# Biological studies and evaluation of *Scymnus coniferarum* Crotch, a predator of hemlock woolly adelgid from western North America

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

Doctor of Philosophy
In
Entomology

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May 1, 2017
Blacksburg, VA

Keywords:
Biological control, Hemlock woolly adelgid, *Adelges tsugae*, *Scymnus coniferarum*, *Tsuga heterophylla*, *Tsuga canadensis*

# Biological studies and evaluation of *Scymnus coniferarum* Crotch, a predator of hemlock woolly adelgid from western North America

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## ABSTRACT
## (ACADEMIC)

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is an invasive pest of eastern hemlock, *Tsuga canadensis* (L.) Carriere and Carolina hemlock *Tsuga caroliniana* Englem. in the eastern United States. A newly reported beetle predator for HWA, *Scymnus (Pullus) coniferarum* Crotch (Coleoptera: Cocinellidae) preys on the pest in the western United States, and was approved for release in the eastern United States for the control of HWA. This research investigated the viability of *S. coniferarum* as a biological control agent of *A. tsugae* in the eastern United States, as well as the ecological dynamics between *S. coniferarum* and host prey species in its native range of western North America.

In objective one, *S. coniferarum* predation, reproductive potential, and survival were evaluated in field-cages on adelgid infested *T. canadensis* in southwestern Virginia. Adult *S. coniferarum* fed on both generations and all life stages of *A. tsugae* at rates comparable to other adelgid-specific predators, and survived for extended periods of time in the field. In objective two, host-range tests for *S. coniferarum* were conducted in a series of no-choice and paired-choice feeding, oviposition, and development studies. *Scymnus coniferarum* adults fed on all adelgid species, and completed development on HWA and *Adelges piceae* Ratz. *Scymmnus coniferarum* oviposition was extremely low. In the final objective, Douglas-fir, *Pseudotusga menziesii* Mirb., Shore pine, *Pinus contorta* Dougl., western white pine, *Pinus monticola* Dougl., and western hemlock, *Tsuga heterophylla* (Raf.) Sarg. host tree species were sampled in Tacoma, Washington to investigate the life history of *S. coniferarum* and associated adelgid prey

species in the western United States. *Scymnus coniferarum* adults were found on both pine species, Douglas fir, and western hemlock, and seemed to move between host tree species seasonally. Each host tree supports a different adelgid species, and a limited diet of strictly HWA in host-range tests could have contributed to low oviposition rates.

This study suggested that *S. coniferarum* is a voracious predator of HWA in the field and laboratory. However, *S. coniferarum* laid very few eggs in laboratory studies, and zero eggs were recovered in field-cage analyses. This suggested that *S. coniferarum* may rely on multiple adelgid species to reproduce and establish in the eastern United States.

# Biological studies and evaluation of *Scymnus coniferarum* Crotch, a predator of hemlock woolly adelgid from western North America

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## ABSTRACT
## (GENERAL AUDIENCE)

Biological control is an economically and environmentally logical approach to pest management, through the introduction of a natural enemy or predator. This dissertation investigated the potential of *Scymnus (Pullus) coniferarum* as a biological control agent of the invasive pest species, hemlock woolly adelgid, otherwise known as HWA. The hemlock woolly adelgid is an invasive pest insect in the eastern United States, responsible for causing widespread death of eastern hemlock trees from southern Maine to Georgia. *Scymnus coniferarum* is a lady beetle that preys on HWA in the western United States, part of HWA's native range.

In objective one, *S. coniferarum* predation, reproductive potential, and survival were evaluated in field-cages on HWA-infested eastern hemlock trees in southwestern Virginia. Adult *S. coniferarum* beetles fed on HWA year-round, at a rate comparable to other successful biological control agents. In objective two, *S. coniferarum* beetles were confined to a selection of insect species similar to HWA and native to the eastern United States. *Scymnus coniferarum* predation, oviposition and development was observed in these tests in order to assess the potential impact of *S. coniferarum* on non-target species in the eastern United States. *Scymnus coniferarum* beetles fed on all adelgid species, and successfully laid eggs and developed to adults while feeding on the balsam woolly adelgid, another pest species in the eastern United States. Adult beetles laid very few eggs, and were very difficult to rear in a laboratory setting. In the final objective, Douglas-fir, Shore pine, western white pine, and western hemlock trees were sampled in Tacoma, WA to investigate the life history of *S. coniferarum* and other adelgid prey

species in the western United States. *Scymnus coniferarum* adults were found on every species of sample tree except for Douglas-fir, and seemed to move among host trees seasonally. Host tree species support different adelgid species, and a limited diet of strictly HWA in host-range tests could have contributed to low oviposition rates.

The lady beetle *S. coniferarum* was a voracious predator of HWA in field and laboratory studies. However, *S. coniferarum* laid very few eggs in laboratory studies, and zero eggs were recovered in field-cage analyses. This suggested that *S. coniferarum* may rely on multiple adelgid species to reproduce and establish in the eastern United States. This had either positive or negative implications regarding its viability to establish, flourish, and impact HWA in the eastern United States.

# ACKNOWLEDGEMENTS

I want to thank my advisor, Dr. Scott Salom, for his encouragement and guidance throughout my graduate career. He always made time for me when my project seemed tumultuous and too much to take on, and helped me understand that I was capable of achieving a Ph.D. in this discipline.

I also want to thank the remaining members of my committee, Drs. James Parkman, Michael Montgomery, Paul Marek and Timothy Kring. Each of these individuals were essential in the development of this project, and I relied heavily on their areas of expertise. I always felt that I could reach out in the midst of conference meetings, finals week, or in times of duress when everyone else was on vacation. Everyone has been exceedingly kind and nurturing in this process.

I could not have accomplished my reseach or maintained my sanity without my lab mates: Katin Mooneyham, Holly Wantuch, Ariel Hemminger, and Amy Snyder (alum.). I also want to thank senior laboratory specialist Tom McAvoy for being there for me when I was just a technician vying for acceptance into the Department of Entomology at Virginia Tech. Tom helped me with experimental design, locate field sites, lab analyses and all manner of trouble-shooting over the last five years. I wouldn’t have been able to do this without his help.

I thank Louie, Blackbird, and Beyoncé for the empathy, jokes, and inspiration when I was at my lowest. Louie, a doctorate is one thing, but you are the true trophy of this experience.

I thank my parents, Sally and Dick, and my brother Alex. Thank you for knowing me, believing in me, and for always having my back. I am what I am because of you goofballs.

# ATTRIBUTION

Several colleagues aided in the writing and research behind the following chapters presented as part of this dissertation. A brief description of their contributions is included here.

**Chapter 2:** Field-cage Evaluation of Survival, Reproduction, and Feeding Behavior of Adult *Scymnus coniferarum* (Coleoptera: Coccinellidae), a Predator of *Adelges tsugae* (Hemiptera: Adelgidae), published in *Environmental Entomology*: (2016, 45(6): 1527 – 1535.

> **Tom J. McAvoy** is a senior laboratory specialist in the Department of Entomology at Virginia Tech. Mr. McAvoy was a co-author on this paper, and assisted in experimental design and data collection.
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> **Carlyle C. Brewster, PhD** is a professor of Quantitative Ecology in the Department of Entomology at Virginia Tech. Dr. Brewster was a co-author on this paper, and assisted in data analysis.
>
> **Scott M. Salom, PhD** is a professor of Forest Entomology in the Department of Entomology at Virginia Tech. Dr. Salom was second author on this paper, and assisted in experimental design, conduct, and data analysis. Dr. Salom was also the principal investigator for the grant supporting the research, and contributed editorial comments.

**Chapter 3:** Host range assessment of *Scymnus coniferarum*, a potential biological control agent of *Adelges tsugae* (Hemiptera: Adelgidae).

> **Scott M. Salom, PhD** (Department of Entomology, Virginia Tech) assisted in experimental design, conduct, and contributed editorial comments.
>
> **Tom J. McAvoy** (Department of Entomology, Virginia Tech) Mr. McAvoy was the primary researcher and data analyst for several studies included in this chapter.
>
> **Michael E. Montgomery, PhD** is a scientist, emeritus for the USDA Forest Service. Dr. Montgomery was the primary researcher and data analyst for several studies included in this chapter. Dr. Montgomery also contributed editorial comments.
>
> **Nathan P. Havill, PhD** is a research entomologist for the USDA Forest Service. Dr. Havill conducted DNA barcoding research and analysis on insect samples referenced in this study.

**Chapter 4:** Phenology and synchrony of *Scymnus coniferarum* (Coleoptera: Coccinellidae) and its host hemlock woolly adelgid (Hemiptera: Adelgidae)

> **Scott M. Salom, PhD** (Department of Entomology, Virginia Tech) assisted in experimental design, conduct, and contributed editorial comments.
>
> **Rachel Brooks,** is a graduate research assistant in the Department of Entomology at Virginia Tech. Rachel was responsible for sampling and shipments in Tacoma, WA for the first half of this study.
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> **Lisa Hannon,** is a Ph.D. student at the University of Washington in Seattle, WA. Lisa was responsible for sampling and shipments in Tacoma, WA for the second half of this study.
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> **Nathan P. Havill, Ph.D.** (Research Entomologist, USDA Forest Service). Dr. Havill conducted DNA barcoding research and analysis on insect samples referenced in this study.

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# CHAPTER ONE

## Introduction and Literature Review

## Risk Assessment and Potential of *Scymnus coniferarum* (Coleoptera: Coccinellidae) as a Biological Control Agent for Hemlock Woolly Adelgid, *Adelges tsugae*

### Introduction

Hemlock woolly adeglid, *Adelges tsugae* (HWA) Annand (Hemiptera: Adelgidae), is an invasive pest insect that is destroying eastern (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*Tsuga caroliniana* Engelmann) forests in the eastern United States. HWA historically colonizes all hemlock species except for those in the eastern United States (McClure 1987, Havill et al. 2006). The first North American report of *A. tsugae* identified the insect on *T. heterophylla* in Oregon, USA and British Columbia, Canada (Annand, 1924). It was first observed in the eastern United States on *Tsuga canadensis,* L. Carrière in Richmond, Virginia in the early 1950's and has since spread from southern Maine to Georgia (Butin et al. 2002; McClure 1987; Souto et al. 1996). Following its introduction into the eastern U.S., *A. tsugae* populations did not build up to damaging levels until the 1980's. At that point, this pest began to have a tremendous impact on both eastern hemlock species, *T. canadensis* and Carolina hemlock, *T. caroliniana* Engelm (Havill et al. 2006).

The extensive loss of hemlock is having devastating impacts on biodiversity and ecological functions, as well as indirect economic consequences (Benton et al. 2015). Hemlocks are late successional, long-lived trees and provide essential shade to native mixed conifer-hardwood forest ecosystems. Hemlocks can influence stream temperature, nutrient cycling, and the surrounding forest community as a whole. Hemlocks provide essential habitats for a wide diversity of wildlife through their influence on fresh water dynamics (Mayfield et al. 2015). Because of their aesthetic nature, hemlock species are also widely planted as ornamental trees, shrubs and hedges. Unchecked, the spread of *A. tsugae* is anticipated to continue throughout the range of eastern and Carolina hemlock (Mayfield et al. 2015), perhaps limited somewhat for now in its northern expansion.

Hemlock woolly adelgid is native to all hemlock ranges except for those in eastern North America (Havill and Foottit 2007). Surveys of hemlock species in western North America show that native trees are commonly infested by *A. tsugae* but at low densities, well below damaging threshold (McClure and Cheah 1999; Havill and Montgomery 2008). The hypothesized reasoning for this ecological equilibrium is two-fold: presence of natural enemies and host-tree resistance (McClure 1989; McClure and Cheah 1999; Havill and Montgomery 2008).

### Biology of *Adelges tsugae*

Hemlock woolly adelgid injures eastern and Carolina hemlock by injecting the stylet bundle through the epidermal cells below the abcission layer of the hemlock needles, sucking the sap of the tree and likely producing toxic saliva at the feeding site (Gonda-King et al. 2012). When densities are sufficiently high, this causes needles on infested branches to desiccate and die off within months. Soon after, the infested hemlocks display reduced terminal branch growth and poor crown condition. These factors significantly weaken tree resistance, and trees often succumb to damage soon after.  The eggs and crawlers are the primary agents of dispersal (Domec et al. 2013). The small size and woolly egg masses allow this insect to be easily dispersed when they are most abundant in the spring and early summer. Common modes of dispersal include wind, human activity (both forestry and nursery-related) and forest dwelling fauna (McClure 1987; USDAFS 2005).

Hemlock woolly adelgid reproduce parthenogenetically and have a complex polymorphic life cycle with two overlapping generations per year. These generations are made up of the asexual generation referred to as the “sistens generation” (present from July to April), and the “progrediens” generation, present from April to June (McClure 1991; USDAFS 2005). HWA life cycle timing and number of eggs laid varies depending on temperature and location (Elkinton et al. 2016). In early spring, overwintering sistens adults lay a clutch of eggs protected by a layer of waxy wool. The average yield of eggs can vary from 75-100 per ovisac (McClure 1987). Nymphal “crawlers” (mobile first instar progrediens) typically hatch around March to May depending on location, and soon insert their mouthparts at the base of the needle and begin feeding. The adelgid will remain here for the entirety of its development, only removing its mouthparts to molt. The progrediens generation of adelgid develops quickly and by mid-June, adults begin laying eggs. Development occurs earlier in the Mid-Atlantic and southern U.S.

The progrediens lay an average of 25-50 eggs per ovisac, though the number of eggs laid by each generation is variable depending on temperature, elevation and weather conditions (McClure 1987; Davis et al. 2012). By early July, the nymphal crawlers hatch (mobile first instar sistens) and soon settle on the tips of new, current year’s growth on hemlock. This generation undergoes aestivation during the summer, and does not resume feeding until mid-October. Nymphal instars feed and develop during the winter and mature by spring, beginning the cycle again (Davis et al. 2012; Vieira et al. 2013). The ability to lay eggs in both spring and summer, the high production of eggs by the sistens generation, and the rapid development of the progrediens are all contributing factors to HWA populations reaching damaging levels. (McClure 1991; USDAFS 2005; Havill and Foottit 2007; Vieira et al. 2013).

## HWA Impacts

The hemlock woolly adelgid is a danger to hemlock stands and the forest landscape of the eastern U.S. As a defoliator; it readily colonizes newly grown foliage, causing dieback, crown reduction, and eventually death of the tree (McClure 1991). Unlike other hemlock species worldwide where *A. tsugae* is native, species of hemlock native to the eastern United States (eastern and Carolina hemlocks) are extremely vulnerable to terminal infestation (Davis et al. 2012). Hemlock woolly adelgid can be fatal to these species at any age, resulting in mortality in as little time as 4 years, regardless of the condition of the tree upon infestation. Modes of dispersal may include birds, deer, humans and wind and are challenging to regulate (McClure 1991; Orwig and Foster 1998). HWA can also cause non-fatal decline in hemlock stands, and there have even been cases of *T. canadensis* recovery in the natural environment. Studies are ongoing to determine the variables contributing to the range of damage HWA can inflict (Elkinton et al. 2016).

Hemlocks are a dominant species in many forest settings in the eastern US. Native hemlocks provide valuable late-succession shade-cover, impact fresh-water dynamics and affect the biodiversity of the forest community (Vieira et al. 2013). Unchecked, the spread of *A. tsugae* could have negatively effect the ecology of forest systems where eastern and Carolina hemlocks occur.

