised from the head capsule and immersed into fixative of 0.1M sodium cacodylate buffer with 5% GTA, 3% formaldehyde, and 2.75% picric acid twice for 15 minutes each time. The sample was then post-fixed in 1% osmium tetraoxide in 0.1M sodium cacodylate for one hour, followed by dehydration in a ethanol concentration gradient, 15%, 30%, 50%, 70%, and 95% for 15 minutes each. Samples were embedded in Spurr's resin. Thin sections were cut in the gold-interference region and stained with 5% uranyl acetate for 12 minutes and lead citrate for five minutes. Thin sections were mounted on Form-var grids and examined using a Jeol 100 CX-II.

Sensilla type and analysis

Sensilla were assigned to groups, and putative function determined as suggested by Altner (1977), Zacharuk (1985) and Keil (1999). Student's t-test was used to compare for sexual dimorphism. No tests were conducted for differences between species.

Results

Laricobius nigrinus

L. nigrinus possess 11 segmented antennae, comprised of the scape, pedicel, and nine annuli (Table 2.1, Fig 2.1a). The scape takes a bulbous form following a constriction distal to its insertion in the antennal socket. Several sensilla of the Bohm form (Jourdan *et al* 1995) are found proximal to the constriction which likely function as mechanoreceptors. We did not focus our attention to these structures. The pedicel inserts into the scape with a highly flexible socket joint and is somewhat smaller than the scape and slightly elongated . The annuli (A) are initially elongate, but grow wider beginning at A7 (Table 2.1). Following A6 the antennae continues to widen to form gradual club shape. Hereafter we pool sensilla counts for males and females, as we were unable to detect any sexual dimorphism (Table 2.2). Sensilla counts drop as one moves distally from the scape to A1, and subsequently increases progressing from A1 to A9 (Fig 2.2).

Table 2.1. Mean and standard deviation of antennal segment length and total antennal length (in mm, n=3) for *L. nigrinus* (Ln) and *P. tsugae* (Pt) males (m) and females (f).

				Segment number										
Sp	Sex	Dimension	Stat	Scape	Pedicel	A1	A2	A3	A4	A5	A6	A7	A8	A9	Total
Ln	M	Length	mean	0.073	0.065	0.061	0.051	0.053	0.044	0.042	0.038	0.052	0.061	0.090	0.628
			st dev	0.009	0.009	0.005	0.006	0.005	0.009	0.006	0.004	0.002	0.016	0.002	0.065
		Width	mean	0.069	0.051	0.031	0.032	0.033	0.038	0.045	0.045	0.062	0.066	0.067	*
			st dev	0.003	0.001	0.002	0.000	0.002	0.001	0.005	0.003	0.006	0.010	0.004	*
Ln	F	Length	mean	0.082	0.069	0.060	0.058	0.056	0.042	0.044	0.040	0.057	0.056	0.091	0.654
			st dev	0.003	0.007	0.005	0.004	0.005	0.004	0.002	0.001	0.003	0.003	0.006	0.009
		Width	mean	0.071	0.054	0.036	0.035	0.040	0.038	0.043	0.046	0.061	0.071	0.068	*
			st dev	0.011	0.002	0.004	0.003	0.002	0.002	0.001	0.000	0.008	0.010	0.006	*
Pt	M	Length	mean	0.049	0.043	0.029	0.014	0.009	0.016	0.023	0.028	0.032	*	*	0.243
			st dev	0.005	0.005	0.006	0.003	0.003	0.004	0.009	0.003	0.007	*	*	0.016
		Width	mean	0.047	0.035	0.017	0.018	0.017	0.023	0.029	0.032	0.034	*	*	*
			st dev	0.008	0.007	0.001	0.003	0.003	0.004	0.005	0.004	0.010	*	*	*
Pt	F	Length	mean	0.058	0.038	0.029	0.016	0.010	0.009	0.045	0.031	0.021	*	*	0.256
			st dev	0.011	0.005	0.003	0.003	0.003	0.003	0.023	0.007	0.006	*	*	0.025
		Width	mean	0.062	0.047	0.028	0.028	0.030	0.033	0.039	0.041	0.052	*	*	*
			st dev	0.008	0.009	0.002	0.000	0.003	0.000	0.008	0.008	0.012	*	*	*

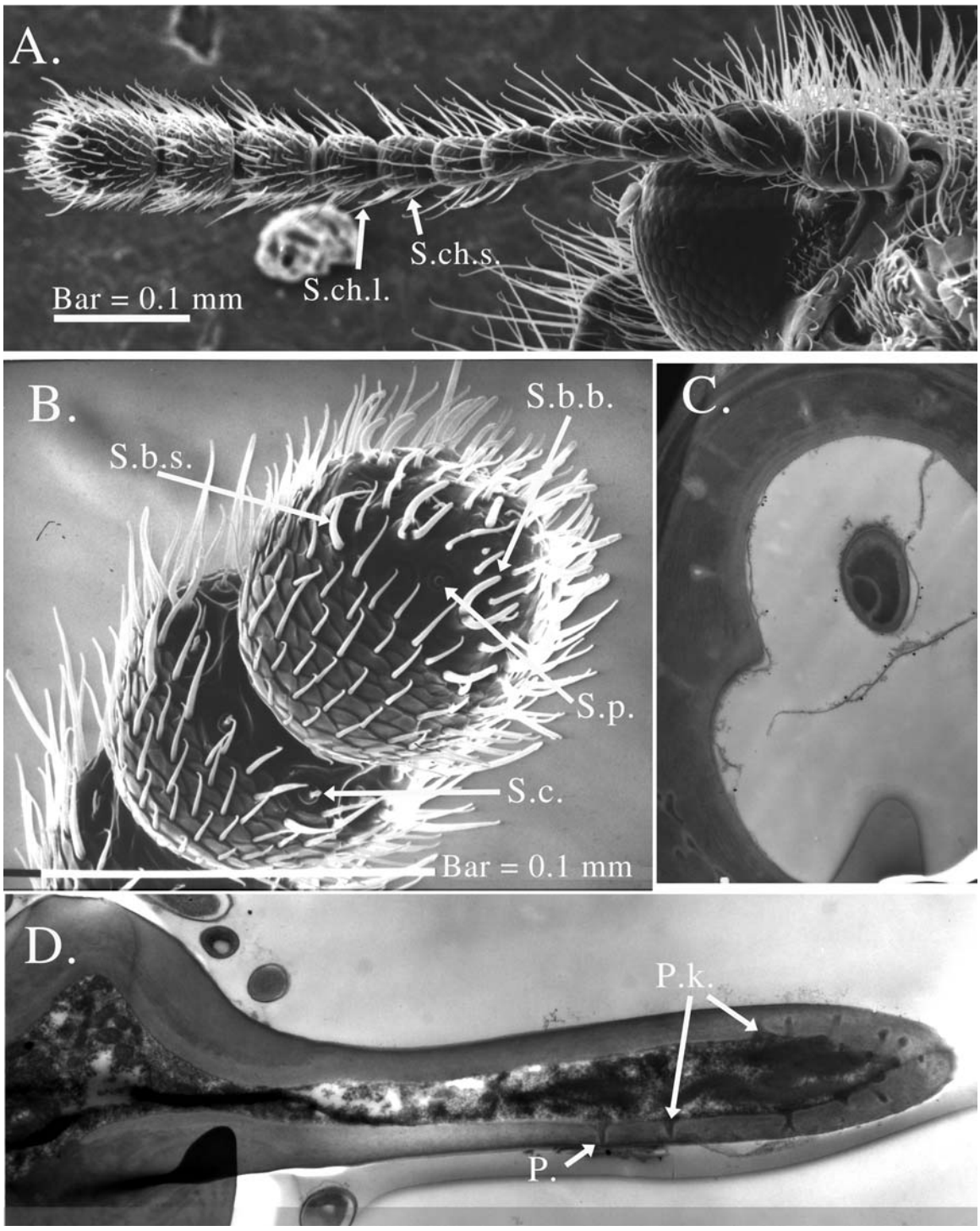

Fig. 2.1. *L. nigrinus* antennae SEM and TEM. A. Whole antennae (S.ch.l. Sensilla chaetica long, S.ch.s. Sensilla chaetica short). B. Annuli 8 and 9 (S.b.b. Sensilla basiconica branched, S.b.s. Sensilla basiconica single, S.c. Sensilla coeloconica, S.p. Sensilla placodea). C. TEM cross section through common base of branched basiconic sensilla. D. TEM longitudinal section through single branch of branched basiconic sensilla.

Table 2.2: Mean and st. dev. for counts of sensilla types by segment results of t-test for sexual dimorphism (n = 3).

			FEMALES		MALES		
SP	SEG	SENS	MEAN	STD	MEAN	STD	p < t
L. nigrinus	Scape	S.ch.l.	20.333	10.116	24.333	7.506	0.612
		S.ch.s.	24.333	5.860	15.000	5.000	0.104
	Pedicel	S.ch.l.	13.000	1.000	27.333	8.737	0.104
		S.ch.s.	39.667	1.528	27.667	11.151	0.203
	A1	S.ch.l.	13.667	2.082	9.333	4.726	0.220
		S.ch.s.	17.000	2.000	22.333	5.774	0.205
	A2	S.ch.l.	13.000	1.000	9.667	3.512	0.189
		S.ch.s.	16.333	0.577	13.000	3.606	0.189
	A3	S.ch.l.	13.667	2.517	9.667	1.528	0.078
		S.ch.s.	12.667	2.082	10.667	5.686	0.598
	A4	S.ch.l.	13.333	1.528	12.333	2.517	0.588
		S.ch.s.	13.333	4.509	14.667	3.786	0.715
	A5	S.ch.l.	15.000	4.583	16.333	2.309	0.676
		S.ch.s.	19.000	2.646	16.667	1.155	0.234
	A6	S.ch.l.	15.333	2.082	14.333	4.042	0.723
		S.ch.s.	23.333	8.505	16.667	4.726	0.301
	A7	S.b.b.	2.667	0.577	2.000	1.000	0.374
		S.b.s.	3.667	2.887	6.000	1.000	0.256
		S.c.	2.667	0.577	4.000	2.646	0.091
		S.ch.l.	30.333	15.695	26.667	12.702	0.769
		S.ch.s.	51.333	5.686	65.000	20.421	0.327
	A8	S.b.b.	5.000	1.000	4.000	1.732	0.435
		S.b.s.	7.667	0.577	5.333	1.155	0.069
		S.c.	7.333	0.577	4.333	3.786	0.304
		S.ch.l.	13.000	6.083	25.000	7.937	0.106
		S.ch.s.	103.000	8.544	100.333	51.675	0.934
	A9	S.b.b.	8.333	1.528	6.667	0.577	0.152
		S.b.s.	12.000	1.000	13.333	4.042	0.609
		S.p.	4.000	0.000	4.000	0.000	1.000
		S.ch.s.	138.333	2.082	122.333	25.146	0.386
P. tsugae	Scape	S.ch.l.	2.333	0.577	2.000	0.000	0.427
		S.ch.s.	6.667	1.528	8.000	1.732	0.374
	Pedicel	S.ch.l.	1.667	0.577	2.667	0.577	0.101
		S.ch.s.	9.000	2.000	7.667	0.577	0.330
	A4	S.ch.s.	6.000	1.000	6.000	0.000	1.000
	A5	S.ch.s.	7.667	1.528	9.333	0.577	0.152
	A6	S.b.l.	3.667	1.155	3.333	0.577	0.678
		S.b.m.	4.667	0.577	4.667	0.577	1.000
		S.ch.s.	5.667	0.577	6.000	1.000	0.643
	A7	S.b.l.	11.500	4.950	4.667	1.155	0.089
		S.b.m.	5.500	2.381	8.667	0.577	0.079
		S.b.s.	4.000	1.000	3.667	0.577	0.643
		S.ch.l.	2.000	0.000	2.333	0.577	0.374
		S.ch.s.	1.667	1.155	1.667	0.577	1.000

* For *L. nigrinus* : S.b.b. Sensilla basiconica branched, S.b.s. S. basiconica single, S.c. S. coeloconica, S.ch.l. S. chaetica long, S.ch.s. S. chaetica short, S.p. S. placodea. For P. tsugae: S.b.l. Sensilla basiconica long, S.b.m. S. basiconica long, S.b.s. S. basiconica short, S.ch.l. S. chaetica long, S.ch.s. S. chaetica short.

Sensilla chaetica short (*S.ch*.s)

S.ch.s. range from 21-28 μm in length and are 1.4-1.7 μm wide at the base (Table 3). They are deeply grooved longitudinally and are freely movable (Fig 2.1a). This sensillum is the most frequently encountered type, present on all segments and annuli (Fig 2.2). They are interspersed with *S.ch*.l. on the scape and pedicel, form a ring around the proximal half of A1-A6, and increase in density with a widening encircling band located on the proximal two thirds of A7-A9. No wall pores were evident in TEM images and most contained one dendrite closely appressed to the interior wall. Some sections contained multiple dendritic units in the lumen of the sensilla.

Sensilla chaetica long (*S.ch*.l.)

S.ch.l. are nearly as common as *S.ch*.s., but are not found on A9 (Fig 2.2). They are 55.0-63.0 μm in length and 1.4-2.0 μm in width at the base (Table 2.3). The only morphological difference between *S.ch*.l. and *S.ch*.s. is in size. The two types are intermingled with each other on the scape and pedicel and *S.ch*.l. form rings around the distal portion of each of A1-A8 (Fig. 2.1A).

Sensilla basiconica single (*S.b*.s.)

S.b.s. possess relatively thick walls permeated with pores with branched pore kettles extending to the lumen. They range from 14.0-30.6 μm in length and taper from a width of 1.7-1.85 μm at the base (Table 2.3) to a blunt tip. They are located solely on the distal portions of A7-A9 and increase in number on A9 (Fig. 2.1B, 2.2).

Sensilla basiconica branched (*S.b*.b.)

S.b.b. are structurally similar to *S.b*.s in wall thickness (Table 2.3) and presence of pores with multiple paths to the lumen, but differ in that they are clustered in groups of 2-5 arising from a common bulbous base and are somewhat shorter in length, ranging from 10.6- 27.0 μm in length (Fig 2.1B,D). They are distributed on the distal portion of the final three annuli, increasing in number from A7-A9 (Fig 2.2).

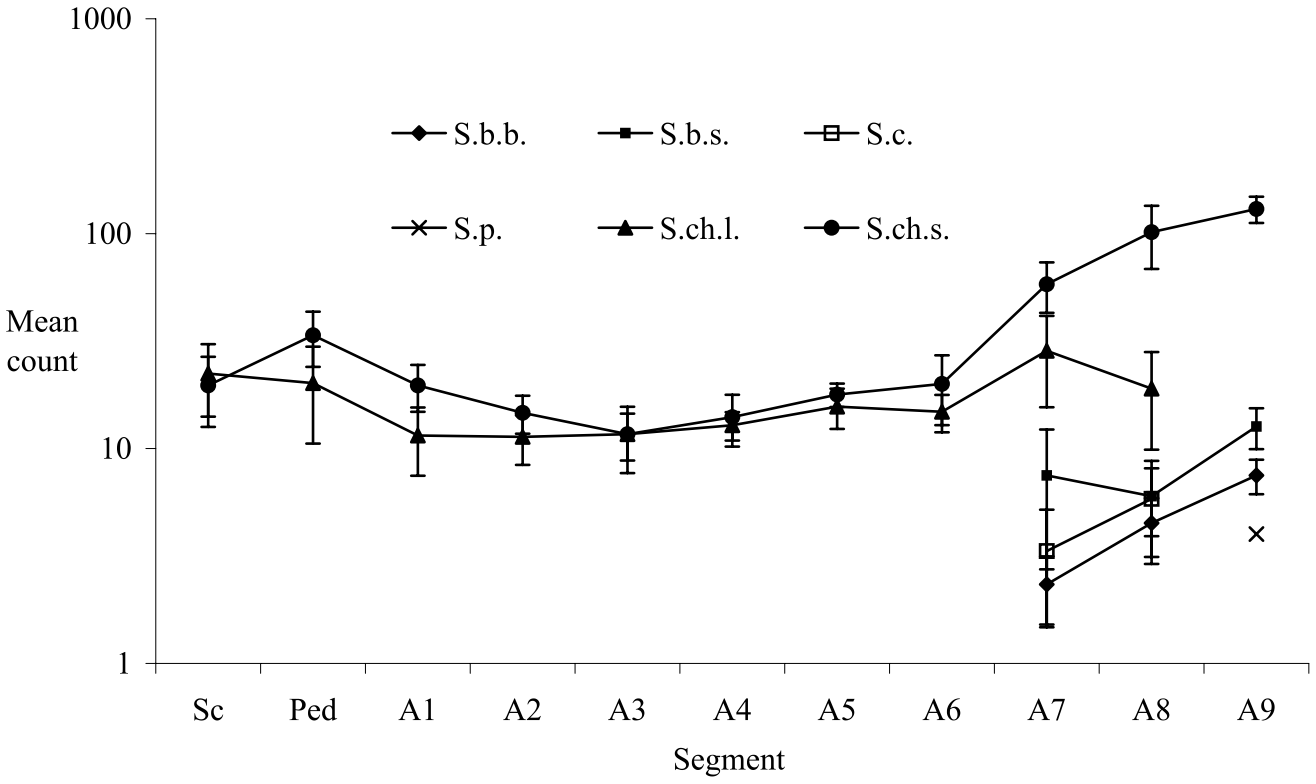

Fig. 2.2. Distribution of sensillum types on *L. nigrinus* antennae. Mean (+/- st.dev.) of males and females: n=6. S.b.b. Sensilla basiconica branched, S.b.s. Sensilla basiconica single, S.c. Sensilla coeloconica, S.p. Sensilla placodea, S.ch.l. Sensilla chaetica long, S.ch.s. Sensilla chaetica short.

Table 2.3. Range of lengths and widths at base of sensilla found on antennae of *L. nigrinus* and *P. tsugae.* Measurements taken from four sensilla of each type from each individual.

	Sensilla*	Length (um)	Width (um)
L. nigrinus	S.c.	4.2 - 5.4	1.4 - 2.1
	S.p.	1.3-1.7	3.0 - 3.2
	S.ch.s.	21.5 - 28.5	1.4 - 1.7
	S.ch.l.	55.0 - 63.0	1.4 - 2.0
	S.b.b.	10.6 - 27.0	1.7 - 1.9
	S.b.s.	14.0 - 30.6	1.7 - 1.8
P. tsugae	S.ch.l.	69.9 - 80.7	1.9 - 2.2
	S.ch.s.	23.6 - 38.0	1.9 - 2.1
	S.b.l.	26.5 - 32.5	1.9 - 2.1
	S.b.m.	17.8 - 20.9	1.3 - 1.7
	S.b.s.	4.3 - 10.9	1.0 - 1.8

* For *L. nigrinus* : S.b.b. Sensilla basiconica branched, S.b.s. Sensilla basiconica single, S.c. Sensilla coeloconica, S.ch.l. Sensilla chaetica long, S.ch.s. Sensilla chaetica short, S.p. Sensilla placodea. For *P. tsugae* : S.b.l. Sensilla basiconica long, S.b.m. Sensilla basiconica long, S.b.s. Sensilla basiconica short, S.ch.l. Sensilla chaetica long, S.ch.s. Sensilla chaetica short

In thin sections through the common base, a distinctly sectioned dendritic sheath is visible comprised of a small compartment containing a tubular body and a larger compartment partially surrounding the smaller (Fig 2.1 D). The bulbous base appears to set on a membranous foundation.

Sensilla placodea (*S.p.*)

S.p. are found solely on the terminal annulus of the antennae (Fig. 1B, 2). Generally, three are located in a triangular pattern on the internal portion of the annulus and one is located opposite the cluster of three. The plates are approximately 3.0 μm in diameter (Table 2.3), dome shaped, and surrounded by a raised ring of cuticle. A nipple centered at the peak of the dome was visible on some images. No pores were visible using SEM, and we were unable to obtain any TEM sections through this type of sensillum.

Sensilla coeloconica (*S.c.*)

S.c. are 4.2-5.4 μm in length and 1.4-2.1 μm in width at the point of insertion (Table 2.3). They are slightly sunken in a depression and surrounded by a raised ring of cuticle (Fig 2.1B). Although the wall appears smooth at the base, following a gradual reduction in width several 'fingers' project upward to the distal tip. *S.c*. were present only on the distal portion of A7 and A8, forming a ring under the projecting subsequent annulus (Fig 2.2). The one TEM section obtained through *S.c.* revealed incomplete fusion of the finger-like extensions, suggestive of a wall pore structure.

Pseudoscymnus tsugae

P. tsugae possesses a very short antennae consisting of a scape, pedicel and flagellum comprised of seven annuli (Table 1. Fig 2.3A). The scape and pedicel are the largest segments. The medial length of the annulus is shorter than that of the lateral, making the distal margin of the first annulus (A1) oblique. The resulting angle directs A2 medially when at rest (Fig 2.3A). Subsequent annuli continue this medial projection, forcing the highly fused A2-A7 into an angled position relative to the line formed by the scape, pedicel, and A1. We did not detect any sexual dimorphism (Table 2.2), thus report

pooled results for sensilla counts. Five types of sensilla were distinguished (Table 2.3, Fig 2.3, 2.4). The scape and pedicel are populated, primarily dorsally, with sensilla counts comparable to that of the terminal two annuli (A). A1-A3 are vacant of any sensilla and counts increase through the final four annuli, though composition changes. The sensilla of the terminal two annuli are concentrated into a distinct cluster.

Sensilla chaetica long (*S.ch*.l.)

S.ch.l. are bristles 69.9-80.7 μm in length and 1.9-2.5 μm in width at the base (Table 2.3). They rest in a flexible socket, are void of wall-pores, and possess longitudinal furrows on the external surface. This type of sensillum can be found on the scape, the pedicel, and A7 (Table 2.2, Fig 2.3A,C). On the scape and pedicel they are interspersed with *S.ch*.s. Two *S.ch*.l. protrude from the tip of the terminal annulus.

Sensilla chaetica short (*S.ch*.s.)

S.ch.s. are 23.6-38.0 μm in length and 1.9-2.1 μm wide at the base (Table 2.3). They possess identical morphology to *S.ch*.l., except in size (Table 2.3, Fig 2.3A). This sensillum is the most ubiquitous type on *P. tsugae*, being found on the scape, pedicel, and A4-A7 (Fig 2.4). On the scape they are found primarily on the dorsal surface, on the pedicel in a ring around the terminal portion of the segment, and generally on the dorsal side of the A4-A7.

Sensilla basiconica medium (*S.b*.m.)

S.b.m. are 17.8-20.9 μm in length and 1.3-1.7 μm in width at the base (Table 2.3). They gradually taper from the base to a blunt tip (Fig 2.3B,C). *S.b*.m. are located solely on the terminal two annuli (Fig 2.4), more densely concentrated on the medial half of each annulus. No wall pores were visible in either SEM or TEM. Thin sections revealed an inner lumen containing a concentrically divided lumen, with the inner lumen containing dendritic tissue (Fig 2.3 E).

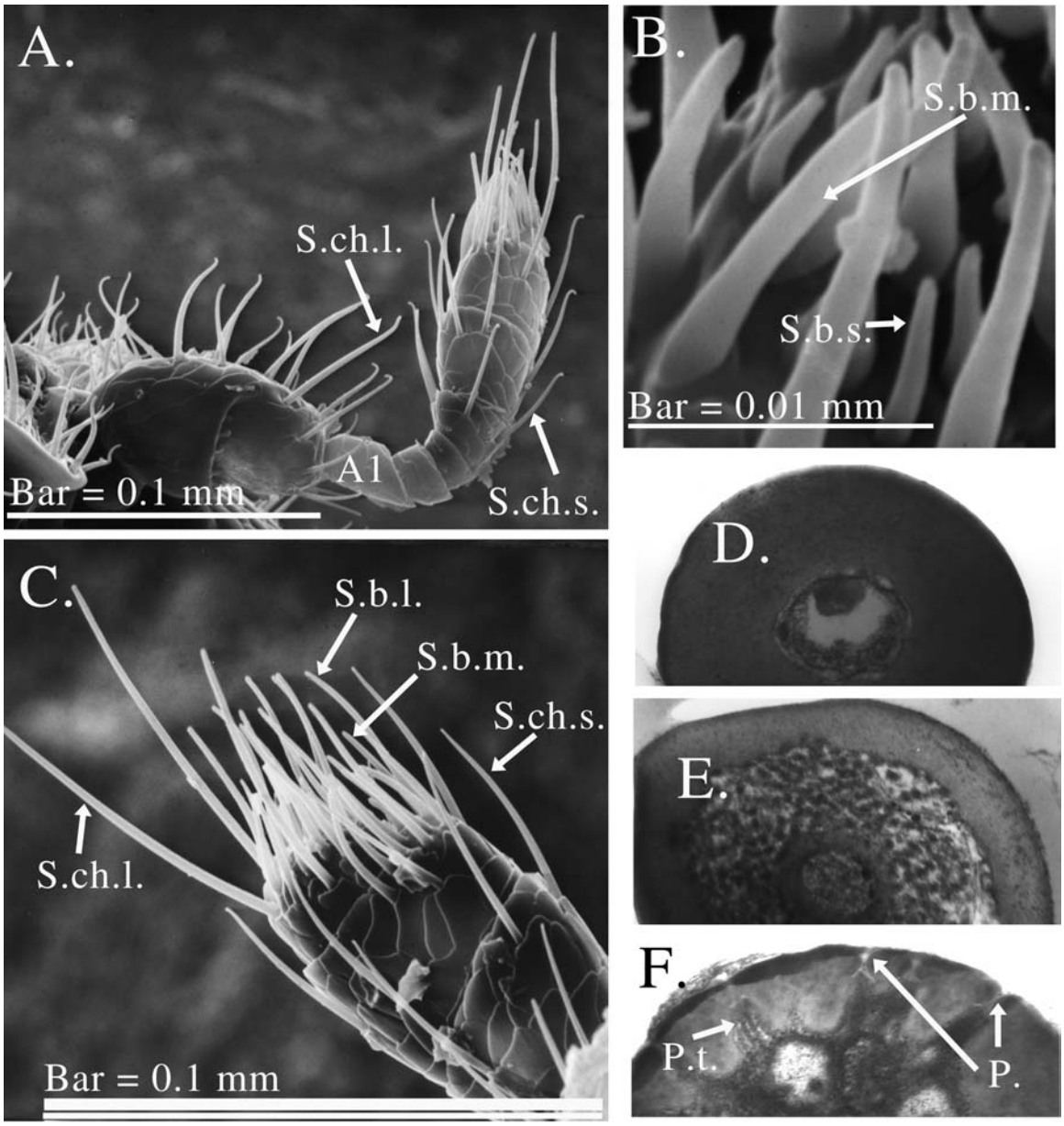

Fig. 2.3: *P. tsugae* antenna, SEM and TEM. A. Whole antenna (S.ch.l. Sensilla chaetica long, S.ch.s. Sensilla chaetica short). B. Short basiconic sensilla clustered under medium basiconic sensilla (S.b.m. Sensilla basiconica medium, S.b.s. Sensilla basiconica short). C. Antennal club: annuli 5-7 (S.b.l. Sensilla basiconica long). D. TEM cross section through long basiconic sensilla. E. TEM cross section through medium basiconic sensilla. F. TEM cross section through short basiconic sensilla (P. wall pore, P.t. Pore tubule).