## Management

Systemic applications of neonicotinoids such as imidacloprid and other products applications are not economically practical and have environmental consequences across the forest ecosystem via bioaccumulation (Cowels et al. 2006; Souto et al. 1996; Cheah et al. 2004a; Cheah and McClure 1995; Davis et al. 2012).

Control methods must be considered and evaluated for both large and small-scale landscapes. One available option is the introduction of biological control agents. Biological control is a method of reducing or mitigating pests through the use of natural enemies. When successful, this method is cost effective and can facilitate long-term, large-scale outcomes through the establishment of a natural ecological balance.

### Biological Control

Native natural enemies have not held *A. tsugae* below injurious levels in the eastern United States (Montgomery and Lyon 1995, Wallace and Hain 2000). However, such a predator complex is thought to exist in the Pacific Northwest, a region where HWA is native (Havill et al. 2006, Havill and Foottit 2007, Kohler et al. 2008). Classical biological control would attempt to addresses the ecological dynamics between *A. tsugae* and its natural enemies in the eastern U.S.

Multiple natural enemies of *A. tsugae* have been identified throughout much of Asia and western North America (Onken and Reardon 2011). Several predators were approved for release into the eastern United States, including *Sasajiscymnus tsugae* Sasajii and McClure (Coleoptera: Coccinellidae) in 1995, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) in 2000, *Scymnus sinuanodulus* Yu and Yao (Coleoptera: Coccinellidae) in 2004, and *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodentidae) in 2010. *Leucopis* spp. (Diptera: Chamaemyiidae) are currently being investigated as potential biological control agents. Both *Leucopis argenticollis* Zetterstedt and *Leucopis piniperda* Malloch have been found in association with *A. tsugae* in the Pacific Northwest region of the United States (Grubin et al. 2011). Both *L. nigrinus* and *L. osakensis* feed on developing sistens generation and progrediens eggs of *A. tsugae* in the winter and early spring. The diapause of these predators coincides with that of first-instar *A. tsugae* sistens in the summer and early fall (Mausel et al. 2008, Mayfield et al. 2015). *Sasajiscymnus tsugae* is active in late spring/early summer, but recovery of this predator at release sites has been inconsistent (Hakeem et al. 2010).

The establishment of *L. nigrinus* and *S. tsugae* continues to be under investigation, and post-release studies indicate impact on *A. tsugae* populations is variable and inconclusive (Cheah et al. 2004b, Vieira et al. 2013). The reasoning behind this limited success could be attributed to the multigenerational lifecycle of *A. tsugae*. Most of the predators mentioned in this section are synchronous with either the progrediens or sistens generation, but not both (Montgomery and Lyon 1995). Even if the establishment of *Laricobius* beetles is successful, this agent only feeds on sistens and progrediens eggs (Vieira et al. 2013). While predators have been released that are active in the summer, their lack of impact thus far, suggest the introduction of additional biological control agents may be necessary to enhance predator impact of *A. tsugae* populations on all stages of development (Mayfield et al. 2015).

*Scymnus (Pullus) coniferarum* Crotch (Coleoptera: Coccinellidae) was first observed as a predator of *A. tsugae* alongside *L. nigrinus* near Puget Sound in 2006 (McDonald 2010). Further observation revealed that *S. coniferarum* completed their lifecycle on the host tree without needing to enter the soil for a period of aestivation, as *L. nigrinus* does. Typical areas where *S. coniferarum* were found included hemlock trees in parking lots, golf courses, bluffs, rocky outcroppings and particularly windy areas, where little needle duff is found under western hemlocks. McDonald (2010) suggests that this may be due to the fact that these types of environments do not favor *L. nigrinus* development, indicating competitive exclusion.

The seasonal occurrence of *S. coniferarum* on western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) was preliminarily studied in the Seattle, Washington area from October, 2010 to June, 2011 (Montgomery et al. 2009; Montgomery and McDonald 2010). More *S. coniferarum* adults were found on western hemlock than *L. nigrinus* adults in early October, with both populations achieving similar densities by the end of the month. Both species continued to be evident throughout the winter. By April, only *S. coniferarum* adults and *L. nigrinus* larvae were evident on western hemlock. At this point, *L. nigrinus* larvae numbers were at their peak, and feeding on the progrediens eggs. *L. nigrinus* larvae then move into the soil to complete development until emergence in the fall. Conversely, *S. coniferarum* completes development on the host tree. The emergent *S. coniferarum* adults feed on eggs laid by the late spring generation (progrediens) of *A. tsugae*. *Scymnus coniferarum* reside on the western hemlocks year-round, thus soil conditions are unimportant (Montgomery and McDonald 2010).

The lifecycles of *S. coniferarum* and *L. nigrinus* are separated through the predation of different generations of *A. tsugae* eggs. The developmental period of the two predators complement each other and do not compete for the same resources. (Montgomery and McDonald 2010; Montgomery and Keena 2011). The introduced biological control complex in the eastern United States still lacks an effective predator for the late spring/early summer. *Scymnus coniferarum* is currently under investigation to determine if this predator could effectively fill this void in seasonal control.

## Biology of *S. conifearum*

**Discovery.** The original species description of *S. coniferarum* was written by George Robert Crotch and published in 1874 and included in *Transactions of the American Entomological Society*, Volume 4. Crotch associated *S. coniferarum* with materials collected from "pine" in California. In this description, *S. coniferarum* appears in the category: *Descriptions of New Species of Coleoptera from the Pacific Coast of the United States* (Whitehead 1967). The taxonomic classification in the original description designates *S. coniferarum* to the order Coleoptera, genus *Scymnus* and the species *coniferarum*. Present day descriptions further classify *S. coniferarum* as being in the family Coccinellidae, subfamily *Scymninae,* tribe Scymnini and subgenus *Pullus* Mulsant 1846 (Gordon 1985).

**Taxonomy.** The genus *Scymnus* is the largest genus of lady beetles, with more than 800 species divided into 8 subgenera. One of these, *Neopullus* has several species in China that are predators of *A. tsugae,* and were imported into North America for biological control, but none of these species have been established (Montgomery and Keena 2011). Most species in the subgenus "*Pullus"* are aphidophagous.

**Morphology.** According to Gordon (1976), the adult male of *S. coniferarum* is a small lady beetle with an average length of 1.75 mm and a width of 1.15 mm. Females are slightly larger; ranging from 1.60 – 2.10 mm in length. The body of the beetle is covered in fine, short pubescence, has a black head and pronotum, a shiny, yellow-brown elytra that is slightly rounded, and is coarsely punctate and black in color along the suture and base. Gordon's description of a male specimen continues as follows: "In male specimens, the triangular area at scutellum and suture are black, and the legs and epipleuron are dark brown. Punctation on head and pronotum are fine and separated by one to two times their diameter. Genatalia simple; basal lobe shorter than paramere, apex curved downward in lateral view; siphon tapered apically to a fine, acuminate point" (Gordon 1976). Female morphology is similar to male, except the apex of fifth sternum was described as 'evenly rounded' and the pronotum can be entirely yellowish brown (Montgomery et al. 2011, Gordon 1985). However, in field and laboratory studies conducted with *S. coniferarum* adult beetles between 2012 – 2015, male and female insects were not sexually dimorphic (personal observation). The length and width of both sexes of beetles vary considerably (personal observation).

The larvae of *S. coniferarum* are covered in a white woolly wax (Whitehead 1967, Gordon 1976). Aside from this observation, no further descriptions have been published detailing the physical dimensions and descriptions of any of the larval intars, pupae or eggs of *S. coniferarum.*

**Range.** The known native geographical range of *S. coniferarum* indicates the species is widely distributed across western North America (Gordon 1985). *Scymnus coniferarum* has been collected on pine and hemlock host trees in British Colombia, Arizona, California, Colorado, Idaho, Nevada, New Mexico, Oregon, South Dakota, Utah, and Wyoming (Gordon 1976). Whitehead stated that he collected large numbers of *S. coniferarum* specimens in California on lodgepole pine (*Pinus contorta* Doug.) and Monterey pine (*Pinus radiata* D. Don) infested with populations of adelgids. Records have even recently indicated populations of *S. coniferarum* occurring on Monterey pine in Chile and Peru, most likely through accidental introduction (Gonzales 2010). Most of the recently recovered *S. coniferarum* adults and larvae were collected from *A. tsugae* -infested western hemlock while collecting *L. nigrinus* in the greater Seattle, WA area (McDonald 2010). A small percentage of *S. coniferarum* adults and a single *S. coniferarum* larva have also been collected on adelgid-infested western white pine in Seattle (Montgomery and McDonald 2010). Whitehead (1976) hypothesizes this is most likely due to artificial transportation through the shipment of nursery stock across the country.

**Life History.** Despite descriptions of *S. coniferarum* dating back to the turn of the century, little is known about the life history traits and phenology of this predatory beetle. The primary publications that target *S. coniferarum* only provide brief taxonomic descriptions (Crotch 1873; Gordon 1976). Further research is needed for a complete understanding of the developmental stages and host feeding preferences of *S. coniferarum.* Most of the information available was documented via field observations made by M. Montgomery and R. McDonald during predator recovery efforts in the Pacific Northwest (Montgomery et al. 2009). The univoltine life cycle of *S. coniferarum* appears to be synchronous with the life cycle of HWA. Adult *S. coniferarum* are present and presumably feed on HWA sistens during the late fall and winter season. In severe winter weather, the adults are known to seek protection by hiding in bark crevices on the bole of the tree. In the laboratory setting, oviposition of *S. coniferarum* begins in early April when the sistens generation of HWA is nearing the end of its oviposition period and progrediens eggs begin hatching. In the same study, *S. coniferarum* oviposition ends in mid-May when 1st – 3rd instar progrediens are present (USDA 2012). The fecundity of *S. coniferarum* females and oviposition length is still unknown. The larval stage has been observed feeding on *A. tsugae* progrediens from May to June, and will most likely continue to feed on the sistens eggs laid by the progrediens generation into July (Montgomery et al. 2011; Kohler et al. 2008). Instead of completing aestivation in the soil, *S. coniferarum* pupates near its host or under tree bark for an undetermined duration of time. The precise timing of adult emergence remains unknown.

**Ecosystem Interactions.** The only documented hosts of *S. coniferarum* are alluded to as ‘adelgids that feed on pine’ (Whitehead 1967; Gordon 1976). Whitehead (1967) postulated that larvae belonging to the Scymini tribe possessed restricted host-specific feeding behaviors; however, supporting data were sparse and inconclusive. Recent observations in the Seattle area have found *S. coniferarum* to be abundant on eastern hemlocks in arboreta, and native western hemlocks infested with HWA. *S. coniferarum* was also recovered to a lesser extent on western white pine (Montgomery et al. 2009; Montgomery and McDonald 2010).

Most species in the subgenus "*Pullus*" are aphidophagous, but the *suturalis* species group contains only three species, all of which feed on adelgids (Whitehead 1967). These species are *S. coniferarum*, native to western North America, *Scymnus impexus* Mulsant, and *Scymnus suturalis* Thunberg, both native to Europe and introduced to the U.S. (Montgomery and Keena 2011).

*Scymnus suturalis* was imported from Europe for the control of the balsam woolly adelgid (*Adelges piceae* Ratz.), and has established and spread in North America (Obrycki and Kring 1998, Montgomery and Lyon 1995). *S. suturalis* has been recovered feeding on adelgid-infested *T. canadensis, P. strobus,* and *Pinus sylvestris,* but favors adelgids associated with *Pinus* species (Montgomery 1997). In laboratory studies, *S. suturalis* has fed on all stages of *A. tsugae.* It is likely that this species moves among these three trees, depending on the presence and life stage/palatability of adelgids available during that time of year. *S. suturalis* also has its oviposition period in the spring, and eggs have been recovered on *T. canadensis.* Larvae of *S. suturalis* have been observed on both *T. canadensis* and *P. sylvestris* (Montgomery and Lyon 1995). *Scymnus impexus* is mostly associated with *A. piceae*, and other fir adelgids, but may feed on other adelgids (Delucchi 1954).

## Rationale

More information is needed on the life history of *S. coniferarum* to determine whether it will be a suitable biological control agent for *A. tsugae* in the eastern United States. Preliminary studies show that *S. coniferarum* feeds and reproduces on *A. tsugae* in both natural and laboratory settings (Chapter 2, Chapter 3). The predator has shown a preference for *A. tsugae* and pine adelgids over other Sternorrhyncha in the laboratory, and appears to be an adelgid specialist (USDA 2012). Because it pupates on the host tree of the prey, soil conditions are of little concern in a rearing setting. The larvae of *S. coniferarum* are present in the late spring and early summer, and feed on the *A. tsugae* progrediens well after *L. nigrinus* has begun aestivation in the soil. The presence of *S. coniferarum* in the predator complex could be additive. *S. coniferarum* impacts a different generation of *A. tsugae* than *L. nigrinus,* thus seasonality of predation between the predators could be complementary. There would be no competition for resources, and both generations of *A. tsugae* would be targeted (Montgomery et al. 2011; Montgomery and McDonald, 2010).

The predator's native range is widely distributed throughout western North America, and has been collected from British Colombia to Chile. Such a wide climate tolerance potential bodes well for the establishment of *S. coniferarum* upon introduction to the eastern United States (Montgomery and McDonald, 2010).

## Research Objectives

*Scymnus coniferarum* is currently under investigation as a potential biological control agent of HWA in the eastern United States. However, there has been limited research investigating the details of *S. coniferarum* development, predation, fecundity and host preference. The purpose of this research is to perform a risk-assessment analysis in an effort to determine the insect's potential and suitability as a biological control agent. If *S. coniferarum* is not considered an undue risk in the eastern United States, release of this species may complement early season predators. The following research objectives were developed to gain a better understanding of *S. coniferarum's* potential for impact and establishment in the eastern United States.

**Objective 1: Assess impact and establishment of *S. coniferarum* in the field.**

Evaluate the survival, reproduction, and feeding potential of *S. coniferarum* adults on *A. tsugae*-infested *T. canadensis.* This work was completed to determine the viability of *S. coniferarum* as a biological control agent in the eastern United States.

**Objective 2: Monitor oviposition and host-feeding preference of *S. coniferarum*.**

A. Determine host specificity of *S. coniferarum.* These analyses evaluate feeding behavior, oviposition timing and development of *S. coniferarum* on *A. tsugae* and ten non-target hemipteran species.

B. Determine the temperature regime(s) for maximum survival and fecundity of *S. coniferarum*. Then use this information to develop a reliable mass rearing procedure for the eventual release of *S. coniferarum* in the eastern United States.

**Objective 3: Phenology and synchrony of *S. coniferarum* and its adelgid prey in Tacoma, Washington.**

Assess seasonal activity in relation to adelgid species of *S. coniferarum* and other natural enemies present on various conifer tree species present in the Pacific Northwest, part of the native range of HWA and *S. coniferarum.*

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# CHAPTER TWO

## Field-cage evaluation of survival, reproduction, and feeding behavior of adult *Scymnus coniferarum*, a predator of *Adelges tsugae* (Hemiptera: Adelgidae)

(Published 17 September 2016: M.N. Darr,[1] T.J. McAvoy, C.C. Brewster, and S.M. Salom. *Environmental Entomology.* (2016) 45(6):1527 – 1535. DOI:10.1093/ee/nvw124).