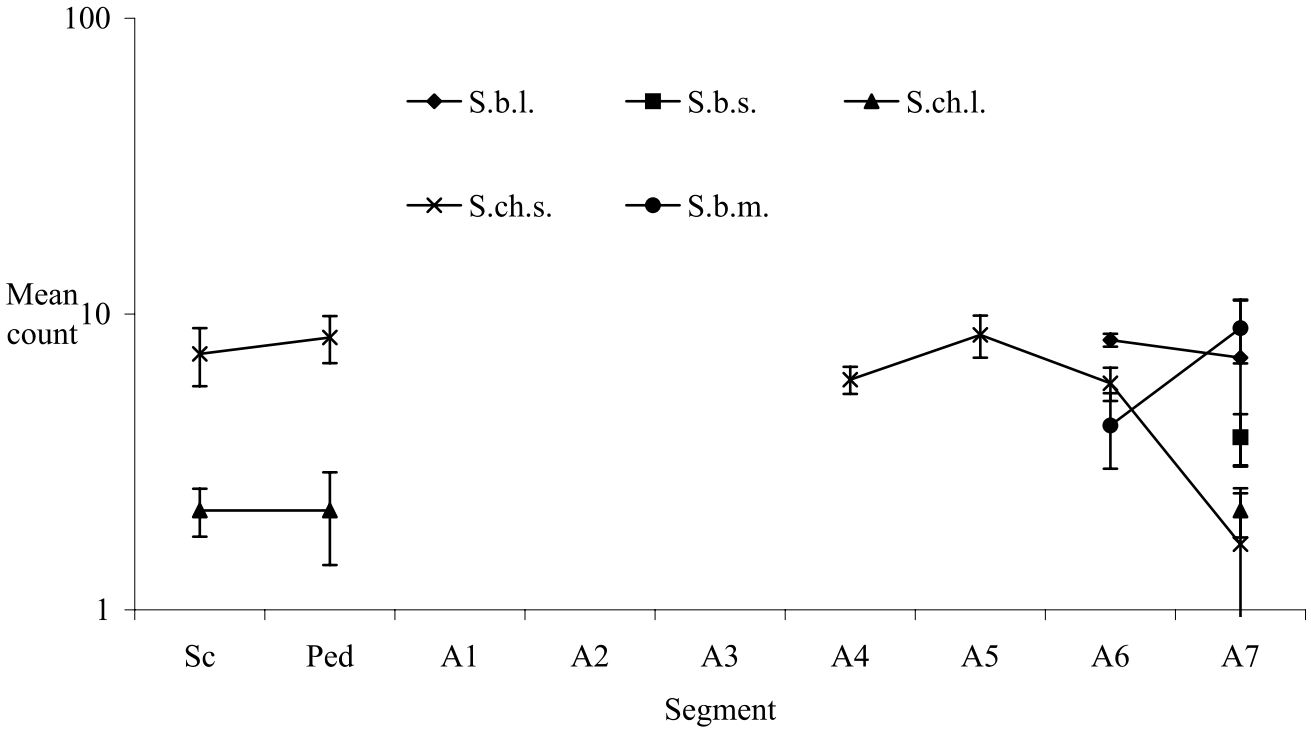

Fig 2.4. Distribution of sensillum types on *P. tsugae* antennae. Mean (+/- St. dev.) of males and females: n=6. S.b.l. Sensilla basiconica long, S.b.s Sensilla basiconica short, S.b.m. Sensilla basiconica medium, S.ch.l. Sensilla chaetica long, S.ch.s. Sensilla chaetica short

Sensilla basiconica long (*S.b*.l.)

S.b.l. are 26.5-32.5 μm in length and 1.9-2.1 μm in width at the base (Table 2.3). They taper only slightly before terminating at a blunt tip and are found only on the terminal annulus (Fig 2.3C). No wall pores were evident in TEM or SEM images. This type of sensilla extends distally beyond all other types on A7 except *S.ch.*l.

Sensilla basiconica short (*S.b*.s.)

S.b.s. are 4.3-10.9 μm in length and 1.0-1.8 μm in width at the base (Table 2.3). This type can only be found on A7 in relatively low numbers and is concealed by the longer *S.b*.m and *S.b*.l (Fig 2.3B). The wall is permeated with pores that extend into the lumen, which contains nervous tissue.

Discussion

L. nigrinus possesses six types of sensilla on densely covered, relatively long antennae. *S.b.s. and S.b.b*. are permeated with wall pores suggestive of an olfactory function. The branched basiconic sensilla seem to serve a dual mechano/chemoreceptive role, as suggested by wall pores, a sectioned dendritic sheath in sections through the common base, and a membranous insertion of the base in the cuticle. Both basiconic types are present on the distal portion of the terminal three annuli in moderate numbers. Keil (1999) attributes an olfactory function to all known placoid sensilla, but we were unable to obtain TEM sections which may have confirmed the presence of wall pores. On *L. nigrinus* the placoid sensilla are situated solely at the terminal tip of the antennae amidst *S.b*.b., *S.b*.s., and *S.ch.*s. It is difficult to imagine this being an appropriate location and orientation for contact chemoreception, being apparently inhibited from substrate contact as a result of their proximity to longer sensilla. Companiform may also function in thermo- or hygroreception. It must also be considered that it is a companiform sensilla with a mechanoreceptive function. This seems unlikely as companiform sensilla are generally sensitive to distortion of the surrounding tissue (Keil 1999) and the terminal annulus is highly sclerotized and contains no musculature.

Thin sections through the coeloconic sensilla did not reveal wall pores in the sense of single wall sensilla, though the external morphology fits the description of

olfactory coeloconic sensilla as described by Keil (1999). Additionally, we did obtain evidence suggestive of incomplete fusion of the 'fingers', and the resultant wall-pore structure. *L. nigrinus* is equipped with two to four potentially olfactory sensilla located solely on the terminal three annuli, offering promise that olfaction could be a modality used for long range host finding. The two types of *S. chaetica* are likely either mechanosensory and/or contact chemoreceptive in function. Some sections revealed a lumen containing one dendrite appressed to the interior cuticle, suggestive of mechanoreception, while others contained multiple dendritic units dispersed throughout the lumen, suggestive of chemoreception. We were unable to conclusively associate innervation type with external morphology.

P. tsugae possesses five types of sensilla on an extremely short antenna characteristic of the tribe Scymnini (Sasaji 1971). One of these types, *S.b*.s., are permeated with wall pores and are present in low numbers solely on the terminal annulus. The medium basiconic sensilla contain a partitoned lumen. The inner lumen contains sensory tissue, the outer has a homogeneous granular appearance, suggestive of a contact chemoreceptive function. The long basiconic sensilla may serve a contact chemoreceptive function, with 1-3 dendritic units visible in cross section through the sensillum. Jourdan *et al* (1995) studied the antennal morphology of *Semiadalia undecimnotata* and report a much longer antennae than that of P. tsugae, a greater diversity of sensillum types, as well as a larger number of total sensilla. Morphological results similar to that of Jourdan *et al* were noted in a study of *Hippodamia convergens* (Hamilton *et al* 1999). *H. convergens* also demonstrated a clear behavioral attraction with intact antennae, and failed to respond to the same odors when the antennae was physically removed (Hamilton *et al* 1999). The olfactory sensilla of *P. tsugae* (*S.b*.s.) may aid in short range host location, but it seems unlikely that they are used in any long-range behavioral orientation due to the paucity of wall pore sensilla. In support of the morphological data, we failed to observe a clear behavioral response to host odors from males or females in an olfactory arena experiment (chapter 3).

CHAPTER 3

Behavioral responses of two hemlock woolly adelgid specialist predators to host volatile emissions.

Introduction:

The exotic hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Homoptera: Adelgidae), is currently advancing through eastern forests at a rate of approximately 15-30 km per year (McClure *et al*. 1996, Souto et al. 1996). This pest is able to decimate individual trees as well as entire stands of both *Tsuga canadensis* (L.) Carr. (eastern hemlock) and *T. caroliniana* Engelm. (Carolina hemlock) in a period as brief as four years (McClure 1991, 1995). This trait has been attributed to a vacant natural enemy complex and an apparent lack of host defense. Material and labor costs of application as well as the ecological sensitivity and patchy distribution of hemlock dominated stands prohibit the effective use of chemical treatment in the natural setting. For this reason biological control shows the most promise. Predators have been imported to eastern North America from HWA's native Asia and from western North America where HWA has been established for at least 75 years (Annand 1924).

Two of the more promising agents discovered thus far are *Pseudoscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae). *P. tsugae* was discovered in the early 1990's and subsequently described as a new species which preyed on HWA in Japan (Sasaji and McClure 1997). Its appetite and reproductive potential suggested potential use as a biological control agent (Cheah and McClure 1998), which resulted in its importation, evaluation under quarantine, and subsequent mass rearing. This species has since been released in locations from North Carolina to Massachusetts (McClure 2001).

L. nigrinus is found associated with HWA on western hemlock, *T. heterophylla* in British Columbia, Canada (Zilahi-Balogh *et al* 2001b). The specificity of this association, as well as the basic biology of the species, is under intensive study. *L. nigrinus* feeds and completes development on only HWA, even when offered other adelgid species. This

specificity, its reproductive potential, and its voracious feeding habits suggest the ability to contribute to a successful biological control program. Caged field trials suggest that *L. nigrinus* has the potential to reduce HWA density on hemlock (Ashley Lamb and S.M. Salom, Va Tech Dept of Entomology, unpbl. data).

P. tsugae is the only mass released control agent as of yet, but the biological palette of control options continues to grow. In addition to *P. tsugae* and *L. nigrinus*, several members of the genus *Scymnus* have been imported from China; one of which is to be reared commercially beginning in 2002 (Rich Pais, Ecoscientific Solutions, pers. comm.) Three new *Laricobius* species have recently been discovered in Nepal and China, with *L. schawalleri* found in a Hemlock/Oak/Rhododendron forest type (Hava and Jelinek, 1999; Hava and Jelinek 2000; Jelinek and Hava 2001). All *Laricobius spp*. are predatory on Adelgidae (Lawrence and Hlavac 1979), offering promise of yet more specialist control agents.

To effectively evaluate the impact of a released population of control agents, researchers and IPM practitioners must accurately trace the dynamics of both pest and predator population levels. Binomial sampling is an ineffective method for estimating HWA population levels (Gray *et al* 1998) and no additional methods have been published. Current sampling methods for predators at release sites of *P. tsugae* employ beating of lower branches to estimate predator numbers. This method is imprecise at best and, in fact, often fails to account for even the presence of predators at southern Virginia release sites.

We wish to elucidate the host-finding mechanisms of *L. nigrinus* and *P. tsugae* with the intent of using this information to more accurately estimate predator presence and population density. We chose *L. nigrinus* and *P. tsugae* as our test species based on their representative nature as HWA biological control agents, as well as availability. While little information is available on host-finding mechanisms in the family Derodontidae, and more specifically the *Laricobius* genus (Franz 1958; Lawrence and Hlavac1979), the coccinellid family has been relatively well studied. Recent literature

suggests that olfaction among coccinellids is a more commonly employed modality than previously supposed. Predatory coccinellids, including *Coleomegilla maculata* (Zhu *et al*., 1999), *Chilocorus nigritus* (Ponsonby and Copland, 1995; Hattingh and Samways 1995), *Harmonia axyridis* (Obata, 1997), and *Cryptolaemus montrouzieri* (Heidari and Copland, 1992), use olfactory cues in host location at some level. Of particular interest is *C. montrouzieri,* as it and all coccinellid predators of HWA discovered in Asia, are members of the same tribe, Scymnini. Based on this recent information and the need to develop an accurate and effective predator sampling method, we set out to test the hypothesis that *P. tsugae* and *L. nigrinus* behaviorally respond to odors of HWA-infested hemlock.

Materials and Methods:

Experimental insects:

A colony of *P. tsugae* was established from individuals obtained from the New Jersey Department of Agriculture, Bureau of Biological Pest Control. *P. tsugae* were reared on field collected HWA-infested hemlock in moistened floral foam contained within one gallon plastic oviposition jars and kept in a room maintained at 25°C, 65% RH, and a photoperiod of 16L:8D (Cheah and McClure 1998). A honey/Wheast® (Planet Natural, Bozeman, MT, Prod # 176) nutritional supplement was provided. Twigs and gauze (for monitoring oviposition) were replaced weekly. Material removed from oviposition jars was then placed in a rearing chamber constructed of Plexiglas (60 x 45 x 45 cm). Each week a new rearing chamber was used. Fresh infested hemlock foliage was provided each week in moist floral foam and newly eclosed adults were collected five weeks after the eggs and foliage were first placed in the chamber.