### Abstract

The hemlock woolly adelgid, *Adelges tsugae* Annand, is an invasive pest of eastern (*Tsuga canadensis* (L.) Carriére) and Carolina hemlock (*Tsuga caroliniana* Engelmann) forests in the eastern United States. *Scymnus (Pullus) coniferarum* Crotch (Coleoptera: Coccinellidae) is a lady beetle that preys on *A. tsugae* in the western United States, where *A. tsugae* infestations on western hemlocks are not lethal. It is thought that *S. coniferarum* could be an important predator that helps keep *A. tsugae* populations from reaching damaging levels in this region. This study assesses the potential of this predator as a biological control agent for *A. tsugae* in the eastern United States. *Scymnus coniferarum* predation, reproductive potential, and survival were evaluated in field-cages on adelgid-infested *T. canadensis* at two sites in southwestern Virginia. Sampling was conducted between December 2012 and June 2014 to evaluate the impact of *S. coniferarum* on both generations of *A. tsuage* (sistens and progrediens). Adult *S. coniferarum* fed on both generations and all life stages of *A. tsugae* during both field trials at rates comparable to other adelgid-specific predators. Evidence of *S. coniferarum* oviposition was minimal, and may be attributed to low temperatures and prey availability. *S. coniferarum* mortality was greatest when exposed to winter temperatures at the higher elevation site in 2013, and least throughout the 2014 spring sample period. *S. coniferarum* demonstrated a high predation rate on *A. tsugae* and survived for extended periods of time at sites in southwest Virginia, indicating that this species could be an effective predator of hemlock woolly adelgid in similar climates.

# Introduction

The hemlock woolly adelgid, *Adelges tsugae* Annand, is an introduced insect that is threatening eastern (*Tsuga canadensis* (L.) Carriére) and Carolina hemlock (*Tsuga caroliniana Engelmann*) forests in the eastern United States (Orwig and Foster 1998, Havill et al. 2006). An infestation of *A. tsugae* was first observed on *T. canadensis* in Richmond, VA, in the early 1950s (Souto et al. 1996). The invasive pest has since spread throughout the eastern United States, covering >50% of the geographic range of eastern hemlock in the United States (USDA Forest Service 2013). The spread of *A. tsugae* to the eastern United States has resulted in the loss of hundreds of thousands of hemlocks from Georgia to Maine (Preisser et al. 2014). The dramatic decline of eastern hemlocks has resulted in losses to biodiversity, habitat for other organisms, and important ecological services (Orwig and Foster 1998, McClure and Cheah 1999), as well as indirect economic consequences (Aukema et al. 2011). The objective of our research is to facilitate the ongoing biological control effort for *A. tsugae*, by evaluating the viability of one of its native predators, *Scymnus (Pullus) coniferarum* Crotch (Coleoptera: Coccinellidae).

*Adelges tsugae* is a hemlock-specific herbivore, native to all *Tsuga* spp. in western North America and Asia (Havill et al. 2006, Havill and Foottit 2007). The adelgid feeds by inserting a thin bundle of stylet mouthparts into the twig and consumes carbohydrates stored in the ray parenchyma cells of the xylem (Young et al. 1995). *Adelges tsugae* damage impedes nutrient uptake, which causes decline in new growth and tree death within several years (Orwig and Foster 1998). *Adelges tsugae* reproduces parthenogenically, and has a complex polymorphic life cycle with two overlapping generations per year (McClure 1991). These generations are made up of the overwintering “sistens” generation, present from July to April in southwestern Virginia, and the spring “progrediens” generation, present from April to June (Gray and Salom 1996).

In the native range of western North America and Asia hemlock species, hemlock trees are commonly infested by *A. tsugae*, but rarely at high densities, making damage and tree mortality uncommon (McClure and Cheah 1999, Havill and Montgomery 2008). This ecological equilibrium is the combined outcome from a complex of natural enemies and evolved host-tree feeding tolerance (McClure 1987, McClure and Cheah 1999, Havill and Montgomery 2008), neither of which are sufficiently represented in eastern hemlock species (Cheah et al. 2005, Salom et al. 2008).

A number of management strategies have been implemented or suggested to control the impacts of *A. tsugae* on *T. canadensis*. These methods include silvicultural thinning (Fajvan 2008), enhancement of host resistance (Montgomery et al. 2009), ex situ host conservation (Jetton et al. 2013), and most commonly chemical and biological control (Cowles et al. 2006, Mayfield et al. 2015). Systemic imidacloprid and horticultural oil are the broad-spectrum insecticides commonly used to control *A. tsugae* infestations (Dilling et al. 2009). While such broadspectrum chemical control methods have proven effective in controlling *A. tsugae* populations on a small scale (such as within small parks and private lands), application is costly, ecologically risky, and infeasible on a large scale (Eisenback et al. 2014, McClure 1987). Imidacloprid application by soil drenches and trunk injections have been shown to reduce abundances of nontarget insects, such as natural enemies, cause secondary pest outbreaks, and even increase the fecundity of various species of spider mites (James and Price 2002, Raupp et al. 2004, Dilling et al. 2009, Szczepaniec et al. 2011). Conversely, biological control can be utilized on a large landscape level, and offers a more sustainable, long-term solution in the forest environment. However, classical biological control can take longer to be effective, and multiple agents may be necessary to match the pest species' reproductive potential. From a management perspective, both short- and long-term strategies can be implemented to target control of *A. tsugae* while reducing the impact on non-target species (Dilling et al. 2009).

Although classical biological control can take years, or even decades before a pest population is controlled, using multiple agents may result in faster, more effective pest suppression. Multiple natural enemies of *A. tsugae* have been identified throughout much of Asia and western North America (Onken and Reardon 2011). Several promising predators have been approved for release into the eastern United States, including *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) in 1995, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) in 2000, *Scymnus sinuanodulus* Yu and Yao (Coleoptera: Coccinellidae) in 2004, but not continuing (Cheah et al. 2004), and *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) in 2010. While the establishment of *L. nigrinus* and *S. tsugae* has been successful (Mausel et al 2010, Hakeem et al. 2011), results of their impacts on *A. tsugae* populations have been limited to only a couple of studies for *L. nigrinus* (Mausel et al 2008, Mayfield et al. 2015). Although many of these biological control agents successfully feed on *A. tsugae*, phenological dynamics between predators and prey may be keeping the current group of natural enemies from sufficiently limiting pest levels in the eastern United States. Both *L. nigrinus* and *L. osakensis* diapause coincides with first-instar *A. tsugae* sistens. Consequently, these predators feed on developing sistens generation and progrediens eggs (Mausel et al. 2008, Vieira et al. 2013). *Sasajiscymnus tsugae* adults and larvae feed on all stages of *A. tsugae* sistens and progrediens, but predator recovery has been low (Cheah and McClure 1998). The introduction of additional biological control agents may be necessary to enhance predator impact on *A. tsugae* populations at all stages of development.

In 2006, *Scymnus (Pullus) coniferarum* was observed feeding on *A. tsugae* in conjunction with the biological control agent *L. nigrinus* near Seattle, WA (McDonald 2010). Sampling with *S. coniferarum* as a target species was conducted 18–21 June, 2010, with the objective of recovering additional natural enemies of *A. tsugae* in their native range (Montgomery and Keena 2011). Densities of *S. coniferarum* were sufficient for mass collection and rearing, with the greatest collections occurring from late spring to early summer. Peak population levels of *S. coniferarum* coincided with the aestivation period of *L. nigrinus*. The observed feeding behavior and relative abundance of *S. coniferarum* indicated that this predator feeds predominantly on the progrediens generation of *A. tsugae* in spring and summer (Montgomery and McDonald 2010, Montgomery et al. 2011), well after *L. nigrinus* moves to the soil to complete development (Mausel et. al 2010). Because *S. coniferarum* is present later in the season, there should be limited competition for resources, and both *A. tsugae* generations would be targeted (Montgomery and McDonald 2010, Montgomery et al. 2011).

This study evaluates the survival, reproduction, and feeding potential of *S. coniferarum* adults on *A. tsugae,* to determine its viability for biological control. Preliminary studies show that *S. coniferarum* appears to be an adelgid specialist. It feeds and reproduces on adelgid-infested conifers in both natural and laboratory settings (Darr, unpublished data), and show preference for *A. tsugae* and pine adelgids over other hemipterans in the laboratory (Montgomery et al. 2011). Because *S. coniferarum* is considered a native predator of *A. tsugae*, it received regulatory approval for release from quarantine in the eastern United States in 2012.

## Materials and Methods

### Location and Timing of Experiments

*Scymnus (Pullus) coniferarm* adults used in this study were field collected on *A. tsugae*-infested *Tsuga heterophylla* (Raf.) Sarg. and adelgid-infested shore pine, *Pinus contorta* subsp. *contorta* Dougl. in Seattle, WA, in 2012 and 2013. They were shipped to the Beneficial Insects Quarantine Laboratory in Blacksburg, VA. Adults were stored in environmental chambers at 15°C, 65–75% RH, and a photoperiod of 12:12 (L:D) h, and fed on *A. tsugae*-infested eastern hemlock branches collected in Virginia and Tennessee. In the shipments received during the sistens trial (18 December 2012–30 May 2013), 8% of the beetles were collected from *Pinus contorta* host trees, and 92% were collected from *T. heterophylla*. In the 2014 progrediens trial, 9% of the *S. coniferarum* beetles were collected from *P. contorta* and 91% from *T. heterophylla* (Dick McDonald, drmcbug@skybest.com). The shipments of *S. coniferarum* were randomly mixed upon arrival, which ensured that there was no differentiation between beetles used in each sample period.

*Scymnus (Pullus) coniferarum* feeding, survival, and reproduction were evaluated in two sites in southwestern Virginia. The first trial, hereafter called the sistens trial, ran from December 2012 to May 2013 at a low and high elevation site. This timeline was selected to observe the impact of *S. coniferarum* on *A. tsugae* sistens. The second trial, hereafter called the progrediens trial, ran from April to June 2014 at the low elevation site, and focused on the impact of *S. coniferarum* on *A. tsugae* progrediens.

The low elevation site was located on Kentland Farm in Whitethorne, VA (37.2078 N, 80.5899 W). It is in plant hardiness zone 6b (USDA-ARS, 2015), on a 16% slope, at an elevation of 574.5 m. Kentland Farm (Virginia Tech research facility) is a 0.73-ha *L. nigrinus* field insectary, established by planting eastern hemlocks in 2001, and infested with *A. tsugae* in 2002 and 2003 (Mausel et al. 2008).

The high elevation site was located in Mountain Lake, VA (37.3656 N, 80.5363 W). It was at an elevation of 1,188 m and in plant hardiness zone 6a (USDA-APHIS 2012). These two sites allowed us to contrast the performance of *S. coniferarum* at different temperature extremes in the first year of the study.

Trees at both locations had high densities of *A. tsugae* and low-hanging branches that could be accessed easily. The trees selected ranged from 3.3–18.4 cm dbh (diameter at breast height). All trees selected were in relatively good health, and able to provide a consistent presence of *A. tsugae* for the duration of the study. Temperature data were recorded at Mountain Lake using an Onset HOBO Pendant data logger, model UA-002-64. The data logger did not have an isolation shield. Instead, we placed the device in the canopy of hemlock tree close to the trunk which limited exposure to direct sunlight. Weather data for the Kentland Farm site were obtained from the farm’s weather station.

The sistens trial began on 28 December 2012, and concluded 30 May 2013 at both sites. Samples were taken monthly, tracking *S. coniferarum* reproduction, predation, and mortality. Adult sistens were present by the third week of February, and had begun oviposition. In March, *A. tsugae* sistens adults were still producing eggs, which developed into visible first-instar nymphs. By April, the adult sistens population was ovipositing, though declining, and the emergence of progrediens nymphs soon followed. In the final sample period, first- and second-instar progrediens nymphs were present. All life stages of the *A. tsugae* sistens (eggs, crawlers, nymphs, and adults) were present during this study.

The progrediens trial began on 28 April 2014 and concluded on 30 June 2014 at Kentland. Sample intervals occurred every two weeks, and tracked *S. coniferarum* reproduction, as well as predation and mortality. All progrediens life stages were present during this sample period.

## Experimental Procedure: Sistens Trial

At the beginning of each sample interval, five trees of the same cultivar, age, shape, growth conditions, and infestation density were selected at each site. The five trees selected each sample period varied throughout the trial, due the fluctuating nature of *A. tsugae* populations. Four branches with high densities of *A. tsugae* were chosen per tree, for a total of 20 branches per site—10 caged branches containing beetles and 10 caged branches without beetles. Starting from the terminal of each selected branch, *A. tsugae* were counted until a total of 200–300 was reached. The branch was marked with flagging tape where the count ended. Each sample branch was associated with a recorded number of *A. tsugae* counted each time a new cage was installed. This precount matched the total *A. tsugae* (alive and dead) counted in laboratory analyses at the end of each sample period. The total number of *A. tsugae* counted when a new cage was set up was also used to calculate hemlock woolly adelgid mortality associated with factors unrelated to *S. coniferarum* predation.

On each tree, two branches were caged with two *S. coniferarum* beetles inside. The remaining two branches were caged without predators, and served as the control. Treatment and control cages with similar pre-counts of adelgid were paired for statistical analysis. Ideally, male and female beetles were paired together in the “treatment” cages. Unfortunately, the gender of *S. coniferarum* cannot be determined by external morphological characteristics. Mating position was used to increase the likelihood of pairing a male with a female in each treatment cage.

The cages used in this study were rectangular bags (72 by 55 cm), made of polyester fabric (white chiffon) to prevent predators from escaping. Cages were secured to flagged branches with plastic zip-ties, tightened 5 cm proximal to the flagging to avoid disturbance of adelgids inside. The branches were forcefully shaken before the installation of the cage to dislodge any predators present. Both treatment and control caged branches were cut at the end of each sample period, and taken to the laboratory for examination.

The feeding rate of *S. coniferarum* was determined by comparing the number of dead *A. tsugae* in cages with predators to the number of dead *A. tsugae* in cages without predators. We used a combination of traits to identify *A. tsugae* predated by *S. coniferarum*. In order to reach *A. tsugae* adults and eggs, *S. coniferarum* chewed a circular hole in the wool of the ovisac. Once the adelgid was accessible, *S. coniferarum* adults partially gored their prey, and fed on the hemolymph. Dried cadavers of *A. tsugae* usually remained in or near the disturbed ovisacs, lacked response to stimulation, and were often ripped to pieces. Therefore, mortality relating the *S. coniferarum* predation was easily identified by the presence of a circular entry hole in a disturbed ovisac containing remnants of desiccated adelgid and dried hemolymph. In the absence of fully developed adult *A. tsugae*, or when eggs and crawlers were abundant, we used the total number of the disturbed ovisacs to represent adelgid mortality. To statistically account for alternate sources of mortality (i.e., extreme temperatures, precipitation, transport damage), we applied the proportion of dead adelgid in control cages to the calculation of *S. coniferarum* feeding rate in treatment cages.

Reproduction was based on the number of *S. coniferarum* eggs and larvae recovered from caged branches at the end of each sample period. *S. coniferarum* mortality was recorded by counting the number of surviving beetles in each cage at the end of the sample period. Surviving beetles were re-used in the following sample period and dead *S. coniferarum* were replaced with new beetles from the colony.

Survival of *S. coniferarum* was analyzed by calculating proportion of individual *S. coniferarum* (both reused and replaced beetles) that survived in each sampling period.