In December 2000, *L. nigrinus* individuals were collected from infested western hemlock in British Columbia, Canada and transported to Virginia Tech for colony establishment. During the months of January to March, adults were held at 12L:12D photoperiod and 8°C day/4°C night temperature regime. In March the photoperiod was extended to 14L:10D and temperature was held at 10°C. Individuals were held at 16º C

for 24 - 72 h prior to use in the behavioral assay to allow acclimation to assay conditions. *L. nigrinus* adults were sexed through observation of mating when possible, or through manual extrusion of the genitalia following the behavioral assay. *P. tsugae* adults were sexed through examination of the penultimate abdominal segment.

Test insects consisted of reproductively mature individuals collected from colony rearing chambers. *P. tsugae* females reach sexual maturity at approximately 32 days with males developing at a similar rate (Cheah and McClure1998). Individuals greater than five weeks old were used and considered sexually mature. *L. nigrinus* emerge as adults the previous fall, thus testing in spring assures reproductive maturity. Twenty-four hours before testing food was removed, and one hour before testing predators were light deprived to stimulate positive phototaxis upon release into the arena. Positive phototaxis induced insects to climb from the entrance port to the floor of the arena, at which time behavioral analysis was initiated.

Behavioral assay:

A four-arm olfactometer similar to that used by L.M. Vet *et al* (1983) was purchased from Syntech® (Hilversum, Netherlands) and used to test behavioral responses of *P. tsugae* and *L. nigrinus* adults. The body of the star-shaped arena is constructed of ground aluminum fitted with a glass top such that under proper flow conditions four distinct fields are created to which experimental odors can be applied. Air was pulled through a center outlet port using a vacuum pump (Doerr®, LR-22132) and each arm was metered to 0.3 L/min (after Vet *et al* 1983) and controlled using flowmeters (Scott Sho-Rate®, Thomas Sci. prod #5083-D35). An additional flow meter was placed between the outlet port and the vacuum source to regulate total flow. Two flasks were attached to each arm with Tygon® tubing, the proximal containing the odor source and the distal containing distilled water for humidifying the airflow. Before entry into the flasks, the lines were merged and passed through a purifying charcoal filter. To confirm quadrant flow delineation, we exposed three of the four airflows to a desiccant in the flasks and the fourth to warm water. Indicating desiccant was spread over the floor of the arena such that the single humidified air quadrant would be made visible by the color change of the

floor desiccant. To minimize potential visual cues the arena was surrounded by black cloth and assays were conducted in a darkened room held at 22°C.

The study was initiated April 15, 2001 and completed May 17, 2001. All assays were run between the hours of 12:00 PM and 6:00 PM. Insects were individually released into the center port and the system was then sealed and behavior recorded on a Panasonic (WV-3240) color video camera for 10 minutes following emergence of the insect onto the floor of the arena. The arena was swabbed with ethanol and hexane between assays and washed with hot soapy water, then rinsed in distilled water, ethanol, and hexane between days.

Hemlock foliage infested with developing HWA was placed in one randomly assigned quadrant for treatment assays, and in none of the quadrants for control assays. Infested hemlock twigs (10 linear inches, cut into 10 portions) under humidified air were used as the treatment odor source. Controls against cut foliage contained only the distilled water humidity source. Thus, treatment assays contained one treatment quadrant and three control quadrants, and control assays contained four control quadrants. This treatment structure was used to test not only for a directional response, but also a non-directional response. As all insects in treatment assays are exposed to hemlock volatiles at the release point, a non-directed behavior may be incited before or at emergence from the entrance port and continued upon entrance to the arena floor. If such non-directional activity were to occur, comparison of behavioral parameters between treatment and control quadrants would likely not be detectable. However, comparison between treatment and control assays should detect such a response.

Statistical design and analysis:

Approximately twenty individuals were tested for each species-gender-treatment combination. For example, 20 *L. nigrinus* males were tested in treatment assays and another 20 *L. nigrinus* males were tested in control assays. Paths were traced from videotape onto transparency paper and behavioral parameters were recorded by quadrant and minute for each assay. The parameters recorded consisted of number of turns,

number of loops, flight attempts, path length on the floor of the assay, path length on the wall of the assay, total path length, walking rate, turn rate, loop rate, flight rate, time in treatment quadrant, and position relative to treatement quadrant at several times (Table 3.1). Path length was obtained by following the traced path using a map measure wheel (ASI, Switzerland: Ben Meadows Cat # 0504041). These parameters were selected due to the relative ease of obtaining measurements without computerized assistance as well as their potential behavioral significance.

Data were analyzed as an incomplete block design with day serving as the block.. Treatment effect was tested for both treatment vs. control assays (assay level comparison) and treatment vs. control quadrants (quadrant level comparison) within treatment assays for each gender within both species. Assay order was completely randomized within blocks. In SAS® (version 6.12) the PROC UNIVARIATE command with the 'normal' option was used to execute the Shapiro-Wilk test for normality; all parameter distributions were indistinguishable from Gaussian. However, the count variables had relatively high p values (greater than 0.9) in this test, suggesting that the distribution was in fact asymmetrical (SAS Institute, Sas Users guide, vers. 6.0). For this reason, assay level count data were analyzed as Poisson data ('/p' option of model statement) subjected to a χ^2 test in PROC GENMOD (SAS® Help, SAS® Institute, version 6.12) and assay level continuous data were subjected to ANOVA using contrast statements in PROC MIXED. For quadrant level comparisons within treatment assays, both count data and continuous data for behavior within the treatment quadrant were transformed to percent of total assay behavior. If behavior within the treatment quadrant were identical to that in the control quadrants, 25% of the total for each assay would occur in the treatment quadrant. Thus, 0.25 was subtracted from the calculated proportion and the difference tested against the null hypothesis of value zero using t-values in PROC MIXED. The central limit theorem for binomial data was employed for constructing confidence limits around the means for position parameters and compared to the null hypothesis of 0.25 (Oliver Schabenberger, Va Tech Dept of Stat. pers. com.). All tests were run at alpha=0.05.

Table 3.1. Description of behavioral parameters recorded in analysis of the effect of hemlock volatiles on *L. nigrinus* and *P. tsugae* behavior.

Parameter	Definition
Turns	Total number of turns completed per assay with turn defined as a deviation of greater than 90 degrees in 5.0 cm or less
Loops	Total number of loops executed during assay with a loop defined as crossing a path previously traced within 10.0 cm
Flight attempts	Total number of flight attempts per assay
Floor path	Total length of path (cm) walked on assay floor
Wall path	Total length of path walked on or along assay wall
Total path	Sum of wall path and floor path, or total path length in assay
Rate	Path (cm) per time (min)
Turn rate	Number of turns per cm of total path length
Loop rate	Number of loops per cm of total path length
Flight rate	Number of flight attempts per total path length
Time	Actual time spent in treatment quadrant of treatment assay: used only for comparison between treatment and control quadrants
Position	Binomial parameter indicating presence (1) or absence (0) in treatment quadrant at time zero minutes, ten seconds, one minute two minutes, three minutes…. ten minutes.

Results

Examination of position data revealed trends suggesting shifts in *L. nigrinus* behavior over the ten minute time period (Fig 3.1). In addition, an overabundance of zero data per minute for count parameters made statistical comparisons by minute imprecise. For these reasons, analyses were conducted per period rather than per assay or minute, with period one represented by the first three minutes of the assay, period two represented by the second three minutes and the final period encompassing the final four minutes. In general, *L. nigrinus* had a higher average walking rate and was more active (Fig. 3.2) than *P. tsugae* at assay conditions. In addition, statistical analyses demonstrated differences between males and females, thus we did not pool within species (data not shown). The time parameter revealed no information beyond that demonstrated with the position parameter and is therefore not presented.

L. nigrinus males:

In the initial time period, *L. nigrinus* males showed a tendency, albeit insignificant, towards the treatment quadrant of treatment assays (Fig. 3.1). This trend reversed in the second period when individuals were significantly more likely to be found in a control quadrant than the treatment quadrant. A slight rebound occurred at minute seven, with likelihood of being found in the treatment quadrant again tailing off toward zero over the last three minutes. No significant effect on turning behavior in response to the treatment was observed at any time period, and only during the second period was there a significant increase at the assay level on looping behavior (Table 3.2). However, flight behavior increased at the quadrant level during the first period, and at the assay level during the second period and simultaneously decreased in treatment quadrants relative to control quadrants of the treatment assays. Although no effect on either the number of loops or path length were observed, the loop rate was decreased in the treatment quadrants relative to the control quadrants. Just as flight was decreased in treatment quadrants, flight rate showed the same trend.

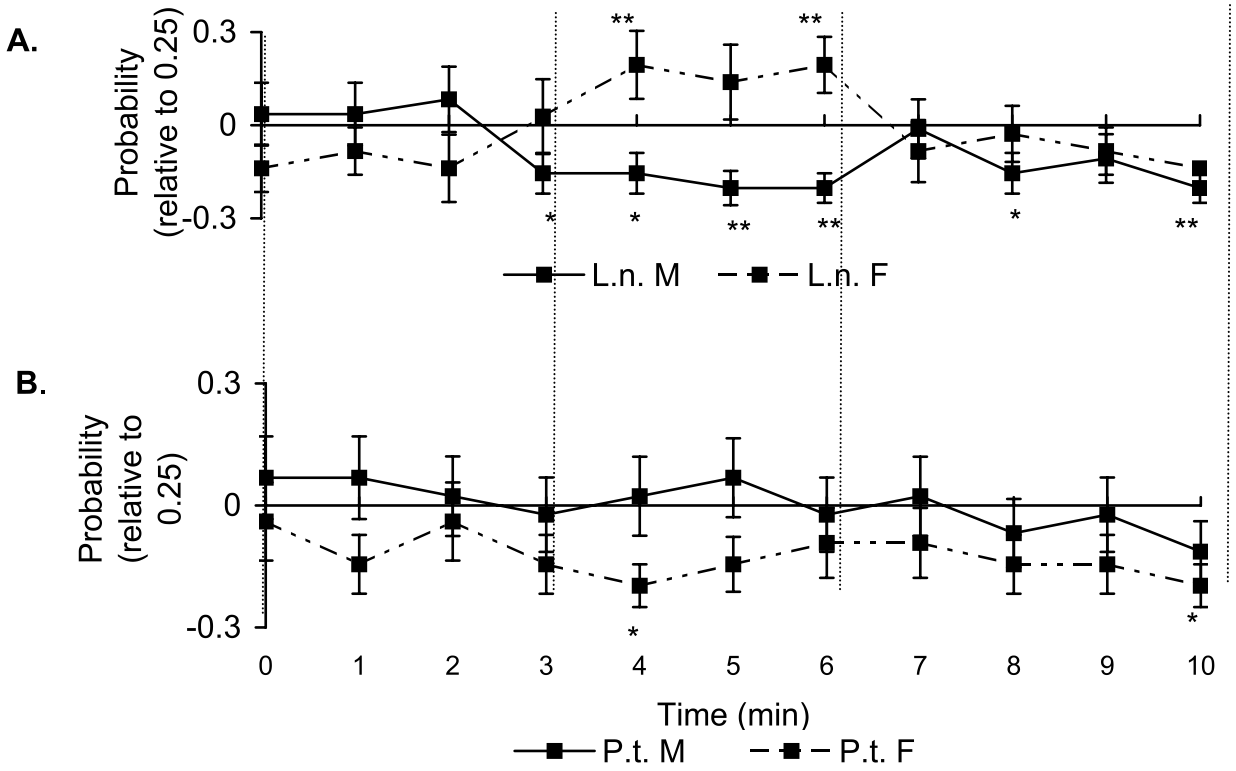

Fig 3.1. Probability of **A.** *L. nigrinus* males and females and **B.** *P. tsugae* males and females to be found in the quadrant containing host odors at specific times over the course of the ten-minute assay. Data are presented as the mean value with standard error bars. The zero line of the y-axis represents a probability of 0.25, the null hypothesis. Positive values represent an increased liklihood of being in the host-odor quadrant (attraction), negative values represent a decreased liklihood (repulsion). $p<0.1$ *, $p<0.05$ **. See methods for statistical tests performed.