### Experimental Procedure: Progrediens Trial

In 2014, this study was conducted only at the Kentland Farm site. Southwest Virginia experienced unusually cold temperatures in January 2014, and at higher elevations, *A. tsugae* winter mortality was 90–100% through February 2014 (T.J.M., unpublished data). Therefore, *A. tsugae* populations were virtually nonexistent at Mt. Lake and we chose to sample only at the Kentland Farm site where a greater density of *A. tsugae* had survived. To increase the likelihood of mating pairs reproducing, four *S. coniferarum* beetles were placed in each treatment cage. Again, we used mating position as an indication of beetle sex as sexual dimorphic characteristics are yet to be identified for *S. coniferarum*.

Sampling was staggered on a weekly basis, and occurred while the progrediens generation of *A. tsugae* was present. Half the trees were sampled each week, resulting in 2-wk sample periods for each cage. Two-week sample periods were maintained to give the predators sufficient time to feed. Sampling dates were staggered so data could be reported on a weekly basis. This was important because the progrediens generation of *A. tsugae* is of relatively short duration, with development occurring much faster than the sistens generation. All other recording methods were repeated from the year before. The number of surviving *S. coniferarum* adults, number of *S. coniferarum* eggs and larvae present, and the number of dead *A. tsugae* (indicated by *A. tsugae* cadavers and disturbed ovisacs) were counted and recorded for each staggered sample period in 20 branch cages, composed of 10 caged branches containing beetles and 10 caged branches without beetles. The 20 cages used in the progrediens trial were distributed across five trees, with four cages per tree. The five trees that were sampled varied throughout the trial, due to the fluctuating densities of hemlock woolly adelgid infestations. All the trees sampled during the progrediens trial were the same cultivar, age, shape, and grown under the same conditions. Surviving beetles in cages containing *S. coniferarum* were counted, recorded, and transferred to replacement cages for continued monitoring in the field. Dead *S. coniferarum* were counted, recorded, and replaced with beetles from the colony.

### Data Analysis

Data collected during sistens and progrediens trials in 2012–2013 and 2014, respectively, were analyzed separately to determine the effects of *S. coniferarum* on *A. tsugae* populations. Each data set was analyzed using a linear mixed model for repeated measures ANOVA with a first-order autoregressive with random effect covariance structure to account for the hierarchical experimental design, i.e., cage nested within treatment and tree, and repeated measurements of the response variable over time (Littell et al. 2000, Preisser and Elkinton 2008, Jones et al. 2015). In the ANOVA model, study site (Kentland and Mt. Lake), treatment (predator and no predator), and sampling time, and their interactions were the fixed effects factors, cage nested within treatment and tree was the random effects factor, and sampling time and cage were the repeated measures parameter and subject, respectively. Because the progrediens trial was only conducted at Kentland, the fixed effects factor, site, was excluded from the ANOVA model for analysis of the 2014 data. Prior to each analysis, the response variable was tested for normality and, where appropriate, was transformed using a log10 transformation (Zar 2010). All statistical analyses were carried out using JMP Pro 12.0.0 (SAS 2013) at a significance level of 0.05.

To compare differences in predation between sites at each sample date, a “predation rate” (dead *A. tsugae*/day/beetle) was calculated to account for the small differences in the duration of each sample interval and number of predators in the sample. To accurately determine the predation rate, the number of dead *A. tsugae* caused by alternate sources of mortality needed to be removed from the feeding rate analyses. Nonpredator-related *A. tsugae* mortality was presumed to be equal among all treatment and control cages. The proportion of dead adelgid in cages without *S. coniferarum* was applied to the total number of dead adelgids in cages containing *S. coniferarum*. The remaining number of dead *A. tsugae* represented the number of *A. tsugae* predated by *S. coniferarum* beetles.

Because all *S. coniferarum* used in this study were field collected, the age of beetles was unknown. For this reason, we were unable to calculate the average life span of *S. coniferarum*. To accommodate the replacement of dead *S. coniferarum* beetles in each sequential sample period, each sample was treated as unique and not a measurement of individual beetles over time. In both sistens and progrediens trials, survival data of adult predators were analyzed with a one-way ANOVA, followed by a Tukey HSD ($P < 0.05$) to determine significant differences in survival among dates and between field sites.

## Results

### Adult Feeding, Reproduction, and Survival—Sistens Trial

*S. coniferarum* predation varied significantly between Kentland Farm and Mt. Lake sites ($F$ (1,75.4) = 16.087, $P = 0.0001$) and by sample period at both sites ($F$ (4,122.5) = 3.014, $P = 0.0207$; Fig. 1).

The mean (±SE) predation rate of adult *S. coniferarum* was 0.97 ± 0.06 adelgids/beetle/day in 2013. Comparison of predation rate showed the consumption of *A. tsugae* to be greatest in May at Kentland Farm (1.95 ± 0.34 adelgids/beetle/day), when progrediens adults and eggs of the sistens generation were present (Fig. 1). Predation was lowest in February at Mt. Lake (0.41 ± 0.07 adelgids/ beetle/day) when nymphs of the sistens generation were developing (Fig. 1). Peak predation corresponded with warmer temperatures and possibly the oviposition period of *S. coniferarum* females (as observed in ongoing laboratory study). It is possible that predation was underestimated in March and April due to the inability to calculate total *A. tsugae* eggs consumed once the ovisac had been disturbed. In the colder sample periods (December–February), mortality rates of *A. tsuage* in cages containing *S. coniferarum* were similar to cages without predators. This indicates that limited predation by *S. coniferarum* took place in the winter.

Evidence of reproduction in the cages was minimal. Only one *S. coniferarum* egg was found in the 1 May–30 May 2013 sample period and two larvae were found in the 22 March–30 April 2013 and 1 May–30 May 2013 samples periods. All eggs and larvae were recovered at the Kentland Farm site.

The extreme minimum temperature at the Kentland Farm and Mt. Lake sites during the sistens trial was -12.7 and -15.9 °C, respectively (Fig. 2a). Beetle survivorship was significantly greater in March (70%) than in January (0%) at the Mt. Lake site (*F* (4,42) = 5.79, *P* = 0.0008; Fig. 3). Time of year did not significantly impact beetle survivorship at the Kentland Farm site (*F* (4,42) = 0.29, *P* = 0.8810).

**Adult Feeding, Reproduction, and Survival— Progrediens Trial**

Cages with *S. coniferarum* adults had a significantly greater mean number of dead *A. tsugae* than cages without the predator in all biweekly sample periods (28 April 2014–30 June 2014) at the Kentland Farm site (*F* (1,41) = 114.09, *P* = 0.0001). Neither sample period (*F*

(7,113.6) = 0.93, *P* = 0.483) nor the interaction of treatment × sample period (*F* (7,113.6) = 1.08, *P* = 0.3828) had a signification effect on *S. coniferarum* predation rate. In 2014, the predation rate of adult beetles averaged 2.47 ± 0.11 adelgids/beetle/day. Predation was greatest in late June at 2.85 ± 0.32 adelgids/beetle/day when progrediens adults, sistens eggs, and first-instar nymphs of the sistens generation were present and entering aestivation. Predation was lowest in early May, when first- and second-instar progrediens nymphs were present (2.23 ± 0.16 adelgids/beetle/day).

In spite of increasing the predator numbers in cages from two to four in this trial, only one *S. coniferarum* egg was found in the 2 June–16 June sample period.

Time of year had a significant impact on beetle survivorship at the Kentland Farm site when mortality and sample date were compared (*F* (7,72) = 12.74, *P* = 0.0001; Fig. 4). Paired comparisons indicated that survivorship remained high until late May (84%), and declined by the end of the study in late June (30%).

## Discussion

The literature describes *S. coniferarum* as an "adelgid specialist" (Montgomery and McDonald 2010). This beetle is documented as a predator of adelgid species primarily associated with pine and hemlock species (Crotch 1874, Whitehead 1967, Gordon 1976, Montgomery and McDonald 2010). Adult *S. coniferarum* beetles were observed feeding on *T. heterophylla* infested with *A. tsugae* and on *P. monticola* infested with an "unidentified pine adelgid" in the Seattle metropolitan area in 2008 and 2009 (Montgomery et al. 2009, Montgomery and McDonald 2010). Large numbers of *S. coniferarum* adults were collected from *P. contorta* and *Pinus radiata* D. Don infested with adelgids on collection trips described more than a century apart (Crotch 1874, Gordon 1976). In 1967, Whitehead collected *S. coniferarum* adults and larvae from *P. contorta* and *P. radiata* infested with “woolly aphids” in Sierra County and Alpine County, CA. This same publication reported that *S. coniferarum* adults fed readily on woolly aphids from *P. contorta*, but would not feed on “pea aphid” in the laboratory (Whitehead 1967). Recent assessments of *S. coniferarum* as a potential biological control agent of hemlock woolly adelgid stated that *S. coniferarum* readily feeds on both generations of *A. tsugae*, but is believed to prey mostly on the spring progrediens generation (Montgomery and McDonald 2010).

*S. coniferarum* is classified within the genus *Scymnus* and subgenus *Pullus*. Most species of the genus *Scymnus* and all *Pullus* species are aphidophagous, and feed on either aphids or adelgids (Gordon 1985, Whitehead 1967). Other than *S. coniferarum,* only two other species in the subgenus *Pullus* are known to be established in North America: *Scymnus (Pullus) suturalis* and *Scymnus (Pullus) impexus*. Both species are native to Europe, were introduced to North America for biological control purposes, and are adelgid specialists. *S. suturalis* was introduced in 1961 for to control *Pineus* spp. attacking pines in the mid-western United States, and is now widely established in the northeastern part of the country (Montgomery and Lyon 1995). *S. impexus* was released in large numbers to control *A. piceae* in eastern Canada, British Columbia, the Pacific Northwest, and North Carolina in the 1950s and the 1960s. Establishment was reported in all areas except North Carolina (Montgomery and Lyon 1995). Like *S. coniferarum*, both *S. impexus* and *S. suturalis* belong to the *Pullus* subgenus, are specific to conifers, and have been reported to feed on both pine and hemlock adelgids (Gordon 1985, Montgomery and Lyon 1995).

The study reported here demonstrated that adult *S. coniferarum* beetles consistently fed on all life-stages and both generations of *A. tsugae* when confined to field cages in a closed-release setting. Adult predation impact was evident at all site locations and sample periods, which indicates that *S. coniferarum* adults could potentially feed on both generations of *A. tsugae* in the southern portion of the geographic range inhabited by *A. tsugae*. *S. coniferarum* predation was significantly impacted by site and sample period in the sistens trial, and was consistent across all sample dates in the progrediens trial. Changes in predation were likely a function of temperature, prey stage, and prey mortality. The effect of temperature on *S. coniferarum* mortality was especially evident at the Mt. Lake site in January and February of 2013, when temperatures dropped to -15.9 C (Fig. 2) and treatment cages suffered near 100% predator mortality. The significant increase in *S. coniferarum* mortality during this sample period could partially be due to the beetle's inability to move beyond the field cage to more protected locations. The geographical location of the study sites in southwest Virginia may have also been a factor. The *S. coniferarum* tested were collected in plant hardiness zone 8a in Tacoma, WA (USDA-ARS 2015). The Mt. Lake site is zoned as 6a, indicating that more studies are needed to determine if this climate is unsuitable for *S. coniferarum* establishment. It should be noted that the coastal strain of *L. nigrinus* also comes from zone 8a and has established well into the mid-Atlantic states (Mausel et al. 2010).

*S. coniferarum* adults fed on all *A. tsugae* life stages in both reproductive generations, including crawlers. Additionally, *S. coniferarum* adults actively fed on *A. tsugae* in the months when *Laricobius* spp. have dropped to the soil to aestivate (Salom et al. 2004, Zilahi -Balogh et al. 2002). Because *A. tsugae* eggs and crawlers are the primary agents of dispersal for each generation, it is especially important that *S. coniferarum* actively fed on these early life stages of the progrediens, and the eggs and crawlers of the sistens (McClure 1987). This feeding behavior by *S. coniferarum* is encouraging, and demonstrates this predator's capacity to prey upon both generations and all life stages of *A. tsguae* in southwest Virginia.

Unfortunately, the oviposition data were inconclusive. A total of two *S. coniferarum* eggs and three larvae were found in the 2013 monthly analyses. In 2014, only a single egg was found in June, during the weekly progrediens trial. Using mating position as an indication of beetle sex may not have been reliable measure, causing the cages to be stocked with majority males or females. This obstacle could be addressed if a reliable method for gender determination could be developed. To date, no sexually dimorphic external characteristics have been identified for this species. It is also possible that *S. coniferarum* requires other adelgid host species to oviposit. *S. coniferarum* adults were found in abundance on adelgid-infested *Pinus contorta* between March and June 2016 in an ongoing population study taking place in Tacoma, WA. This time frame synchronizes with the oviposition period of *S. coniferarum* females, which indicates that *S. coniferarum* may require alternate adelgid species in addition to *A. tsugae* for reproductive success (unpublished data).

The discrepancy in survival between the Mt. Lake and Kentland Farm sites in January 2013 was likely due to the unusually low temperatures, and the higher elevation of this site. At the Kentland Farm site, 40% of all specimens died in the 18 December–21 January sample period and 20% of adult *S. coniferarum* beetles survived through the May 2013 sample period. Only 30% of adult *S. coniferarum* survived past the December and January sample period at the high elevation Mt. Lake site. This indicates that *S. coniferarum* is capable of withstanding low temperatures at relatively high elevations, but are limited by extreme temperatures (Fig. 2). The immediate drop in survival during the December–January sample period suggests the need for an acclimation period in the laboratory, so as not to shock insects in storage with ambient winter temperatures upon introduction to the outside environment.

Based on the results reported here, it is possible to estimate the potential impact of one *S. coniferarum* adult. An adult *S. coniferarum* that begins feeding in December and dies at the end of June could consume an average total of 334.5 *A. tsugae*, with 65% of the total *A. tsugae* consumed in May and June when *A. tsugae* progrediens adults are present. It should be noted that the field cages used in this experiment provided almost complete protection from other insects, and may have reduced competition for resources. This projected predation rate exceeds the estimated feeding potential of *L. osakensis* (163 *A. tsugae* adults) in a similar field cage analysis conducted from December 2010 to May 2011 (Vieira et. al 2013).

These studies show that *S. coniferarum* can successfully impact *A. tsugae* populations in sleeve cages at varying elevations in southwest Virginia. Active winter and spring feeding, a voracious predation rate, and robust survivorship indicate that *S. coniferarum* has the potential to be an effective biological control agent, and an important addition to the control complex of predators introduced in the eastern United States. Ongoing studies are focusing on the suitability of other hemipteran species present in the eastern United States for *S. coniferarum*. Additionally, if open releases became operational, efforts will need to be made to evaluate this beetle’s ability to establish, disperse, and eventually impact *A. tsugae* populations.

## Acknowledgments

We thank Dr. L. Vieira for advising in field cage installation procedure. Thanks to Drs. M. Montgomery and P. Parkman for assistance in experimental design, and to L. Manden for her assistance in field sampling and lab analyses. We thank Dr. Richard McDonald for the collection and shipment of beetle specimens. This work was supported by SAES-422 Multistate Research Project, Biological Control of Arthropod Pests and Weeds, NE- 1332. Specific funding was provided by USDA Forest Service, Grant 12-CA-11420004-069.

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## Figures

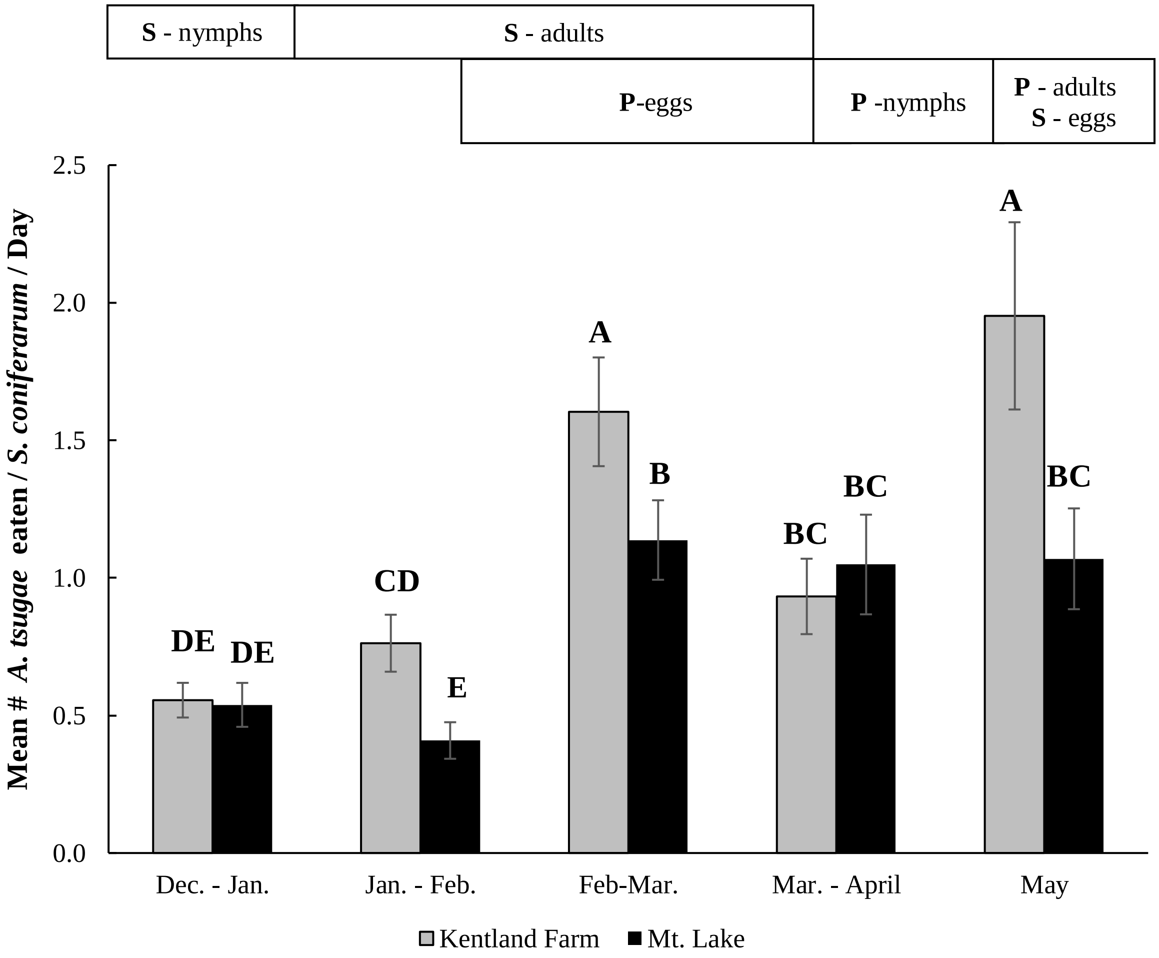

**Fig. 1.** Mean predation rate (dead *A. tsugae*/day/beetle) ± SE of *S. coniferarum* preying on *A. tsugae* at Mt. Lake and Kentland Farm sites for the sistens trial (December 2012–May 2013). Mean predation rates were compared among all sample periods and sites with a linear mixed model for repeated measures ANOVA followed by a Tukey HSD ($P < 0.05$) comparison test. Significance differences are represented with different capital letters.

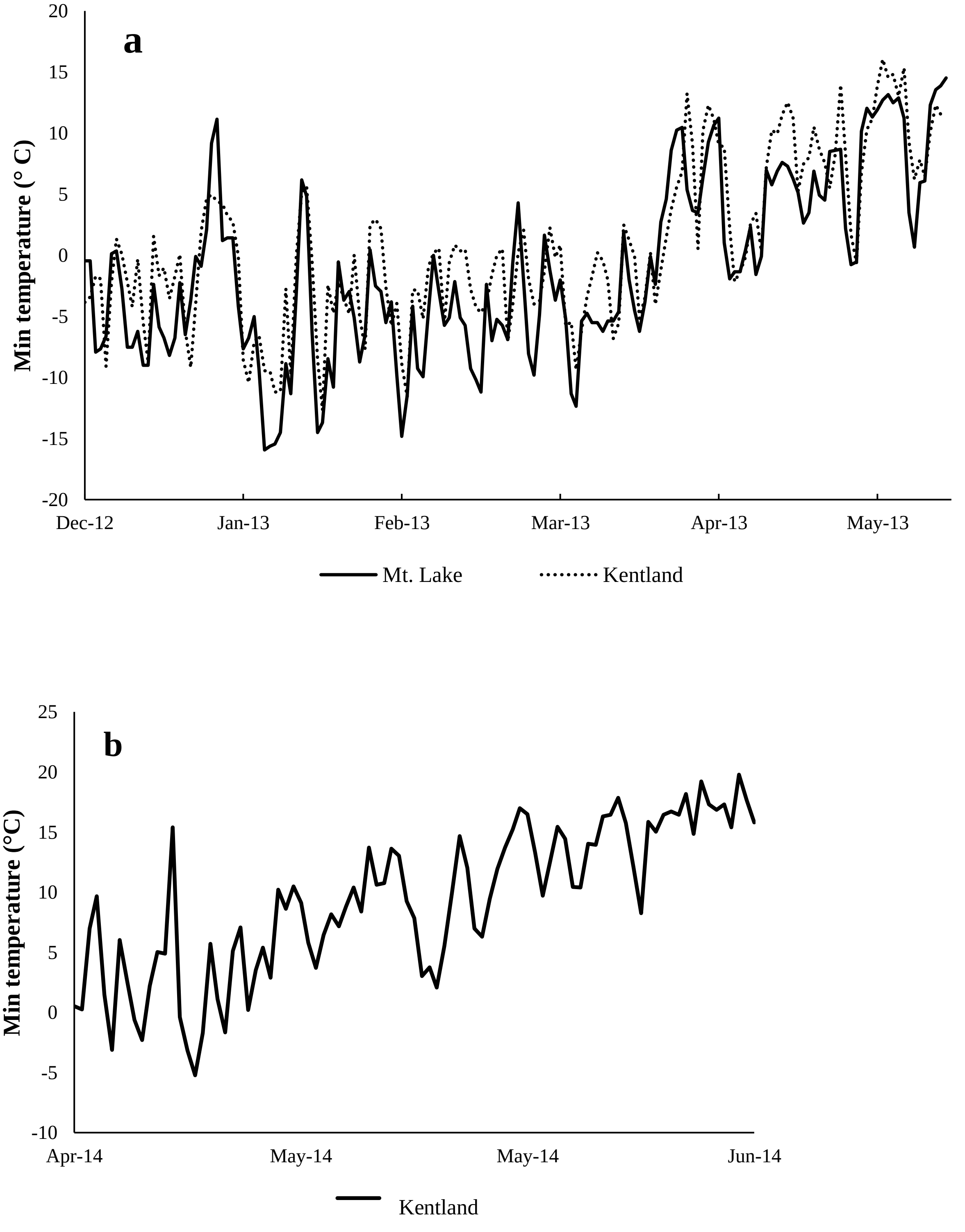

**Fig. 2.** Minimum temperatures during this study at (a) Kentland Farm and Mt. Lake during sistens trial (Dec. 2012–May 2013) and (b) progrediens trial (April–June 2014).

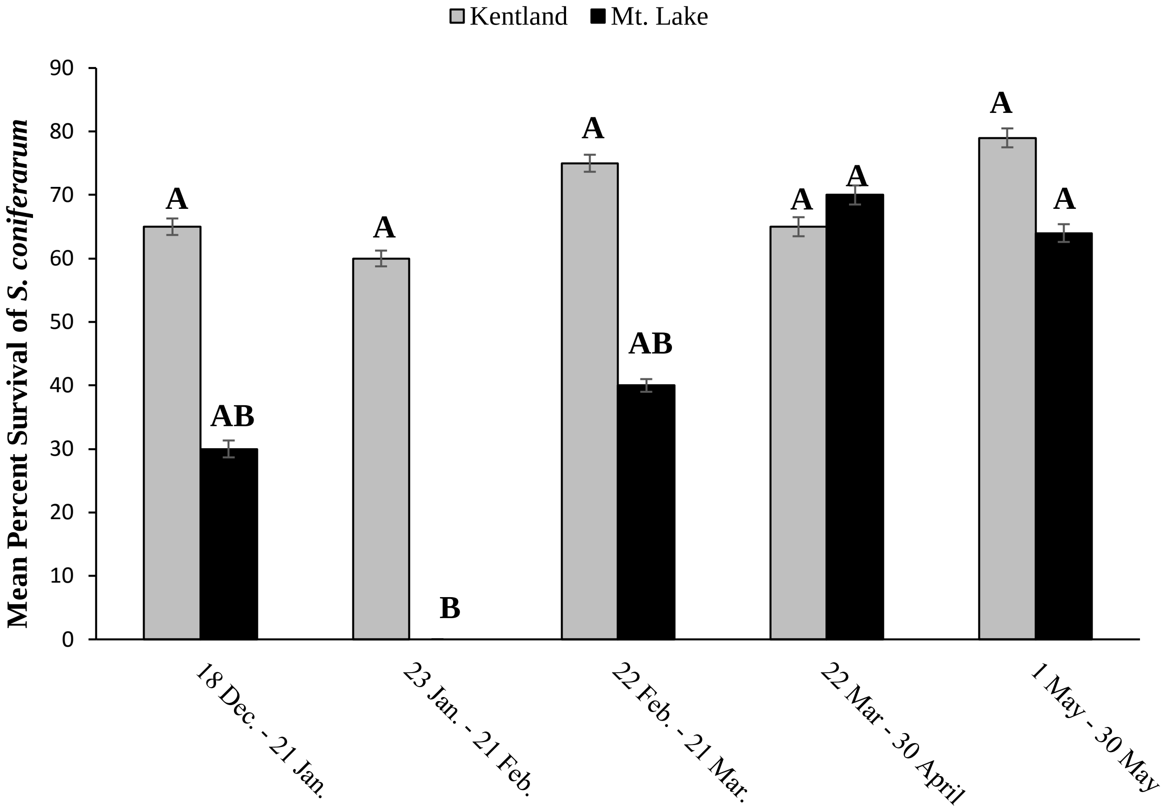

**Fig. 3.** Mean percent survivorship ± SE of individual *S. coniferarum* at Kentland Farm and Mt. Lake sites for the sistens trial from December 2012 to May 2013. Values not sharing the same letter are significantly different, according to Tukey's HSD Test ($P < 0.05$). Significant difference indicated by capital letters for the Mt. Lake field site.

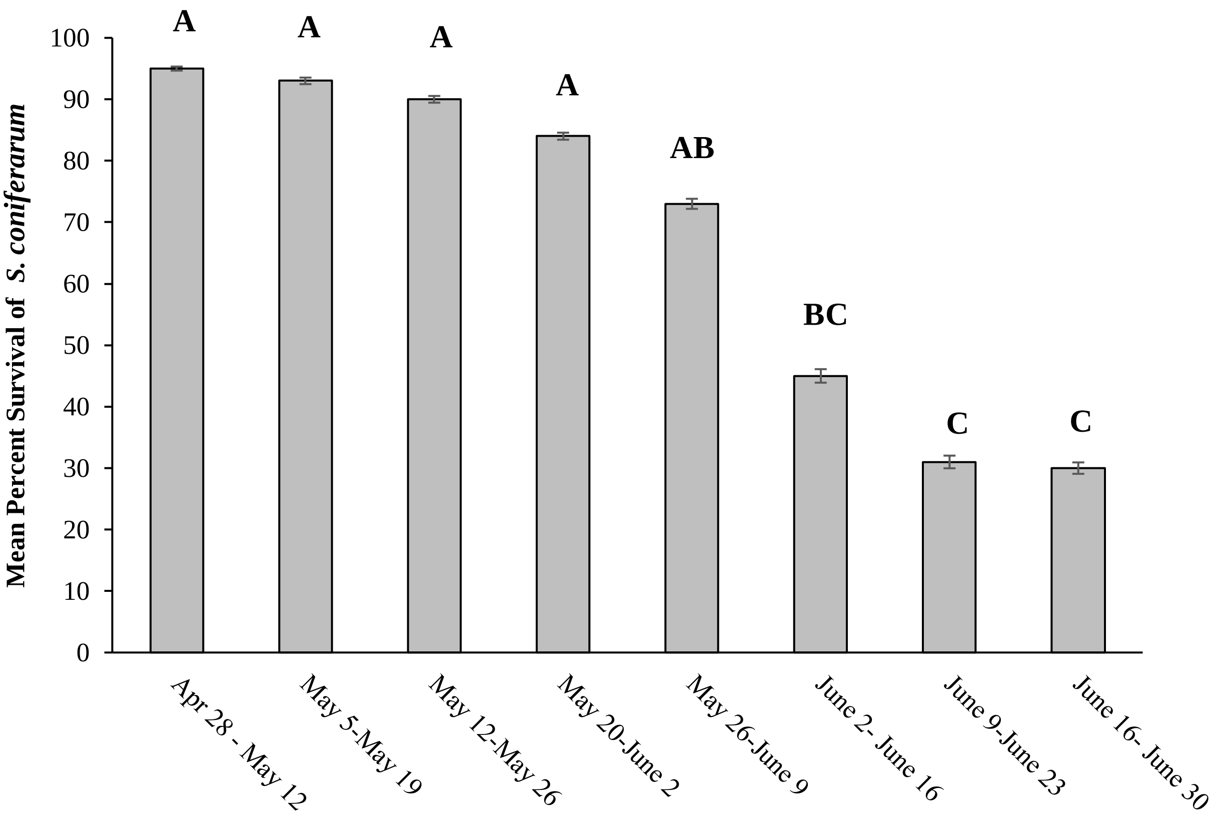

**Fig. 4.** Mean percent survival ± SE of individual *S. coniferarum* at the Kentland Farm site for the progrediens trial from April 2014 to June 2014. Values not sharing the same letter are significantly different, according to Tukey's HSD Test ($P < 0.05$). Significant difference indicated by different capital letters.