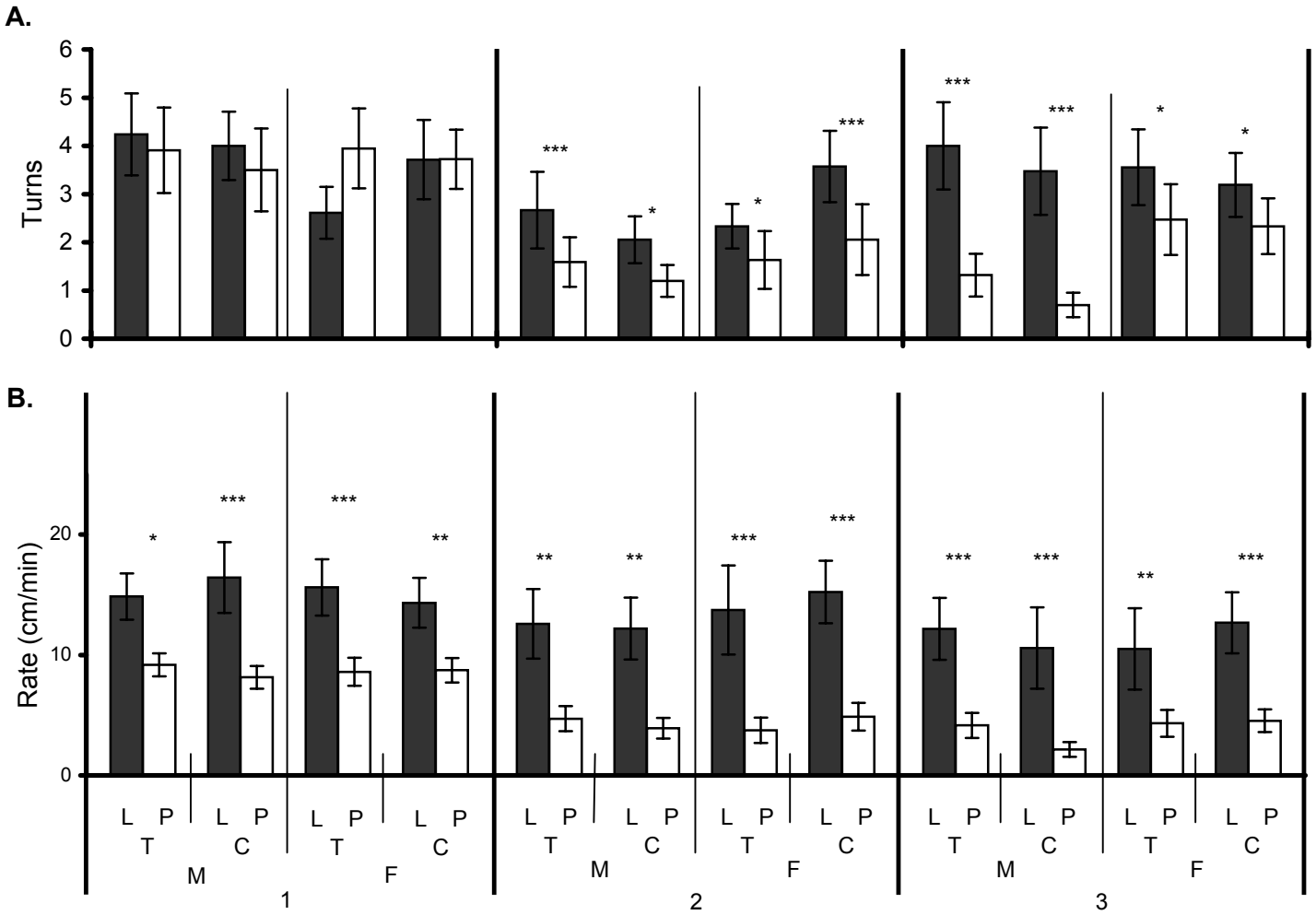

Figure 3.2. Species comparisons by period holding gender and treatment constant in **A.** turning behavior and **B.** walking rate parameters. Bars represent assay level means per period +/- S.E. $p < 0.1$ *, $p < 0.05$ **, $p < 0.01$ ***. See methods for description of statistical tests. n = 18-21. 'L' represents *L. nigrinus*, 'P' represents *P. tsugae*, 'T' for treatment quadrant, 'C' for control quadrant, 'M' for males and 'F' for females. 1, 2, and 3 represent the three time

Table 3.2. Statistical summary of the effect of hemlock volatiles on predator behavior by period. “M” and “F” stand for male and female respectively. "Q" indicates a comparison between treatment and control quadrants, which would detect a directional response to treatment odors. "A" represents comparisons between treatment and control assays which would detect a non-directional response to treatment assays. "+" indicates an increase in parameter value in response to the treatment and "-" a decrease in parameter values in response to the treatment. $p < 0.1$ *, $p < 0.05$ **, $p < 0.01$ *** with plus and minus signs replacing asterisks.

Sp*Sex	***L. nigrinus*** **male**						***L. nigrinus*** **female**						***P. tsugae*** **male**						***P. tsugae*** **female**					
Period	1		2		3		1		2		3		1		2		3		1		2		3	
Level	Q	A	Q	A	Q	A	Q	A	Q	A	Q	A	Q	A	Q	A	Q	A	Q	A	Q	A	Q	A
Turns							-	-		- -								+						
Loops				+							-	+++										++		
Flight	+++		-	++					++	-		+										-		
Wall																			- -					
Floor					-						-											-		
Path																					- -			
Turn rate																		+			- -			
Loop rate			- -												-	-								
Flight rate			- -								- - -													
Time		X	- -	X	-	X		X		X		X		X		X		X		X	- -	X	-	X
Walking rate																								

L. nigrinus females:

The position parameter for females showed an exact opposite trend to that of conspecific males during the first six minutes of the assay, with an initial trend towards repulsion followed by a significant attraction (Fig. 3.1). The final four minutes showed a similar tailing off from the null hypothesis of probability equals 0.25 toward zero. Host odor decreased turning behavior at both the quadrant and assay level in the first period and the assay level in the second period (Table 3.2). No effect on the loop parameter was seen until the third period, in which the treatment increased the number of loops at the assay level while decreasing the number of loops at the quadrant level. In the second period, flight attempts were lower in treatment assays than control assays, while within the treatment assays more flight attempts were executed in treatment quadrants than control quadrants. The only path parameter showing a significant change due to treatment was the floor parameter in the third period. The composite flight rate parameter significantly decreased in treatment quadrants as compared to control quadrants in the third period, although there was no effect at this period on either flight or path parameters.

P. tsugae males:

P. tsugae males demonstrated no consistent patterns in position relative to the treatment position (Fig. 3.1). In fact, at only one time (ten minutes) was the probability to be found in the treatment quadrant statistically distinguishable from 0.25. Very few tests indicated a significant effect of treatment on behavioral parameters (Table 3.2). An increase in both turns per assay and turn rate per assay was observed in the third period, while a decrease in loop rate was observed in the second period at both the assay and quadrant levels.

P. tsugae females:

P. tsugae females were significantly repelled by the treatment location at four and ten minutes, and showed an insignificant trend towards the control quadrants at every other time period but ten seconds (Fig. 3.1). Females responded to the host odors with an increase in loop behavior and a decrease in flight behavior at the assay level during the

second time period (Table 3.2). Also in period two, the floor path length was shorter in treatment assays as compared to control assays and total path, turn rate, and total time were lower in treatment quadrants relative to control quadrants within treatment assays. Proportionally more of the wall path occurred in control than treatment quadrants in the first period.

Discussion

The life histories of the species are important to the analysis of the results. HWA passes through two asexual generations per year, a long (9 months) sistens and a short (3 months) progrediens generation (McClure 1989, Gray and Salom 1996). The sistens generation begins in early summer when nymphs settle on newly expanded hemlock foliage, where they enter an aestival period lasting throughout the summer. In early fall, the nymphs break aestivation and resume development throughout the winter months. In early March, they mature and lay the eggs of the progrediens generation, which quickly develop in the warm spring temperatures to lay sistens eggs in late May to early June.

L. nigrinus eggs are laid in HWA ovisacs and on hemlock twigs in early spring to early summer when HWA enters its summer aestival period (G.M.G. Zilahi-Balogh *et al*, 2001a). First instar larvae hatch and feed on all stages of HWA and develop through four larval instars. Fourth instar larvae drop to the soil to pass through a prepupal stage before pupating in the soil for 2 to 3 wk and emerging as adults. Newly emerged adults remain in the soil until mid autumn, when they emerge and begin feeding on HWA sistens throughout the winter months.

P. tsugae passes through two generations per year, with maximum oviposition and development occurring during those periods in which HWA eggs are present. Whereas *L. nigrinus* pupates and aestivates in the soil, *P. tsugae* pupates on the tree for 5-10 days before emerging as an adult (Cheah and McClure 1998). The entire life cycle is completed on the tree. Although individuals of both species live for up to one year in the lab, their longevity in the field is unclear.

L. nigrinus was more active at assay conditions than *P. tsugae* (Fig. 3.2). This difference can be explained in part by the experimental temperature relative to that at which the insects are adapted, reared, and held. *P. tsugae* is reared at 25ºC and *L. nigrinus* was reared at 10ºC and then 16ºC. The cooler temperature preference *of L. nigrinus* is representative of the genus *Laricobius* and the Derodontid family (Lawrence and Hlavac 1979, Zilahi-Balogh 2001). Assay conditions were held at 22ºC, below rearing conditions for *P. tsugae* and above rearing conditions for *L. nigrinus*. However, assay conditions are representative of conditions that would be experienced in the field at the time of year assays were conducted. In addition to *L. nigrinus* behavioral adaptation to cool weather, they are approximately 50% larger than *P. tsugae* adults. These facts can explain much of the difference in rate and path length between species. However, *P. tsugae* adults were observed to spend much of the ten-minute period idle, whereas *L. nigrinus* was in motion a larger portion of the time.

L. nigrinus males exhibited a non-significant trend toward initial attraction with increased flight behavior within treatment quadrants followed by repulsion coupled to decreased flight behavior within the treatment quadrants. Assays were run in mid to late spring, which corresponds to the egg stage, hatch, and settling period of the sistens generation of HWA and the mid- to late-stages of the *L. nigrinus* oviposition period. As the food source becomes inadequate, individuals might cease foraging on HWA and initiate a flight-dependent behavior such as dispersal. An alternative explanation, yet not necessarily independent of the first, is that the volatile concentration in the treatment quadrant was too high to be considered suitable as forage. Although the concentration of hemlock volatiles was not quantified, the partitioning of the twig into sections may have resulted in a supra-optimal release rate. In the first period, only at the quadrant level was an increase in flight attempts observed, implying that a directed response was initiated. By the second period however, flight attempts, loop and flight attempts per centimeter of path, and proportion of time spent in the treatment quadrant decreased relative to control quadrants. The decreased time spent and loop-rate in the treatment quadrant suggest repulsion from the treatment odors during this time period. At the same time, the assay

level loops and flight parameters possess higher values in treatment assays than control assays. Degradation of treatment associated behavioral patterns seem to occur by the third period, although overall activity levels seem to be maintained over the ten minute period.

L. nigrinus females show a pattern in which the difference between treatment and control behaviors are most pronounced in the second period. In the first period, a lower occurrence of turning behavior in response to the treatment is observed at both the quadrant and assay level. As decreased turning in the treatment quadrant would carry the individual out of the quadrant, repulsion by the odor source is demonstrated in the position data. The quadrant level response may have been strong enough in itself to be reflected in the assay level response. By the second period, turning behavior was still decreased in treatment assays as compared to control assays, however, both flight behavior and the position parameter were high in treatment quadrants relative to control quadrants. In the third period, flight attempts per treatment assay were significantly higher than those of the control assay and treatment assay flight attempts increased in this period when compared to the first and second. All of these data may suggest an avoidance or dispersal mode triggered by the treatment odor. Supporting this hypothesis is the tendency for more loops to be executed and more of the floor path to occur in the control quadrants. Each of these behaviors would have the effect of keeping the individual out of the treatment quadrant. The treatment odor consisted of ten one-inch portions of infested hemlock; the number of cut twig surfaces may have released enough volatile emissions to result in this apparent repulsion. Repulsion and eventual dispersal from a location with hemlock/HWA volatile concentration too high might serve an adaptive function as a response to an unsuitable oviposition location or food source. Additionally, the effect of cutting the twigs has an unknown effect on the chemical composition of the volatile blend.

P. tsugae males failed to respond to hemlock/HWA odors except for an increase in turning behavior parameters in treatment assays relative to control assays in the final time period and an increase in the loop rate at both quadrant and assay level in the second

period. It is unlikely that these responses are biologically significant as three of the four tests exhibiting significance are tests for evidence of a non-directed response (assay level), while all of the parameters that demonstrate significance (turning and looping) would tend to support a directed response. In addition, the turn parameter and the turn rate parameter are highly dependent on each other as are the loop rate at the assay and quadrant levels in the same period, further reducing the biological significance of the results.

P. tsugae females are unresponsive to the treatment in the initial time period except for decreased wall path in treatment quadrant as compared to control quadrants. By the second period however, individuals exposed to treatment assays completed more loops, fewer flights and a shorter floor path than control assays. This finding seems to suggest an arrestant effect of the odor. However, within those treatment assays, more time was spent in the control quadrants than would occur randomly, more of the total path occurred in control quadrants, and the turn rate was higher in control quadrants. This pattern of behaviors suggests a repulsion pattern similar to that of *L. nigrinus* females. However, while *L. nigrinus* females increase this behavioral pattern in the third period, *P. tsugae* females abandon any recognizable response to the treatment.

L. nigrinus is thought to live only one year, with mortality occurring at the time of HWA sistens diapause. The behavior exhibited by both males and females suggests that even near the purported end of the life cycle, behavioral modifications to potentially unsuitable nutritional and ovipositional sites are evident. Flight behavior is the most notable response to treatment odors, suggesting a dispersal mode or treatment-induced flight activity coincidentally at a time of year when the host is declining in nutritional quality.

P. tsugae males and females spend the summer months on the tree, feeding lightly on settled HWA nymphs (McClure 2001). There is no evidence from our results that males use olfactory cues to find the host at this time of year. *P. tsugae* females did demonstrate an apparent weak repulsion as suggested by the position parameter at times

four and ten minutes (Fig 3.1). The avoidance response by females may be due to a concentration of hemlock/HWA volatiles too high to be recognized as suitable for feeding or oviposition and therefore avoided. However, the isolation of apparent behavioral response to only the second period arouses some doubt as to whether the response is real. In addition, electron microscopic analysis of *P. tsugae* antennae suggest that olfaction is not an important mechanism in host finding due to the small number of wall pore sensilla (Chapter 2). It seems unlikely that *P. tsugae* are dependent on olfaction in long-range host-finding behavior, although a short range mechanism may explain the apparent arrestant effect displayed by females.