# CHAPTER THREE

## Host range assessment of *Scymnus coniferarum*, a potential biological control agent of *Adelges tsugae* (Hemiptera: Adelgidae)

### Abstract

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an invasive pest of eastern (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*Tsuga caroliniana* Engelmann) forests in the eastern United States. *Scymnus (Pullus) coniferarum* Crotch (Coleoptera: Coccinellidae) is a lady beetle that preys on *A. tsugae* in the western United States, where *A. tsugae* infestations on western hemlocks are not lethal. *Scymnus coniferarum* is being considered as a potential biological control agent for *A. tsugae*. While preliminary studies have shown its release to be of limited risk, more information is needed on the suitability of *S. coniferarum* as a biological control agent for HWA in the eastern United States. Host-range tests for *S. coniferarum* were conducted in quarantine facilities in Blacksburg, VA and Hamden, CT in a series of no-choice and paired-choice feeding, oviposition and development studies. These tests involved *A. tsugae*, *Pineus pini* (Marcquardt) (Hemiptera: Adelgidae), *Eucallipterus tilliae* (L.) (Hemiptera: Aphididae), *Paraprociphilus tesselatus* Fitch (Hemiptera: Adelgidae), *Fiorinia externa* Ferris (Hemiptera: Diaspididae), *Dysmicoccus wistariae* Green (Hemiptera: Pseudococcidae), *Pineus strobi* Hartig (Hemiptera: Adelgidae), *Adelges piceae* Ratzeburg (Hemiptera: Adelgidae), *Adelges laricis* Vallot (Hemiptera: Adelgidae), and *Pineus floccus* Patch (Hemiptera: Adelgidae). Overall, *S. coniferarum* fed on all adelgid species only, and completed development only on HWA and *A. piceae*. The results of this host-range assessment should not exclude *S. coniferarum* as a potential biological control agent of *A. tsugae.*

## Introduction

Hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an introduced insect that is destroying eastern (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*Tsuga caroliniana* Engelmann) forests in the eastern United States (Havill and Foottit 2007, Orwig and Foster 1998). Infestations of HWA are rarely lethal to hemlock stands in its native range of Asia and the western United States (Havill and Foottit 2007). It is possible that along with natural resistance, a complex of natural enemies plays a role in maintaining *A. tsugae* populations below injurious levels in these regions (Cheah and McClure 1996, Montgomery and Lyon 1996).

There are no known natural enemies of HWA in the eastern United States, and chemical control methods are impractical in a large-scale forest setting (Cowles et al. 2006, Wallace and Hain 2000). A coordinated research effort began in 1993 to investigate potential biological control agents of HWA in the eastern United States (Reardon and Bullard 1996). Multiple natural enemies of HWA were identified in eastern Asia and western North America, and several predators were approved for release (Onken and Reardon 2011). The natural enemies imported for the biological control of HWA are *Sasajiscymnus tsugae* Sasaji and McClure (Coleoptera: Coccinellidae) in 1995, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) in 2000, *Scymnus sinuanodulus* Yu and Yao (Coleoptera: Coccinellidae) in 2004, and *Laricobius osakensis* Montgomery and Shiyake (Coleoptera: Derodontidae) in 2010. Both *L. nigrinus* and *S. tsugae* have successfully established, but HWA populations still remain at injurious levels across the eastern United States. It is possible that phenological gaps between predators and prey may be keeping the current group of natural enemies from sufficiently controlling this pest in its introduced range. The introduction of additional biological control agents may be necessary to enhance predator impact on HWA populations at all stages of development.

*Scymnus (Pullus) coniferarum* Crotch (Coleoptera: Coccinellidae) was observed feeding on HWA while making collections of the biological control agent *L. nigrinus* near Seattle, Washington in 2006 (McDonald 2010). Because *S. coniferarum* was considered a native predator of HWA, it received regulatory approval for release from quarantine in the eastern United States in 2012, even though only limited preliminary host-range studies had been conducted (Montgomery and Keena 2011, Darr et al. 2016). In this chapter, I report on a series of host-range studies carried from 2008-2015 for *S. coniferarum*, designed to provide a robust risk assessment of this insect.

The objectives of this study are two-fold:

1. Determine host specificity of *S. coniferarum.* These analyses evaluate feeding behavior, oviposition preferences and development of *S. coniferarum* on HWA and ten non-target hemipteran species.
2. Determine the temperature regime(s) for maximum survival and fecundity of *S. coniferarum*. Develop a reliable mass rearing procedure for the eventual release of *S. coniferarum* in the eastern United States.

## Materials and Methods

### Overview of Host Range Tests

Prey acceptance and host suitability tests were conducted over three separate periods between 2008 and 2015. These separately conducted groups of tests are identified as “Connecticut”, “Virginia1”, and “Virginia2” (Table 1).

In Connecticut from 2008-2010, no-choice and choice feeding tests and paired oviposition tests were conducted. Species tested were HWA, *Pineus pini* Marguardt (Hemiptera: Adelgidae), *Pineus strobi* Hartig (Hemiptera: Adelgidae), *Eucalypterus tilliae* (L.) (Hemiptera: Aphididae), *Paraprociphilus tesselatus* Fitch (Hemiptera: Adelgidae), *Fiorinia externa* Ferris (Hemiptera: Diaspididae), *Dysmicoccus wistariae* Green (Hemiptera: Pseudococcidae), and *Pineus pinifoliae* Fitch (Hemiptera: Adelgidae) (Table 1).

In Virginia in 2010 (Virginia1), no-choice feeding tests were conducted with HWA, *P. tesselatus,* and *Adelges piceae* Ratzeburg (Hemiptera: Adelgidae). Development tests were conducted with *P. strobi* (Table 1).

Additional tests were completed in Virginia between 2011 and 2015 (Virginia2). No-choice feeding tests were conducted with HWA, *A. piceae,* and *A. laricis.* Paired-choice feeding tests were conducted with HWA, *A. piceae,* and *A. laricis.* No-choice oviposition tests were conducted with HWA, *A. piceae,* and *Pineus floccus* Patch (Hemiptera: Adelgidae). Development tests were conducted with HWA, *P. strobi,* and *P. floccus* (Hemiptera: Adelgidae) (Table 1).

The *S. coniferarm* adults used in these experiments were field-collected in the Seattle-Tacoma, WA area on HWA-infested *Tsuga heterophylla* (Raf.) Sarg. and to a lesser extent on shore pine, *Pinus contorta* subsp. *contorta* Dougl. and *Pinus monticola* Dougl. Specimens were shipped to the Beneficial Insects Quarantine Laboratory in Blacksburg, VA or to the USDA Forest Service, Hamden CT. Adults were stored in environmental chambers at 15° C, 12:12 (L:D), and 65-75% RH and fed on HWA infested eastern hemlock branches collected in Virginia and Tennessee or Connecticut or Massachusetts, respectively. All specimens used in 2010 and 2011 were field collected from *T. heterophylla.* In the shipments received in 2013-2015, 8-14% of the beetles were collected from *P. contorta*, and the remainder collected from *T. heterophylla* (Dick McDonald, drmcbug@skybest.com). The shipments of *S. coniferarum* were mixed together prior to shipment.

**Test Prey Species**

The ten alternate prey species were selected for the host range tests based on taxonomic or ecological similarity to HWA, and ecological importance (Table 2). The species used in these experiments occur naturally in the coniferous forests of Virginia, and would likely encounter *S. coniferarum* upon release. The target and all alternate prey species belong to the order Hemiptera, from six genera in four families. Comparison within the family Adelgidae was emphasized to determine host-specificity of *S. coniferarum.*

All life stages (eggs, nymphs or adults) were offered in the feeding, oviposition, and development tests, either as a group of the stage collected in the field at the time of collection, or a single stage. To minimize damage to test prey in the Adelgidae and Diaspididae families, all specimens were left intact on their host plant during testing. For most prey, twigs with foliage were stored with cut end in water in a cooler. The *P. strobi* and *A. piceae* tested in VA were on bark removed from a large tree limb, the back of which was coated in melted paraffin wax immediately after harvesting to prevent desiccation. For testing, a small portion of the host plant with prey was trimmed to fit the test chamber and excess prey removed.

### Prey Evaluation Tests

Prey acceptance of adult *S. coniferarum* was recorded through a series of no-choice and paired-choice feeding tests using adults, nymphs, or eggs of each test prey species. These tests included no-choice feeding comparisons among HWA and alternate prey species, no-choice feeding and survivorship rates over time at different temperatures on HWA, and paired-choice feeding tests among species. For each of these tests, consumption of host species was determined by the number of live prey offered minus the number of live prey remaining at the end of the test. The adult beetles both chew and suck prey and the carcass of large prey especially may remain at the end of the test. To compare predation across trials of different durations, predation rate (consumption/day/beetle) was calculated at each sample date. Prey suitability for development and reproduction was evaluated by confining beetles on prey for longer periods.

Unless otherwise noted, each replicate used one *S. coniferarum* adult, which was starved for 24 h before each test. The assay chamber was a 100 × 25 mm polystyrene Petri dish (Nalgene Lab-Tek), with a layer of 90 mm diameter filter paper (GE Healthcare Whatman) on the bottom. No-choice feeding tests contained one test prey species, and paired-choice tests contained HWA and an alternate test prey species. A dissecting microscope was used to count the number of eggs, nymphs, and adults of each test species before and after exposure to *S. coniferarum* adults to determine the predation rate. Excess eggs, nymphs, and adults of each test species were removed from the host plant to facilitate an accurate count. Dishes were checked after 1-3 days, and the number of eggs, live nymphs, or live adults were counted. In long-term tests, the host plant foliage was replaced when the prey was depleted or foliage was desiccated, usually every 4-6 days. Tests were conducted at 15°C and a photoperiod of 12:12 (L:D) h., unless otherwise specified.

### No-choice Feeding Tests

#### Study 1 – No-choice Feeding Tests, Connecticut.

**Study 1a –** Adult *S. coniferarum* collected in Kings County, Washington State were received 8 April 2008 at the USDA Forest Service laboratory in Hamden, CT. They were maintained and tested at 18°C. The trial took place on 26 April 2008 for 24 hr., while *A. tsugae* sistens adults and progrediens eggs were present. All tests were done with individual beetles in 50 mm diameter by 9 mm high clear plastic dishes with the bottom covered by the circular piece of filter paper. A portion of an infested terminal, less than 0.5 cm in length, was selected that had a set number of HWA adults, eggs, and instar II or III nymphs. Excess of each stage was removed with forceps to achieve 10-15 eggs, 10 nymphs and 2-3 adults. After confined with a test beetle for ca 24 h, the foliage was again examined under a microscope and the number of each stage recorded.

**Study 1b – No-Choice Feeding Tests, Connecticut.** In 2010, *S. coniferarum* adults that had been held in the laboratory for 1-3 months were confined singly to an alternate test prey species: *P. pini, P. strobi, E. tiliae, P. tesselatus, F. externa,* and *D. wistariae.* Each test had 10 replicates and was accompanied by control dishes with an HWA adult and 10-15 eggs. All assays were maintained at 15°C for 24 h.

**Study 2 – No-choice Feeding Tests, Virginia1.** From 2 – 18 November 2011, stems with *P. tessellatus* colonies on smooth alder were collected near Blacksburg, VA. *P. tessellatus* is viviparous and all stages except eggs were present when collected. *P. tessellatus* nymphs are fairly large compared to *S. coniferarum* adults and HWA. *P. tessellatus* first instars are 1.1 and 1.8 times the length of *S. coniferarum* adults and HWA in October (2nd and 3rd instars), respectively. Adult *P. tessellatus* are over 6 times the length of HWA 2nd and 3rd instars and nearly 4 times the length of *S. coniferarum.*

From 13 October – 27 November 2011, 10 replicates with one *S. coniferarum* adult were isolated with 5-10 HWA 2nd and 3rd instar nymphs in each dish. All tests were maintained at 15°C. The number of HWA nymphs before and after exposure to *S. coniferarum* adults were counted every 48 h to determine the predation rate.

### Study 3 – No-choice Feeding Tests, Virginia2.

**Study 3a:** From 15 April – 21 June 2013, *S. coniferarum* adults collected in Seattle WA, in March of that year, were confined individually with *A. piceae* and HWA (Table 1). Frasier fir bark swatches hosting *A. piceae* were collected from a stand in Blacksburg, VA. *A. piceae* eggs, crawlers and nymphs were all present for the duration of this study. On 15 April 2013, a total of 5 adult *S. coniferarum* were placed in 5-cm Petri dishes, at 15°C. A 3×3 cm bark swatch populated with *A. piceae* was placed in each Petri dish containing a *S. coniferarum* adult. The total number of *A. piceae* consumed, the associated life stage present, and the total number of days in each dish were recorded every 2-4 d to monitor *S. coniferarum* adults. At this time, a newly harvested bark swatch containing *A. piceae* replaced each desiccated square of bark. Each 3-cm square of bark typically contained 20-30 adelgids at various life stages. The study was terminated at the end of June, when no surviving *S. coniferarum* remained in the dishes (Table 3).

**Study 3b:** This next series of tests examined feeding on eggs of *A. piceae* and *A. laricis*. From 4 June – 8 July 2014, 10 *S. coniferarum* adults were isolated in 5-cm Petri dishes, at 15$^{o}$C, and fed exclusively *A. laricis.* Adults and nymphs were removed, and only *A. laricis* eggs were present for these studies. *A. laricis* eggs were laid at the base of *Larix laricinia* (Du Roi) needles, and sprigs of *L. laricinia* were provided in assays containing one *S. coniferarum* adult. *A. laricis* eggs were counted at the beginning and end of each test period to document *S. coniferarum* predation.

Ten additional *S. coniferarum* adults were isolated in 10 Petri dishes and fed exclusively *A. piceae* eggs at the same temperature and photoperiod. A 3×3 cm bark swatch populated with *A. piceae* was placed in each Petri dish containing one *S. coniferarum* adult. The number of eggs or adelgids before and after exposure to *S. coniferarum* adults was counted. At this time, a newly harvested bark swatch containing *A. piceae* replaced each desiccated square of bark. Each 3 cm$^{2}$ of bark typically contained 20-30 adults and eggs.

For both studies, the total number of *A. piceae* and *A. laricis* consumed, and the total number of days in each dish were recorded every 2-4 days to determine the number of adelgid eggs eaten/day/beetle. Each prey acceptance test had 10-15 replications, and one adult *S. coniferarum* was used per replicate.

**Study 3c:** Predation rate studies at different temperatures on HWA were conducted from 27 January – 30 June 2014, in three growth chambers, and were set up under three different temperature/photoperiod combinations, mimicking an accelerated version of the ambient environment in their native Seattle range (15°C, 20°C, and 25°C). All life stages of both sistens and progrediens generations of HWA were present for the duration of this study. *S. coniferarum* predation was recorded in an effort to determine the optimal conditions for rearing *S. coniferarum* in the laboratory. The total number of HWA was recorded every 2-4 days to determine the number of adelgid eggs eaten/day/beetle. Each prey acceptance test had 10-15 replications, and one adult *S. coniferarum* was used per replicate.

**Study 3d** No-choice predation tests were conducted with HWA aestivating nymphs from 14 August - 23 September 2014 and from 11 August to 15 October, 2015. From August to September, only diapausing neosistens of HWA are present in the field. Infested terminals were collected weekly, the number of aestivating nymphs counted on the twig and a single *S. coniferarum* were confined for 5-7 days. At this point, the nymphs were re-counted and foliage was replaced if necessary.

**Study 4 – Paired-choice Feeding Tests, Connecticut.**

**Study 4a:** From 9 June – 18 August 2009, adult *S. coniferarum* were confined to a choice of *P. strobi* adults and eggs, and HWA aestivating nymphs. *P. strobus* foliage containing *P. strobi* was collected in North Carolina and Georgia on 9 June 2009, and refrigerated for 2-4 weeks. Number of prey eaten was recorded.

**Study 4b:** A 24 h study was conducted on 1 June 2010, which compared *S. coniferarum* predation between *P. pini* eggs and HWA aestivating nymphs. *Pineus pini*-infested *Pinus sylvestris* (L.) foliage was collected in CT. Number of eggs eaten was recorded.

**Study 4c:** A separate 24-h, paired-choice study was completed on 29 June 2010, which isolated *S. coniferarum* adults with *P. pini* eggs and *A. tsugae* aestivating nymphs. *Pineus pini*-infested *Pinus mugo* Turra. was collected in Washington.

For each of these tests, adult *S. coniferarum* where confined for 1 - 4 days in a Petri dish or 3.8-L oviposition jar at 15°C with a choice of *T. canadensis* or a species of pine on a potential prey species that occur in the eastern United States. A minimum of 10 individual beetles or individual containers were used for each trial. The adelgids were tested on their respective host plant, and counts made prior to and after the test. For accurate counting, low densities were chosen and excess adelgids were sometimes removed to provide comparable numbers of prey. The first trial used only un-infested foliage. All but two trials (noted) used beetles that had been reared or held on eastern hemlock foliage infested with HWA. Beetles were starved overnight prior to the start of the test.

#### Study 5 – Paired-choice Feeding Tests, Virginia2.

To compare *S. coniferarum* feeding preference, two host-feeding tests were devised with a choice between *A. piceae* and HWA and *A. laricis* and HWA from 9 June – 7 July 2014. Frasier fir bark swatches hosting *A. piceae* were collected from a stand in Blacksburg, VA. *Adelges piceae* and *A. laricis* eggs and crawlers were present for the duration of this study. This study included two replicates of 10, one replicate per host species. All replicates were held at 15°C, and dishes were checked every 2-4 days for the number of adelgids consumed per species.

### Oviposition Tests

No-choice (single-prey) and paired-choice tests were conducted to evaluate acceptance and preference of test prey by *S. coniferarum* for oviposition. Tests were conducted in 100 × 25 $mm^2$ (Nalgene Lab-Tek) plastic Petri dishes lined with a single layer of 90 mm diameter filter paper (GE Healthcare Whatman) at the base. *S. coniferarum* females were randomly assigned to dishes that contained a single test prey species with their associated plant host. One female *S. coniferarum* was used for each replicate. Assays were checked for *S. coniferarum* eggs every 3-8 days. Because there was not a reliable method for identifying *S. conifarum* sex morphologically, we had to rely on the presence of eggs associated with an adult prior to the study to determine if a living beetle was female.

**Study 6 – Paired-choice Oviposition Tests, Connecticut.** Host preference for oviposition was examined from 3–7 May 2010 by placing 10 *S. coniferarum* in 3.8-L clear plastic, rectangular jar with a choice of HWA on *T. canadensis* and *P. pinifoliae* on *P. monticola*. In this test, one branch of HWA infested foliage and test prey foliage, each about 100 cm$^2$, were placed 4-5 cm apart in the oviposition jar. The adult beetles had been collected seven days prior to the test from *P. monticola* and were held at 5°C prior to the study to the study. The study was initiated by warming the beetles to the assay temperature of 15°C and selecting active beetles of unknown sex. After placement in the jars, the location of the beetles was observed continuously for 4 hrs and then twice each day to the end of the test after 4 days.

**Study 7 – No-choice Oviposition Tests, Virginia2**.

**Study 7a:** From 15 April – 9 June 2013, *S. coniferarum* beetles were placed in dishes containing HWA, *A. piceae*, or *P. floccus* to be monitored for oviposition. HWA eggs and adults; *A. piceae* eggs and adults, and *P. floccus* adults were all present for these assessments. These assays were held at 15°C and dishes were checked every other day for *S. coniferarum* eggs, *S. coniferarum* mortality, and the adelgid life stage present.

**Study 7b:** In 2014, we continued to investigate how temperature impacted *S. coniferarum* oviposition pattern and overall fecundity while feeding on HWA. From 28 January – 31 July 2014, three growth chambers were set up with 3 different temperature settings (15, 20, and 25°C), all to remain constant throughout the experiment.

### Prey Suitability

Survivorship of each stage of *S. coniferarum*, from egg to adult, was assessed on *P. strobi* in 2010, and HWA, *A. piceae,* and *P. floccus* in 2013. Individual *S. coniferarum* eggs were transferred with a fine brush onto test prey host plant samples. Eggs were examined daily until they hatched, at which point larval stages were examined every 2-3 d for survivorship at each life stage. Fresh prey was added at each check. The number of days/life stage were recorded until *S. coniferarum* eggs completed development or died.

**Study 8 – No-choice Prey Suitability Tests, Virginia1.** From 7 April – 11 August 2011, *S. coniferarum* eggs oviposited by adults feeding on HWA were removed from hemlock twigs and placed onto *P. strobi* – infested *P. strobus* bark squares to determine if *S. coniferarum* larvae can develop solely on this species. Eggs were checked every other day for hatching, and if egg hatch occurred, more *P. strobi* was added as needed.

**Study 9: No-choice Prey Suitability Tests, Virginia2.**

**Study 9a:** From 15 April – 9 July 2013, *S. coniferarum* eggs were individually placed in single dishes containing either HWA or *P. strobi.* HWA and *P. strobi* eggs and adults were present for the duration of the study. The *S. coniferarum* eggs used in *P. strobi* prey-suitability tests were produced by *S. coniferarum* females that were fed exclusively on *P. strobi* eggs and adults.

**Study 9b:** From 9 April – 9 June 2013, *S. coniferarum* eggs were individually placed in single dishes containing *P. floccus* adults and eggs and monitored for development.

### Data Analysis

All data were tested for normality using goodness-of-fit tests, and for heterogeneity of variance through plots of the residuals. All statistical tests were carried out using JMP software. Comparisons between three or more independent means were analyzed using a two-way analysis of variance (ANOVA) and, if significant, the Tukey-Kramer range test was used to determine significant differences among treatments. In feeding and oviposition paired-choice tests, paired *t*-tests were used to determine prey preference. Contingency tables and the Chi-square statistic were used to evaluate equal distribution of counts between two groups and two or more categorical variables. Larval development was summarized by the number of each stage that *S. coniferarum* reached and the mean number of days per life stage calculated for each test prey species. Other than the standard error, statistical tests for the development data and no-choice prey consumption were not made because the data were not collected at different times, had unequal variances and/or very unequal sample sizes.

## Results

A summary of the host-range studies conducted for *S. coniferarum* is outlined in Table 1. The plant hosts, origin, occurrence of life stages and reason for selection of each host prey species are described in Table 2.

### Prey Evaluation Tests

Prey evaluation tests included no-choice feeding comparisons of among species, no-choice feeding and survivorship rates over time at different temperatures while feeding on HWA, and choice-feeding tests among species. Because studies occurred at different time periods, under varying conditions, mean predation could not be compared statistically among species in no-choice feeding tests.

#### No-choice Feeding Tests

**Study 1a:** In the 2010 no-choice tests, HWA eggs were preferred by adult *S. coniferarum* over the other host stages available, with 3 times more eggs being fed upon than nymphs and more than 11 times more than adults (Table 3). However, there is a difference in biomass among the three life stages.

**Study 1b:** *S. coniferarum* adults fed on *P. pini* and *P. strobi* adults and eggs, but did not consume any *E. tilliae, P. tesselatus, F. externa,* or *D. wistariae* (Table 3).

**Study 2:** *S. coniferarum* adults fed on HWA nymphs, but did not feed on any *P. tesselatus.*

**Study 3a:** In no-choice tests from 15 April – 21 June 2013, *S. coniferarum* consistently fed on both HWA and *A. piceae*. There appeared to be no preference to species or lifestage for either species (Table 3).

**Study 3b:** Between 4 June - 8 July 2014, *S. coniferarum* adult beetles consumed a mean (± SE) of (3.02 ± 0.19) *A. piceae* eggs/day and (1.94 ± 0.18) *A. laricis* eggs/day (Table 3).

**Study 3c:** *S. coniferarum* predation of HWA was monitored again from 27 January – 30 June 2014 at 15, 20, and 25°C. Temperature and time of year significantly affected the predation rates of *S. coniferarum* (df = 16, $F$ = 69.05, $P < 0.0001$) (Fig. 1). The beetles held at 25°C consumed the greatest average number of HWA/day, nearly twice the predation rate of *S. coniferarum* stored at 15°C. There was no difference in predation rate between 15°C and 20°C in April and May. Overall, predation rate was greatest in May at 25°C. At 20°C, highest predation took place April – June, and in April and May at 15°C (Fig. 1).

The mean (±SE) percent mortality of *S. coniferarum* was also compared among all three temperature treatments during the same sample period. *S. coniferarum* mortality varied according to storage temperature (df = 40, $P < 0.0001$). All beetles died by June at 20 at 25°C, but only half the *S. coniferarum* maintained at 15°C died by this time (Fig. 2).

**Study 3d:** From 14 August– 23 September 2014, 45 *S. coniferarum* adults were isolated in Petri dishes and fed exclusively HWA aestivating nymphs. The mean number (± S.E.) of aestivating nymphs consumed/*S. coniferarum* beetle/day (predation rate) was 0.02 ± 0.06 (Table 3). *S. coniferarum* predation of HWA aestivating nymphs was analyzed the following year, from 11 August – 15 October 2015. The average number of HWA consumed/*S. coniferarum* beetle/day (predation rate) was 0.04±0.04 in a series of single-choice tests (Table 3). Regardless of these low predation rates, adult *S. coniferarum* remained alive and active for the duration of both studies.

**Study 4a:** *S. coniferarum* were given a choice of HWA and *P. strobi* eggs and adults from 9 June – 18 Aug. 2009. Beetles consumed significantly more *P. strobi* adults (0.69 ± 0.12) than HWA adults (0.15 ± 0.06) in this study ($P$ = 0.0003). No difference was observed in feeding of eggs between both species (Table 4).

**Study 4b:** A 24 h study was conducted on 1 June 2010, which compared *S. coniferarum* predation between *P. pini* and HWA eggs. Both species were consumed at a similar rate ($P$ = .220) (Table 4).

**Study 4c:** A separate 24-h paired-choice study was completed on 29 June 2010, comparing *S. coniferarum* predation with *P. pini* eggs and HWA eggs. In this case, *S. coniferarum* consumed more *P. pini* eggs than HWA eggs ($P$ = 0.012) (Table 4).

**Study 5:** To compare *S. coniferarum* feeding preference, two host-feeding tests were conducted with a choice between *A. piceae* and HWA and between *A. laricis* and HWA from 9 June – 7 July 2014 (Table 4). Eggs and crawlers were available for each test prey species. More HWA were consumed than *A. piceae* ($P$ = 0.0013), while no differences were observed between HWA and *A. laricis* ($P$ = 0.157) (Table 4).

## Oviposition

**Study 6:** Host preference for oviposition was examined from 4-7 May 2010 by isolating 10 *S. coniferarum* with a choice of HWA on *T. canadensis* and *P. pinifoliae* on *P. monticola.* When unsexed *S. coniferarum* beetles were confined in oviposition jars with both western white pine and eastern hemlock infested with adelgids, about four times more eggs were laid on HWA-infested hemlock than on pine infested with pine adelgid (t = 29, df = 4, *P* = 0.493) (Table 5). Most of the eggs on hemlock (80%) were found in the opened hemlock strobili (spent male flower cones). Since both species of adelgid were present, the beetle could have feed on either species but sought out the spent flowers for oviposition.

**Study 7a:** From 15 April – 9 June 2013, a total of ten *S. coniferarum* beetles were placed in dishes containing HWA, *A. piceae,* or *P. floccus* to be monitored for oviposition. Out of a total 90 eggs laid between 17 April and 9 July 2013, 86 were oviposited when HWA was a host. The first egg was recorded the week of 4 March 2013. Peak egg laying occurred from 19 – 25 April. Egg production seemed to be highest when HWA sistens eggs and crawlers were present. Spent hemlock flowers were provided, but *S. coniferarum* adults did not show a preference for these as an oviposition location.

**Study 7b:** Upon entering the 2014 season, southwest Virginia experienced unexpectedly low temperatures. HWA mortality in the field was extremely high (90 – 100%) through February 2014 as a result of these low temperatures. The poor food quality available may have affected *S. coniferarum* fecundity during their oviposition period. Out of 20 total *S. coniferarum* observed from 28 January – 31 July 2014, only 4 eggs were laid. All oviposition took place in the third week of April. One egg was laid at 20°C, 2 eggs at 15°C, and 1 egg at 25°C (Figure 3).

## Development

**Study 8:** In 2011, a total of 52 *S. coniferarum* eggs oviposited on HWA-infested hemlock were placed on *P. strobus* bark containing *P. strobi* (Table 6). *S. coniferarum* females oviposited about 0.1 egg per female per day. Nineteen of these eggs hatched (37%) after a mean of 18.8 days. A total of 6 larvae (16%) developed to the pupal stage in 32.7 days. Three of these pupae emerged as adults. Therefore, 6% of the eggs developed to the adult stage.

**Study 9a:** From 15 April – 9 July 2013, *S. coniferarum* eggs were individually placed in single dishes containing either HWA or *A. piceae. S. coniferarum* beetles successfully completed development on both HWA (40%) and *A. piceae* (5.9%) in the no-choice development assays observed in 2013. The number of days/life stage were recorded from oviposition to teneral adulthood for both test species (Table 6).

**Study 9b:** From 9 April – 9 June 2013, *S. coniferarum* eggs were individually placed in single dishes containing *P. floccus* adults and eggs and monitored for development. There was a 36.7% survival from egg to larvae, but no *S. coniferarum* larvae survived past the fourth instar when feeding on *P. floccus.*

## Discussion

In laboratory analyses, *S. coniferarum* fed on all life stages and both generations of HWA. They also oviposited and developed on HWA. Since all stages of *S. coniferarum* are collected from HWA-infested hemlock in Washington State, *A. tsugae* seems to be a suitable host for this lady beetle. However, both field collections and laboratory host range evaluation of oviposition, feeding, and larval development indicate that *S. coniferarum* is not specific to HWA, as it feeds and develop on several species in the family Adelgidae. Although testing of prey in other families was limited, there is not evidence to support biologically meaningful predation on Hemiptera other than Adelgidae.

Within the family Adelgidae, inconsistent results make it difficult to establish species preferences, but do indicate that several species of adelgid may be suitable prey. The balsam woolly adelgid is a good example. In no-choice trials, *S. coniferarum* consumed more *A. piceae* per day, but when provided a choice between *A. piceae* and *A. tsugae*, no feeding preference was found. In paired-choice predation studies conducted in 2009 and 2010, sometimes pine adelgids were preferred, and at other times HWA. Occasionally, no preference was shown.

In temperature-controlled studies evaluating feeding and mortality of HWA, 20°C appears to be the optimal temperature for *S. coniferarum* beetles. In all temperature treatments, feeding rates were lowest in January and February. Feeding rates increased by April, but oviposition was extremely low. In no-choice feeding studies with aestivating HWA nymphs, *S. coniferarum* adults survived for 1-2 months without feeding. This suggests that *S. coniferarum* can go for long periods of time without feeding. In the 24-h studies presented in studies “4b” and “4c”, *S. coniferarum* predation of eggs varied in terms of host preference. In study 4b, both *P. pini* and HWA eggs were consumed at a similar rate. In study 4c, *S. coniferarum* adults consumed more *P. pini* eggs than HWA eggs. Again, *S. coniferarum* does not seem to demonstrate species prefence among adelgid species.

The foliage used in these analyses was collected in North Carolina, Georgia, and Washington state. The seasonal presence of pine adelgids on branch samples indicates that pine adelgids are active in these locations during the summer, when HWA is in diapause. Because *S. coniferarum* and pine adelgids (*P. strobi* and *P. pini*) are active during the summer, this predator may feed on multiple pine adelgid species (Table 2).