Repellency due to high concentration of otherwise attractive volatile compounds is not uncommon. The southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Scolytidae), pheromone, verbenone, serves as a synergistic attractant at low concentrations and as a inhibitor of attraction at higher concentrations (Rudinsky 1973). Anderson and Fisher (1960) demonstrate a similar phenomenon for the white pine weevil, *Pissodes strobi* (Coleoptera: Curculionidae) in response to white pine bark extract and one of its components, α-pinene. Adult females of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae) find low doses of α-farnesene attractive, but are repelled by the same compound at higher doses (Hern and Dorn 1999). Bhasin *et al* (2000) found low concentrations of lactic acid to be an attractant to two *Culicoides spp*. (Diptera: Ceratopogonidae), while higher concentrations repelled the same species. With this behavioral mechanism employed in such taxonomically and ecologically diverse species, we see no reason to believe that HWA predators might not use concentration of host odors as a cue in determination of host suitability.

Although the data do not support our hypothesis that predators locate host material through olfactory cues, they do suggest that *L. nigrinus* are able to detect volatile emissions from host material. The timing of the study and the concentration of the volatile treatment may have masked any behavioral attraction. Further research needs be conducted in fall and early spring with a more dilute volatile source to better evaluate the role olfaction plays in host finding behavior.

CHAPTER 4

Effect of hemlock woolly adelgid (Homoptera: Adelgidae) on volatile emissions of eastern hemlock, *Tsuga canadensis*.

Introduction:

Eastern hemlock, *Tsuga canadensis* Carriere, is an ecologically important late succession species, with about 800,000 ha of forest characterized as hemlock or hemlock/white pine (Orwig and Foster 1998). Hemlock plays a unique role in forests of eastern North America, being extremely long lived, shade tolerant, and present in either pure stands or in combination with white pine and deciduous hardwoods. Undisturbed stands are characterized by a cool damp microclimate, low light levels, sparse understory vegetation, and a relatively stable forest composition due to hemlock's dense overstory and acidic litter. These stands are found on both exposed slopes as well as more sheltered locations such as along streams. Hemlock creates structural diversity and provides cover for a number of wildlife species (Godman and Lancaster 1990).

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, has been recognized as a major pest of eastern hemlock and the less frequently encountered Carolina hemlock, *T. caroliniana* Engelm. (McClure *et al*. 2001). HWA is presumed to have been introduced to western North American from Asia (McClure 1987) in 1924 (Annand). It was later discovered in the eastern United States in the early 1950's in Richmond, VA and has spread as far north as Massachusetts and south and west to North Carolina and West Virginia (McClure 2001). On the east coast, HWA passes through two generations per year (McClure 1989, Gray *et al.* 1998). HWA damages hemlock by feeding on ray parenchyma cells via long stylets, causing needle loss, bud mortality, and branch and tree mortality (Young *et al.* 1995). Curiously, western and Asian hemlocks are generally not fatally injured (Furniss and Carolin 1977, McClure 1995). Exposing the mechanism of this resistance may prove valuable to understanding the apparent lack of defense in eastern and Carolina hemlock.

HWA is able to decimate individual trees as well as entire stands of both eastern and Carolina hemlock in a period as brief as four years. This ability has been attributed to a vacant natural enemy complex and an apparent lack of host defense (McClure 1991, 1995). Although pesticides are an effective method for control of HWA, material and labor costs of application as well as the ecological sensitivity and patchy distribution of hemlock dominated stands prohibit the effective use of chemical treatment in the natural setting. For this reason biological control agents are actively being pursued in its native Asia and in western North America where HWA has been established for at least 75 years (Annand 1924).

Currently *Pseudoscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) is being mass reared and released, and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and *Scymnus spp*. (Coleoptera: Coccinllidae) are nearing the release stage (McClure 2001, Salom *et al.* 2001). Successful biological control requires the ability to accurately track the population dynamics of the predator and prey species. To estimate *P. tsugae* population numbers at release sites, researchers sample by beating lower branches on and near the release tree and counting the number of predators that fall onto a collection sheet. This method is imprecise and often misleading; predators are often undetected even when present. As a means of improving the sampling method, we are attempting to uncover the host finding mechanisms of the predators. It is our hypothesis that predators use olfactory cues. Therefore, we wish to identify and quantify volatile emissions of the hemlock/HWA complex that might serve as cues to specialist predators.

Foliar and twig chemistry studies revealed both intrinsic and released volatile compounds (Table 4.1) in several conifer species. Muzika *et al*. (1990) identified major constituents of blue spruce, *Picea pungens* Engelm., and grand fir *Abies grandis* Dougl. McClure and Hare (1984) analyzed intrinsic foliar terpenoids of *T. canadensis* and *T. sieboldii* Carr. and identified and quantified 15 compounds through steam distillation of plant material. Subsequent analysis revealed no significant defensive wound response to infestation by two scale insect speceies, *Fiorinia externa* and *Nucalaspis tsugae* (Homoptera: Diaspididae). However, fecundity of the scale species was impacted by

specific terpenoid concentrations, suggesting an adaptive advantage of increasing production of certain terpenoids. Von Rudloff (1975) summarized his work on the chemosystematics of conifers, including analysis of *T. canadensis*, *T. heterophylla* and *T. mertensiana*. His studies have resulted in the most comprehensive analysis of hemlock terpenoids (see Table 4.1).

A volatile sampling technique was utilized by Wibe *et al.* (1998) to determine the enantiomeric composition of monoterpenes emanating from seedlings of *Pinus sylvestris* L., branches of *Juniperus communis* L., and seedlings of *Picea abies* L. (Karst.). Shu *et al*. (1997) analyzed *Pinus strobus* L. volatile release and identified the most abundant monoterpenes. To our knowledge, *Tsuga spp*. volatile profiles have not been characterized. As it is specifically the volatile emissions of hemlock and/or HWA that predators might utilize, we have attempted to identify and quantify the volatiles associated with these two trophic levels and determine whether a *T. canadensis* wound response is expressed in the volatile profile.

Methods:

Volatile collection:

Twenty-four eastern hemlock trees were selected from a nursery plot at Price's Fork Research Center, Blacksburg, VA (UTM 17 - 545,275 E; 411,844 N). Half of the trees were treated systemically with Thiamethoxam® to eliminate HWA populations two years previous to the study. The remaining were left untreated. All trees selected were two to three meters tall and apparently of sound health, having sent out new shoots during the previous spring despite infestation with HWA. Samples were collected between 11:00 and 13:00 hours and taken while HWA sistens were in diapause, from 20 August 2001 to 28 Aug 2001.

Table 4.1: Foliar extracts and volatile emission of some conifer species. 'X' indicates presence.

Species	Camphor	Borneol	Bornyl acetate	Cadinene	Camphene	3-Carene	B-Caryophyllene	a-Copaene	a-Cubibene	Cymene	p-Cymene	Geranyl acetate	Germacrene	a-Humulene	Limonene	Linalool	Longifolene	y-Muurolene	Myrcene	a-Ocimene	cis-Ocimene	trans-Ocimene	a-Phellandrene	B-Phellandrene	a-Pinene	B-Pinene	Sabinene	a-Terpinene	y-Terpinene	a-Terpineol	Terpinen-4-ol	Terpinolene	a-Thujene	Tricyclene	Source
Compound																																			
Foliar extracts																																			
Picea pungens	X		X		X	X									X				X						X	X			X			X			Muzika et al. 1990
Abies grandis	X		X		X										X				X					X	X	X						X		X	Muzika et al. 1990
Tsuga seiboldii		X	X		X					X		X			X	X			X				X		X	X		X	X	X	X				McClure and Hare 1984
Tsuga canadensis		X	X		X					X		X			X	X			X				X		X	X		X	X	X	X				McClure and Hare 1984
Tsuga canadensis		X	X		X	X				X	X	X			X	X			X		X	X	X	X	X	X	X	X	X	X	X	X		X	Von Rudloff 1975
Pinus tropicalis					X										X				X					X	X	X						X		X	Valterova et al. 1995
*Pinus caribaea var. 1***					X	X				X	X				X				X				X	X	X	X			X			X	X	X	Valterova et al. 1995
*Pinus caribaea var. 2***			X	X	X	X	X	X	X		X		X	X	X		X	X	X	X			X	X	X	X	X		X						Barnola et al. 1997
Pinus maestrensis					X					X	X				X				X				X	X	X	X			X			X		X	Valterova et al. 1995
Pinus cubensis					X					X	X				X				X		X	X		X	X	X		X	X			X		X	Valterova et al. 1995
Volatile extracts																																			
Picea abies					X	X									X									X	X	X	X								Wibe et al. 1998
Pinus sylvestris					X	X									X									X	X	X	X								Wibe et al. 1998
Juniperus communis					X	X									X									X	X	X	X								Wibe et al. 1998
Pinus strobus	X	X	X		X	X									X				X					X	X	X	X					X			Shu et al.

**var. 1 = *caribaea*
var. 2 = *hondurensis*

Volatile collection was performed by cutting 24 hemlock branch tips (approximately 10.0 cm each) from each tree. To eliminate potential variability due to aspect or relative crown position, two tips were removed from the upper-crown, two from mid-crown, and two from the lower-crown from each of four cardinal directions. As a result of the stringent nature of tip selection and the patchy in-tree distribution of HWA, not all branch tips were infested. Priority was given to branch tip location. Once removed from the tree, the cut end was immediately inserted into a disc of floral foam (Oasis® floral products: 2 cm, 5 cm diameter) saturated with distilled water. The floral foam disc containing 24 twigs was placed into a one pint glass mason jar, and sealed with a size 13 rubber stopper containing an inlet and outlet port of glass pipette connected via Teflon® tubing. A flow of compressed air (0.5 L/min) was established through a charcoal filter, over the cut foliage, and through a glass pipette containing 0.6 g of Porapak® Q adsorbent backed on either side by glass wool. The adsorbant trap was exposed to the airflow for 60 minutes. No trap breakthrough was evident using this regimen.

In trails previous to this experiment, methanol extracts yielded no consistent peaks. At the end of the collection period, volatile compounds were extracted from the trap with 2.5 mL of 0.01 mg/mL (+)-longifolene (internal standard see peak 15, fig.1) in hexane applied in 0.5 mL aliquots. The solvent extract was then reduced to 0.1 mL under a gentle nitrogen flow and stored at -60°C until analyses were performed. The use of the less volatile sesquiterpene, (+)-longifolene, as an internal standard likely resulted in a slight underestimation of monoterpene loss while concentrating under nitrogen flow. However, it was necessary to choose a compound that did not interfere with hemlock volatiles on either column. Branch tips were weighed immediately following volatile collection for determination of wet weight. Although the weight of the twig may have been slightly altered during the volatile collection period due to the uptake of water, we considered the immediate insertion of the twig into a water source important in preserving the integrity of the sample. Additionally, the weight would likely be altered consistently in both infested and uninfested samples. We randomly selected 12 of the 24 branch tips and recorded branch tip length and number of adelgids present to estimate

adelgid density (HWA density) per sample and recorded the percent of each twig and sample that was current year growth (percent new growth).

Separation and identification:

A two microliter splitless injection was made on a Perkin Elmer® Autosytem gas chromatograph; separation was executed using a DB-5 column (30m, 0.32 mm O.D., 0.25 um film, J and W Scientific®) with an initial temperature of 50°C (8 min) ramped at 2°C/min to 70°C (0 min) and then to 200°C (20 min) at 20°C/min. Isomeric content was examined using a β-Dex 120 capillary CG column (30 meters, 0.25 mm, 0.25 um film, O.D., Supelco®). Oven temperature was initially set to 70°C (8 min) and then ramped at 3°C/min to 175°C and held for 15 minutes. Column pressure was set to 35 psi. All samples were run within 8 weeks of collection. Concentration was determined through comparison of peak areas in collected samples to a dilution series of known concentration, with adjustments made based on the loss of internal standard. Tricyclene concentration was calculated using the α-pinene dose response curve, as high purity tricyclene is commercially unavailable. This calculation is reasonable because flame ionization detector response is dependent on carbon number and saturation level (McNair and Miller 1997). α-pinene and tricyclene are structurally very similar, with identical molecular weight and similar retention times. After obtaining a total release, we adjusted the release by dividing total release by wet weight of the sample to standardize between samples.

Constituent compounds were tentatively identified on a HP 5790A Series gas chromatograph coupled to a VG Analytical Mass Spectrometry system utilizing electron impact ionization (70kev) and quadrupole mass separation. Identification was confirmed by comparing retention times of hemlock samples to commercially available standards (see table 4.2) on DB5 and β-Dex 120 columns. We were unable to obtain optically pure α-phellandrene for isomeric identification, thus we report the pooled plus and minus isomers simply as α-phellandrene. β-phellandrene was also unobtainable, thus our

Table 4.2. Compounds used for identification and quantification of volatiles emitted from *T. canadensis* twigs.

Compound	Source[A]	Purity (%)
(-) bornyl acetate	1	97
(-)-camphene	1	80
Camphene	1	95
Hexanes	4	100
Limonene	2	95
(+)-longifolene	1	98
Myrcene	1	99
α-phellandrene	2	98
(1S)-(-)-α-pinene	1	98
α-pinene	1	98
(1s)-(-)-β-pinene	1	99
(+)-β-pinene	1	98
Terpinolene	2	89
Tricyclene*	3	18

[A] 1. Aldrich, Milwaukee, WI. 2. Hercules, Wilmington, DE. 3. Rodney Croteau, Washington State University. 4. Mallinckrodt, Thomas scientific, product number C38920. *Tricyclene was used only for retention time confirmation, see methods.

identification is reliant on the mass spectra and the work by von Rudloff (1975) on hemlock consituent terpenes.

Statistical design and analysis

A generalized randomized block design was used, with two samples of each treatment being taken during each of six blocks (days). Thus the stistical model contained a main effect (HWA), a block (Day) and an interaction term (HWA*Day). Analysis was executed in PROC MIXED (SAS® v 6.12) to compare the release rate of infested and uninfested trees for each compound and total monterpene emission rate. Each individual compound was converted to a mass proportion of total monterpene emission and treatment vs control trees were compared by conducting χ^2 tests in PROC MIXED. Isomeric proportions were compared in the same fashion.

Results

HWA density was extremely variable in the infested category, with densities on individual twigs ranging from 0 to 7.27 HWA/cm of twig within a sample from an individual tree. The range of values of mean HWA-density per sample (or tree) was smaller, from 0 - 1.01 HWA/cm of twig. Although some of our samples did not contain HWA on the twigs examined for HWA density estimates, the twigs were taken from trees supporting HWA populations. We therefore continue to consider them infested samples. None of the twigs sampled from the uninfested category contained adelgids.

Of the total twig sample from individual trees, samples from infested trees ranged from 93 to 100 percent new growth (mean ± s.d. = 98.34 ± 2.24), while those from uninfested samples contained 98 to 100 percent new growth (mean ± s.d. = 99.64 ± 0.68). Despite the seemingly small difference between the two values, there was a significant negative relationship between HWA population density and percent of sample that is current year growth ($r^2 = 0.3023$, $\beta = -0.0779$, $p = 0.0080$). This result is to be expected, as HWA-feeding on eastern hemlock results in a reduction in growth at branch tips (McClure 1991).

Ten compounds were identified as being consistent components of the hemlock volatile profile (Fig 4.1, Table 4.3). α- and β-pinene, camphene, limonene and α- and β-phellandrene can exist as (+)- or (-)-isomers. It appears that all were expressed as both isomers, although one β-pinene and one α-phellandrene isomer coeluted, making confident identification and quantification impossible. We thus report only the isomers we confidently identified. In addition, a peak immediately following the limonene peak had a retention time suggestive of an optical isomer, however the mass spectra of the second peak possessed extra ions not in the standard, thus we only consider the first peak (Fig. 4.1, peak 9). Isomeric composition was not significantly altered by HWA for any identified compound (not shown).

Infested hemlock tended to have a higher release rate for all compounds (Table 4.3), but due to high variability, many compounds failed to demonstrate a statistically significant change at the 0.05 level. Many of those not significant at the 0.05 level were significant at the 0.10 level, and only the test for bornyl acetate resulted in a p-value larger than 0.15. The treatment*block interaction was significant for minus and total α-pinene and myrcene. In addition to the increased release due to HWA, there is a slight but significant change in the relative proportion of certain compounds (Table 4.3). The portion of the terpene profile represented by total α-pinene and its minus isomer was lower for infested foliage as compared to uninfested foliage. Terpinolene and β-phellandrene proportions were higher for infested foliage.

Inclusion of HWA density or percent new-growth as covariables failed to improve the analysis of variance. However regression analysis demonstrated a significant correlation between release rates of several compounds and percent new growth (Table 4.4).

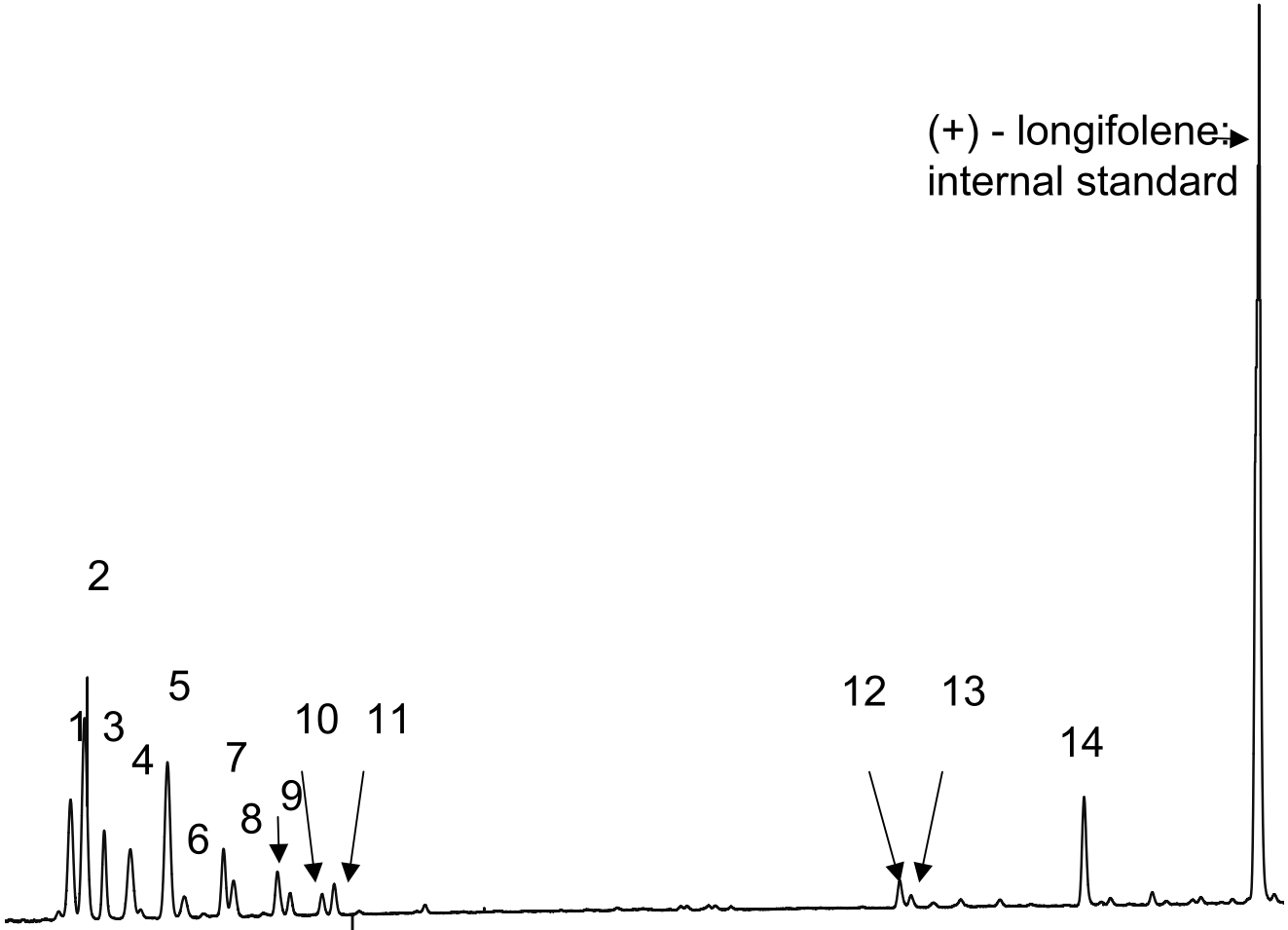

Fig 4.1. Chromatographic trace of hemlock volatiles on Betadex120 column. See table 4.3 for peak identification

Table 4.3: Volatile emissions from eastern hemlock foliage infested with and uninfested with HWA: A. release rate for each compound (ng/g wet wt/hr). B. Proportion of total profile represented by each component (ng compond rate / ng total terpenes rate).

A.	Mean release rate (ng/g wet wt/hr)				Statistical results			
	Infested foliage		Uninfested foliage		HWA main effect		HWA*Block	
Compound	mean	s.e.	mean	s.e.	F	p>F	F	p>F
(-)-α-pinene	239.02	64.19	163.02	35.56	2.49	0.148	3.47	0.045
(+)-α-pinene	355.18	87.99	205.03	22.57	3.12	0.108	1.50	0.273
Total α-pinene	594.20	145.55	368.04	53.07	4.08	0.071	2.96	0.068
Myrcene	115.16	26.46	66.71	12.14	5.38	0.043	2.99	0.067
Tricyclene	39.12	13.16	10.36	1.76	3.05	0.111	0.56	0.732
(-)-camphene	115.99	39.36	31.86	4.97	3.03	0.113	0.58	0.715
(+)-camphene	8.59	2.54	2.66	0.78	3.12	0.108	0.45	0.807
Total camphene	124.58	41.85	34.52	5.62	3.05	0.112	0.57	0.722
α-phellandrene	60.08	16.23	26.89	3.14	4.59	0.058	1.60	0.246
(-)-β-pinene	33.18	8.89	16.58	3.50	3.38	0.096	1.62	0.242
Limonene	9.49	2.25	5.42	1.17	3.16	0.106	1.86	0.189
β-phellandrene	46.29	10.72	20.34	6.66	5.05	0.048	1.51	0.269
Terpinolene	2.03	0.78	0.00	0.00	5.32	0.044	0.73	0.617
Bornyl acetate	24.21	4.64	14.22	2.37	2.35	0.157	0.56	0.732
Total terpenes	1048.33	261.91	563.09	79.41	4.41	0.062	2.04	0.158

B.	Mean proportion of total monoterpenes				Statistical results			
	Infested foliage		Uninfested foliage		HWA main effect		HWA*Block	
Compound	mean	s.e.	mean	s.e.	χ^2	p>χ^2	χ^2	p>χ^2
(-)-α-pinene	0.23	0.02	0.28	0.02	3.52	0.061	4.57	0.510
(+)-α-pinene	0.35	0.01	0.38	0.02	2.53	0.112	8.07	0.242
Total α-pinene	0.57	0.02	0.66	0.02	11.35	0.001	6.51	0.260
Myrcene	0.12	0.01	0.12	0.01	0.00	0.965	4.78	0.443
Tricyclene	0.03	0.01	0.02	0.00	2.18	0.140	0.94	0.967
(-)-camphene	0.09	0.02	0.06	0.01	2.69	0.101	0.69	0.984
(+)-camphene	0.01	0.00	0.00	0.00	0.99	0.320	1.76	0.881
Total camphene	0.10	0.02	0.06	0.01	2.62	0.105	0.67	0.984
α-phellandrene	0.06	0.01	0.05	0.00	0.99	0.321	4.31	0.505
(-)-β-pinene	0.03	0.00	0.03	0.01	0.17	0.683	7.08	0.215
Limonene	0.01	0.00	0.01	0.00	0.68	0.410	5.16	0.397
β-phellandrene	0.05	0.01	0.03	0.01	3.37	0.096	3.19	0.671
Terpinolene	0.00	0.00	0.00	0.00	23.03	0.000	10.86	0.054
Bornyl acetate	0.03	0.00	0.03	0.00	0.07	0.789	2.27	0.802

Table 4.4. Summary of regression analyses of percent new growth of eastern hemlock samples vs. compound release rate

Compound	β	r^2	p < t
Total terpene	-234.781	0.3470	0.0039
(-)-α-pinene	-68.098	0.4621	0.0005
(+)-α-pinene	-70.931	0.2901	0.0097
Total α-pinene	-139.028	0.3987	0.0016
Myrcene	-25.465	0.3759	0.0024
Tricyclene	-7.771	0.1500	0.0749
(-)-Camphene	-23.952	0.1606	0.0646
(+)-Camphene	-1.802	0.2027	0.0355
Total Camphene	-25.754	0.1637	0.0618
α-phellandrene	-14.504	0.3469	0.0039
(-)-β-Pinene	-7.962	0.3340	0.0048
Limonene	-1.867	0.2711	0.0130
β-Phellandrene	-9.863	0.2924	0.0094
Terpinolene	-0.612	0.2489	0.0181
Bornyl acetate	-1.952	0.0673	0.2436

Discussion

Given the high levels of variability in monterpene release rate, we relaxed our definition of statistical significance. Release rate from infested foliage as compared to uninfested foliage was considerably higher for all compounds, but analysis of variance was generally not able to separate the two treatments at the 0.05 level. However, every test for altered release rate resulted in $p < 0.15$ except bornyl acetate. We consider the extremely high levels of significance for terpinolene a statistical artifact. This compound was present in doses slightly above the quantifiable limit of detection in infested samples. It was likely not entirely absent in uninfested samples, simply below the limit of detection. Despite high variability, the trend is clear, infested foliage tends to release monoterpenes at a higher rate than uninfested foliage. It is unclear why variability was so high, although natural biological variation is a likely candidate. Von Rudloff (1975) was able to characterize geographically isolated populations of *T. canadensis* through solvent extraction of foliage from as few as ten individuals. Muzika *et al.* (1990) demonstrated that variability is affected by extraction techniques, with two techniques demonstrating coefficients of variation for foliar concentration frequently as high as 100%. We observed a similar level of variation in release rate in this study.

Our identification is qualitatively in agreement with the results of solvent extracts of *T. canadensis* foliage (McClure and Hare 1984, von Rudloff 1975), although we expectedly encountered fewer compounds at quantifiable levels. The percent composition is altered in our study as compared to those utilizing solvent extracts, with less volatile oxygenated monoterpenes representing a much lower proportion in the volatile samples. For example, McClure and Hare found that bornyl acetate comprised approximately 33% of the extracted terpenoids, whereas the volatile profile is only 3% by mass bornyl acetate.

T. canadensis may actively respond with an increase in terpene production. A herbivore-induced response in ponderosa pine (*Pinus ponderosa)* increased monoterpene cyclase activity without increasing the intrinsic concentration of foliar terpenes, likely

due to a higher rate of volatilization (Litvak and Monson 1998). The increased monoterpene release rate from *T. canadensis* may be due to a similar process.

Alternatively, the observed changes in release rate may be an indirect result of HWA feeding. HWA-feeding results in a decrease in new growth at branch tips (McClure 1991). Release rate was highly correlated with percent new growth of the sample. Thus, as HWA population levels increase, and new growth decreases, the volatile profile must necessarily change.

The differential response exhibited by various compounds and isomers suggests that changes in production of monoterpenes might be governed by more than one metabolic pathway. Myrcene and (+)- and (-)-α-pinene release show the smallest increase (approx 1.7 times) following exposure to HWA feeding (table 4.5), while tricyclene and (+)- and (-)-camphene showed the greatest increased (approx 3.4 times). *A. grandi* contains at least seven monterpene cyclases, the products of which account for a large portion of monoterpenes produced in this species (Bohlmann *et al.* 1997, 1999). It is possible that a few to several paths may contribute to the observed hemlock profile as well (Table 4.5). More work needs to be done to uncover the effect of HWA on eastern hemlock physiology.

Regardless of the biochemical mechanism driving the observed changes in the volatile profile, we have demonstrated that the changes could be used as indicators of the presence of HWA, if predators use olfactory cues. In related experiments, the specialist biological control agent *Laricobius nigrinus* was shown to possess several types of olfactory sensilla (Chapter 2) and behaviorally respond to HWA-infested hemlock odors (Chapter 3), offering promise that we may be able to utilize this host-finding mechanism to develop a more accurate sampling procedure.

Table 4.5. Ratio of mean release rate of total terpenes from *T. canadensis* foliage: infested (I) foliage to uninfested foliage (U).

Compound	I/U ratio
(-)-α-pinene	1.466
(+)-α-pinene	1.732
Myrcene	1.726
Limonene	1.751
(-)-β-pinene	2.001
α-phellandrene	2.234
β-phellandrene	2.276
(+)-camphene	3.229
(-)-camphene	3.640
Tricyclene	3.776

CHAPTER 5
Conclusions and Summary

L. nigrinus possesses several antennal sensilla that may serve an olfactory function on the three terminal annuli. In addition, this predator displayed a behavioral response to olfactory cues from HWA-infested hemlock foliage. This response consisted of altered flight behavior in both males and females and decreased turning behavior in females. The altered flight behavior offers promise that the observed behavioral response is part of a long-range host location mechanism. However, we did not observe a clear behavioral attraction to the olfactory cues. Although we did not demonstrate behavioral attraction, our data suggest that *L. nigrinus* does modify its behavior following exposure to volatile cues. More behavioral assays should be conducted as adults emerge from aestivation and with varying concentrations of hemlock/HWA volatiles to determine whether an attractive volatile blend exists.

P. tsugae was found to possess one type of sensillum that contained numerous wall pores suggestive of an olfactory function. This type of sensillum was present in rather low numbers on the terminal annulus, suggesting olfaction is not a primary host-finding modality. In concordance with the morphological data, we were unable to demonstrate behavioral modification as a result of exposure to hemlock/HWA odor. It is possible that the time of year or volatile concentration prevented behavioral attraction. However, this scenario seems unlikely due to the apparent lack of any behavioral modification at assay conditions.

Given that one of the predator species potentially uses olfactory cues in long range host location, volatile emissions from the hemlock/HWA complex were identified and quantified. We were unable to detect any cues emitted directly from HWA, although it is impossible to rule out a release rate of compound(s) below our minimum detection level. Ten compounds were consistently expressed in the volatile profile. Infested branch tips released volatile monoterpenes at a rate 1.7 to 3.4 times higher than that of uninfested branch tips. Due to high variability, mean separation tests were sometimes

inconclusive at the 0.05 level. In addition, the percent composition of the monoterpene profile is altered slightly, with α-pinene comprising 57% of total terpene emission in infested foliage as opposed to 66% in uninfested foliage. These results should encourage further research to attempt to elucidate the function of this change, whether the response is exclusive to HWA infestation, and why HWA is unaffected by the increased production of often toxic monoterpenes.

Incorporating all of the data provides us with a framework from which we can focus future research. It is possible that *L. nigrinus* uses olfactory cues in long range host location, but additional behavioral tests are needed to demonstrate attraction and electrophysiological examinations are necessary to determine which compounds are being used. *P. tsugae* is likely using a sensory modality other than olfaction in long range host selection. Examinations focusing on visual cues such as color and shape may improve understanding of the innate host-finding mechanism. Predators able to detect olfactory cues might be able to utilize the altered release rate or subtle change in percent composition as a cue for the presence of a HWA or herbivore-induced stress. We have identified the major compounds emanating from hemlock foliage, which will allow for a synthetic hemlock odor to be produced. This synthetic odor has the potential to simplify sampling for HWA biological control agents such as *L. nigrinus* that may use olfaction in long-range host location.

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Zilahi-Balogh, G.M. 2001. Biology of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and its Potential as a Biological Control Agent of the Hemlock Woolly Adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae) in the eastern United States. PhD dissertation. Virginia Tech, Blacksburg, VA. 121 pp.

Zilahi-Balogh, G.M.G., L.T. Kok and S.M. Salom. 2001. Host specificity of *Laricobius nigrinus* Fender (Coleoptera; Derodontidae), a potential biological control agent of the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). Biological Control (accepted - 23 Oct 2001).

Corey David Broeckling
216 Price Hall
Department of Entomology
Blacksburg, VA 24061
(540) 231-4029
cbroeckl@vt.edu

BORN: August 13, 1976 Albers, Illinois

RESEARCH INTERESTS: Chemical ecology of insect-plant interactions, plant defense against herbivores, general natural history.

ACADEMIC DEGREES:

B.S. Environmental Science with Chemistry minor, 1998
Quincy University
GPA: 3.2

M.S. Entomology, 2001
Virginia Polytechnic Institute and State University
Thesis: "The Role Olfaction Plays in Host Finding for Two Specialist Predators of Hemlock Woolly Adelgid"
GPA: 3.30

RESEARCH EXPERIENCE:

M.S. candidate (August 1999- 2002)
Virginia Polytechnic Institute and State University
Advisor: Dr. Scott Salom
Researched the role of olfaction in host-finding by specialist predators of hemlock woolly adelgid using gas chromatography/mass spectrometry, electrophysiology, electron microscopy, and behavior assays. Established and maintained a colony of *Pseudoscymnus tsugae* beetles as well as gas chromatography and electrophysiology equipment for the Entomology Department.

Biological Technician (September 1998- August 1999)
U.S. Department of Agriculture, Dr. Joseph Dickens
Research focused on developing an olfactory attractant for the insect pests *Lygus lineolaris* and *Leptinotarsa decimlineata* using behavior and electrophysiology assays.

Biological Technician (June 1998- August 1998)
University of Notre Dame, Dr. David Lodge
Examined the effects of waterfowl herbivory on aquatic plant communities by field observation and data collection and analysis. Also researched the scientific literature on aquatic invasive species in the Great Lakes basin for incorporation into a predictive model.

GIS Technician (June 1997- September 1997)
City of Quincy, IL
Generated a comprehensive database on municipal flora in city right-of-ways using a lap-top computer equipped with Arcview GIS.

RESEARCH GRANTS:

Graduate Research Development Project
"Olfaction in Specialist Predators of Hemlock Woolly Adelgid"
Virginia Polytechnic Institute and State University
December 2000: $300

MEETING PRESENTATIONS:

Broeckling, C.D. Investigations of HWA predators using olfaction to locate host habitat. Hemlock woolly adelgid symposium: Feb 5-7, 2002. East Brunswick, New Jersey.

Broeckling, C.D. and S.M. Salom. "Antennal morphology and behavioral response to host volatiles of *Pseudoscymnus tsugae* (Coleoptera: Coccinellidae) and *Laricobius nigrinus* (Coleoptera: Derodontidae), two specialist predators of Hemlock Woolly Adelgid, *Adelges tsugae* (Homoptera: Adelgidae)." Annual meeting of the Entomological Society of America, December 2001. Presidents Prize talk.

Broeckling, C.D., M. McClannan, S. Satterlee and K.L. Tabor. 2001. "Resistance management for *Bt* genetically modified crops is insufficient." Presented as part of the student debate in the plenary session of the Annual meeting of the Entomological Society of America, December, 2001.

Salom, S.M. and C.D. Broeckling. "Effect of Hemlock Woolly Adelgid feeding on volatile emissions of Eastern Hemlock (*Tsuga canadensis*). Annual meeting of the Entomological Society of America, December, 2001.

Broeckling, C.D. and S.M. Salom. "Examination of the Role of Olfaction in HWA Predator Host Finding." Southern Forest Insect Work Conference, July 21-26, 2001.

Broeckling, C.D. and S.M. Salom. "Olfactory capabilities of *Pseudoscymnus tsugae*, a specialist predator of hemlock woolly adelgid." Southern Appalachian Forest Entomologist and Pathologist Workshop, February 15-16, 2001.

Broeckling, C.D. and S.M. Salom. "Olfaction in *Pseudoscymnus tsugae* (Coleoptera: Coccinellidae), a specialist predator of hemlock woolly adelgid (Homoptera: Adelgidae)." Annual Meeting of the Entomological Society of America, December 3-6, 2000.

Broeckling, C.D. and S.M. Salom. "Olfaction in *Pseudoscymnus tsugae* (Coleoptera: Coccinellidae), a predator of hemlock woolly adelgid." Southern Appalachian Forest Entomologist and Pathologist Workshop, February, 2000.

Broeckling, C.D. and S.M. Salom. "Olfaction in specialist predators of Hemlock Woolly Adelgid." Southern Forest Insect Work Conference, July, 2000. Jekyll Island, GA.

SEMINARS

Virginia Christmas Tree Growers Association. White Top, VA; March, 2001. "Can We Stop the Hemlock Woolly Adelgid?"

Entomology seminar. Virginia Tech, January, 2000. "Research Proposal: Olfaction in *Pseudoscymnus tsugae*, a specialist predator of Hemlock Woolly Adelgid."

PUBLICATIONS:

Broeckling, C.D. and S.M. Salom. 2002 . Behavioral Responses of Two Hemlock Woolly Adelgid Specialist Predators to Host Volatile Emissions. In: Proceedings of the second hemlock woolly adelgid symposium: Feb 5-7, 2002. East Brunswick, NJ. USDA-FS-FHTET. (in press).

Broeckling, C.D. and S.M. Salom. 2002 Abstract: Effect of HWA Feeding on Volatile Emissions of Eastern Hemlock, *Tsuga canadensis*. In: Proceedings of the second hemlock woolly adelgid symposium: Feb 5-7, 2002. East Brunswick, NJ. USDA-FS-FHTET.(in press).

Broeckling, C.D., M. McClannan, S. Satterlee and K.L. Tabor. 2001. "Resistance management for *Bt* genetically modified crops is insufficient." Summary article submitted to "American Entomologist"

TEACHING EXPERIENCE:

Informal Lecture Series (Summer 2001)
Virginia Polytechnic Institute and State University
Techniques in the chemical ecology of insect olfaction.
Provided instruction to members of the forest entomology group at Virginia Tech on gas chromatography, behavioral assays and analysis, electrophysiology, and mass spectroscopy.

Teaching Assistant (2000, 2001)
Virginia Polytechnic Institute and State University
Pest and Stress Management of Trees, Entomology section
Responsibilities included: set-up of lab exercises, supervision of students during lab sessions, administration and grading of homework and exams, and provided instruction to lab students in introductory forest entomology.

Biology and Ecology Tutor (August 1997- May 1998)
Quincy University
Responsibilities included teaching principles of biology to non-science majors and assisting biology majors with concepts in ecology.

Lab Assistant (January 1997- May 1998)
Quincy University
Introduction to Biology
Responsibilities included: setup of lab exercises, supervision of students during lab sessions, and writing and administration of quizzes and written exams.

PROFESSIONAL SERVICE:

W.B. Alwood Entomological Society, Virginia Tech
Secretary, 1999-00
Entomology Department computer maintenance, 2001

PROFESSIONAL MEMBERSHIPS:

Entomological Society of America
W.B. Alwood Entomological Society

AWARDS AND HONORS:

Winner of the President's prize for student presentations at 2001 National Meeting of the Entomological Society of America
Outstanding Senior in Environmental Science, awarded May 1998. Quincy University, Quincy, IL 62301.
Quincy University Scholarship, awarded 1994-1998. Quincy University, Quincy, IL 62301.
Moorman Science Scholarship, awarded 1996-1998. Quincy University, Quincy, IL 62301.

SKILLS:

Hardware and software experience in both PC and MAC formats
Gas Chromatography (several systems) hardware and software
Electron Microscopy (Scanning and Transmission)
Mass Spectrometry
HTML code
Electrophysioligical techniques
Behavioral analysis
Statistical design and analysis
GIS