Oviposition studies showed a significant difference in oviposition rate between target and non-target host species. *S. coniferarum* adults readily fed on all alternate prey host species, but demonstrated a higher oviposition rate when feeding on HWA. However, it is important to note that paired studies were limited. *S. coniferarum* also oviposited when *P. strobi* was a host. In one study, most of the *S. coniferarum* eggs laid on hemlock were found in the spent male flower cones. It is possible that a suitable substrate must be present for optimal *S. coniferarum* oviposition.

In addition to HWA, *S. coniferarum* beetles successfully developed to adults on *P. strobi* and *A. piceae* in no-choice assays in 2011 and 2013, confirming both *P. strobi* and *A. piceae* are viable hosts to *S. coniferarum*. It appears that *S. coniferarum* is not a specialist predator, and can develop to the adult stage on multiple adelgid species found in the eastern United States.

### Host Specificity of *Scymnus* spp. of Interest

Three species in the subgenus *Pullus* are known to be established in North America: *S. coniferarum, S. (Pullus) suturalis* and *S. (Pullus) impexus* (Montgomery and Keena 2011). Both *S. suturalis* and *S. impexus* are adelgid specialists, and it appears *S. coniferarum* is as well (Gordon 1985). *S. suturalis* feeds on *Pineus* spp. in the Northeast and central U.S., and *S. impexus* feeds on *A. piceae* in Canada and the Pacific Northwestern region of the U.S. (Gordon 1985, Montgomery and Lyon 1995). All of the *Scymnus (Pullus)* species described in the literature have been found feeding on multiple adelgid species.

Three species of Chinese *Scymnus* lady beetles belonging to the subgenus *Neopullus* were evaluated as potential biological control agents. These predators were *Scymnus (Neopullus) camptodromus* Yu and Liu, *S. (N.) sinuanodulus* Yu and Yao, and *S. (N.) ningshanensis* Yu and Yao. These beetles were all recovered from native hemlock host trees. However, *S. sinuanodulus* was also consistently collected from *Pinus armandii* var. *amamiana* (Koidz.) Hatus., infested with *Pineus armandicola* Zhang, Zhong, and Zhang. Importantly, when HWA was in diapause, higher numbers of *S. (N.) sinuanodulus* was collected from *P. armandii* than on hemlock (Montgomery and Keena 2011). Additionally, larvae of *Scymnus (Pullus) yunshanpingensis* Yu and *S. (P.) gemius* Yu and Montgomery were recovered in large numbers from *P. armandii* (Montgomery and Keena 2011).

The information presented above indicates that these *Scymnus* species are adelgid predators, and do not show a preference among adelgid species. Just like *S. coniferarum* recovery described in this chapter, higher numbers of *S. sinuanodulus* were collected from *P. armandii*, infested with pine adelgid, *P. armandicola* when HWA was in diapause. It is possible that *Scymnus* species rely on different adelgid species throughout the rear, based on the lifestage and abundance available.

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# Tables and Figures

**Table 1.** Summary of host-range studies conducted for *Scymnus coniferarum* from 2008-2015.

| Group | Study # | Study Type | Prey | Host plant[1] | Source |
|---|---|---|---|---|---|
| Connecticut[2] | Study 1a | Feeding (no-choice) | *Adelges tsugae* | *Tsuga canadensis* | NY & PA |
| | Study 1b | Feeding (no-choice) | *Pineus pini*<br>*Pineus strobi*<br>*Eucallipterus tilliae*<br>*Paraprociphilus tesselatus*<br>*Fiorinia externa*<br>*Dysmicoccus wistariae* | *Pinus sylvestris*<br>*Pinus strobus*<br>*Tilia cordata*<br>*Alnus serrulata*<br>*Tsuga canadensis*<br>*Taxus brevifolia*<br>*Taxus spp.* | Hamden, CT<br>Hamden, CT<br>North Haven, CT<br>Northhampton, MA<br>Hamden, CT<br>Hamden, CT |
| | Study 4 (a, b, c) | Feeding (paired-choice) | *Adelges tsugae*<br>*Pineus strobi*<br>*Pineus pini*<br>*Pineus pini* | *Tsuga canadensis*<br>*Pinus strobus*<br>*Pinus mugo*<br>*Pinus sylvestris* | Hamden, CT<br>Boone, NC<br>Seattle, WA<br>Hamden, CT |
| | Study 6 | Oviposition (paired-choice) | *Adelges tsugae*<br>*Pineus pinifoliae* | *Tsuga canadensis*<br>*Pinus monticola* | Hamden, CT<br>Seattle, WA |
| Virginia1[3] | Study 2 | Feeding (no-choice) | *Adelges tsugae*<br>*Paraprociphilus tesselatus*<br>*Adelges piceae* | *Tsuga canadensis*<br>*Alnus serrulata*<br>*Abies balsamea* | Blacksburg, VA<br>Blacksburg, VA<br>Blacksburg, VA |
| | Study 8 | Prey suitability (no-choice) | *Pineus strobi* | *Pinus strobus* | Blacksburg, VA |
| Virginia2[4] | Study 3 (a, b, c, d) | Feeding (no-choice) | *Adelges tsugae*<br>*Adelges piceae*<br>*Adelges laricis* | *Tsuga canadensis*<br>*Abies balsamea*<br>*Larix spp.* | Blacksburg, VA<br>Blacksburg, VA<br>Blacksburg, VA |
| | Study 5 | Feeding (paired-choice) | *Adelges tsugae*<br>*Adelges piceae*<br>*Adelges laricis* | *Tsuga canadensis*<br>*Abies balsamea*<br>*Larix spp.* | Blacksburg, VA<br>Blacksburg, VA<br>Blacksburg, VA |
| | Study 7 (a, b) | Oviposition (no-choice) | *Adelges tsugae*<br>*Adelges piceae*<br>*Pineus floccus* | *Tsuga canadensis*<br>*Abies balsamea*<br>*Picea rubens* | Blacksburg, VA<br>Mt. Lake, VA<br>Mt. Lake, VA |
| | Study 9 (a, b) | Prey suitability (no-choice) | *Adelges tsugae*<br>*Pineus strobi*<br>*Pineus floccus* | *Tsuga canadensis*<br>*Pinus strobus*<br>*Picea rubens* | Blacksburg, VA<br>Blacksburg, VA<br>Mt. Lake, VA |

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[1] Host plant species on which adelgid species were collected.

[2] M. Montgomery lead, L. Schwartzburg (Study 1a), G. Bradford, D. Cole, M. Keena (Studies 1b, 4, 6).

[3] T. McAvoy (Studies 2, 8)

[4] Darr (Studies 3, 5, 7, 9)

**Table 2.** Host prey species used in host range tests for *S. coniferarum*. Plant hosts, origin, occurrence of stages (eggs, dormant nymphs or eggs, wingless adults and winged adults) in eastern North America, and reason for selection are listed.[1]

| Prey | Plant Host | Origin | Eggs | Dormant period and life stage | Progrediens (# generations) | Sexupara flight | Selection rationale |
|---|---|---|---|---|---|---|---|
| **Adelgidae**<br>Hemlock woolly adelgid<br>*Adelges tsugae* | Primary: *Picea* (Asia)<br>Secondary: *Tsuga* | Japan | Feb. – July | Aestivosistens: July – Oct. | June – July<br>(1) | June | Target |
| Balsam woolly adelgid<br>*Adelges piceae* | Primary: None<br>Secondary: *Abies* | Europe | June – Oct. | Aestivosistens: June – Nov.<br>Heimosistens: Nov. – Apr. | rare | June (rare) | Taxonomic similarity |
| Larch woolly adelgid<br>*Adelges laricis* | Primary: *Picea*<br>Secondary: *Larix* | Europe | July – Oct.<br>Apr. – June | Heimosistens: Aug. – March | May- July<br>(1-3) | June – July | Taxonomic similarity |
| Pine bark adelgid<br>*Pineus strobi* | Primary: None<br>Secondary: *Pinus* | Europe | Mar. – Oct. | Heimosistens: Dec. – Apr. | All year<br>(many) | May – June | Taxonomic similarity |
| Pine woolly aphid<br>*Pineus pini* | Primary: None<br>Secondary: *Pinus* | Europe | Mar. – Oct. | Heimosistens: Dec. – Apr. | May – Oct.<br>(2+) | May – June | Taxonomic similarity |
| Red spruce adelgid<br>*Pineus floccus* | Primary: *P. rubens*<br>Secondary: *P. strobus* | Eastern N. America (native) | unknown<br>March – Oct. | unknown | All year<br>(unknown) | May | Taxonomic similarity |
| Pine aphid<br>*Pineus pinifoliae* | Primary: *Picea*<br>Secondary: *Pinus* | N. America (native) | August<br>Apr. – June | Heimosistens: Aug. – Apr. | May – July<br>(2) | May | Taxonomic similarity |
| **Aphididae**<br>Woolly alder aphid<br>*Paraprociphilus tessellatus* | Primary: *Acer*<br>Secondary: *Alnus* | N. America (native) | October<br>Viviparous | Eggs overwinter on *Acer.* | May – Sept.<br>(several) | July – Sept. | Ecological importance |
| Linden aphid<br>*Eucallipterus tiliae* | *Tilia* | Europe | October<br>Viviparous | Eggs overwinter on *Tilia.* | June – Oct.<br>(several) | July – Oct. | Ecological dissimilarity |
| **Diaspididae**<br>Elongate hemlock scale<br>*Fiorinia externa* | Primary: *Tsuga, Picea, Abies* | Japan | July<br>Sept. – Apr. | Fertilized female or eggs overwinter. | June – March<br>(2) | Males: July | Ecological similarity |
| **Pseudococcidae**<br>Taxus mealybug<br>*Dysnicoccus wistariae* | Primary: *Taxus, Tsugae* occasionally | Worldwide | Viviparous | Overwinters as nymph. | June – Aug.<br>(1) | Males: June – Aug. | Ecological similarity |

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[1] Sources: Annand 1928, Börner and Heinze 1957, Blackman and Eastop 1994, Havill and Foottit 2007.

[2] Plant host species commonly associated with adelgid species.

[3] Terms apply to adelgids only, active or flight period for other families.

**Table 3.** Prey consumed by adult *S. coniferarum* in no-choice feeding tests.

| Researcher / Study # | Prey species | Temp (°C) | Dates | n (replicates) | Stage tested | Mean # host (±SE) consumed/*Sc.*/day |
|---|---|---|---|---|---|---|
| **Connecticut**<br>Study 1a | *Adelges tsugae* | 18 | Apr. 26 & June 16, 2008 | 13 | Adult | 1.00±0.21 |
| | | | | | Egg | 11.69±0.57 |
| | | | | | Nymph II | 3.42±2.16 |
| **Connecticut**<br>Study 1b | *Pineus pini* | 15 | June 22, 2010 | 10 | Adult | 0.6±0.37 |
| | | | | | Egg | 1.23±0.68 |
| | *Pineus strobi* | 15 | July 29, 2010 | 10 | Adult | 1.41±0.41 |
| | | | | | Egg | 2.42±1.78 |
| | *Eucallipterus tilliae* | 20 | July 8, 2010 | 10 | Adult | 0.0 |
| | *Paraprociphilus tesselatus* | 15 | Apr. 5, 2010 | 10 | Nymph I | 0.0 |
| | *Fiorinia externa* | 20 | Oct. 22, 2010 | 10 | Adult | 0.0 |
| | *Dysmicoccus wistariae* | 20 | Oct. 15, 2010 | 10 | Nymphs | 0.0 |
| **Virginia1**<br>Study 2 | *Paraprociphilus tesselatus* | 15 | Nov. 2 – 18, 2011 | 15 | Nymph I | 0.0 |
| | *Adelges tsugae* | 15 | Oct. 13 – Nov. 27, 2011 | 10 | Nymphs II/III | 0.36±0.12 |
| **Virginia2**<br>Study 3a | *Adelges tsugae* | 15 | Apr. 15-June 21, 2013 | 7 | Egg | 2.70±0.87 |
| | | | | 3 | Crawler | 2.63±0.31 |
| | | | | 8 | Nymphs II-IV | 4.74±0.92 |
| | | | | 5 | Adult | 2.92±1.04 |
| | *Adelges piceae* | 15 | Apr. 15-June 21, 2013 | 30 | Nymph | 3.05±0.23 |
| | | | | 11 | Adult | 3.44±0.36 |
| | | | | 3 | Egg | 2.47±1.07 |
| Study 3b | *Adelges piceae* | 15 | June 4 – July 8, 2014 | 48 | Egg | 3.02±0.19 |
| | *Adelges laricis* | 15 | June 4 – July 8, 2014 | 50 | Egg | 1.94±0.18 |
| Study 3d | *Adelges tsugae* | 15 | Aug. 14 – Sept. 23, 2014 | 148 | Aest. Nymphs | 0.02±0.06 |
| | *Adelges tsugae* | 15 | Aug. 11 – Oct. 15 , 2015 | 144 | Aest. Nymphs | 0.04±0.04 |

**Table 4.** Consumption of *A. tsugae* and alternate prey by adult *S. coniferarum* in paired-choice tests.

| | | | | **Mean ± SE prey consumed per day/*S. coniferarum* adult[1]** | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Researcher/Study | Alternate prey | Life stage present | n[2] | *A. tsugae* | Alternate prey | Difference | t | df | *P*[3] |
| **Connecticut**<br>**Study 4a**<br>June 9 –Aug 18, 2009 | *Pineus strobi* | Eggs | 39 | 0.87±0.31 | 1.41±0.35 | -0.54±0.45 | 1.20 | 38 | 0.237 |
| “ | “ | Adults | “ | 0.15±0.06 | 0.69±0.12 | 0.54±0.15 | 3.94 | “ | 0.0003 |
| **Study 4b**<br>June 1, 2010 | *Pineus pini* | Eggs | 10 | 5.50±1.25 | 3.00±1.22 | 2.50±1.90 | 1.32 | 9 | 0.220 |
| **Study 4c**<br>June 29, 2010 | *Pineus pini* | Eggs | 13 | 3.62±1.24 | 9.62±2.30 | -6.00±2.03 | 2.96 | 12 | 0.012 |
| **Virginia2/**Study 5<br>June 9 – July 7, 2014 | *Adelges piceae* | Eggs + crawlers | 35 | 1.91±0.1 | 1.43±0.1 | 0.48±0.14 | 3.34 | 69 | 0.0013 |
| | *Adelges laricis* | Eggs + crawlers | 30 | 1.98±0.32 | 1.35±0.32 | 0.63±0.45 | -1.43 | 59 | 0.157 |

[1] *Pineus strobi* host was *P. strobus*; *Pineus pini* host was *Pinus sylvestris* for June 1 trial, *Pinus mugo* for June 29 trial.

[2] “n” indicates the number of *S. coniferarum* beetles present in each study.

[3] Two-tailed P-value of statistical significance between *A. tsugae* and alternate prey; P<0.05 is considered statistically significant.

**Table 5.** Comparisons of oviposition by unsexed samples of *S. coniferarum* on various hosts.

| Researcher / Study | Test prey | Dates | Temp (°C) | Reps × n | Eggs laid (Mean±SE/beetle/day) |
|---|---|---|---|---|---|
| **Connecticut**<br>Study 6 | *Adelges tsugae*<br>*Pineus pinifoliae* | May 4 – 7, 2010 | 15<br>15 | 5 ×10 | 1.16±0.37<br>0.28±0.11 |
| **Virginia2**<br>**Study 7a** | *Adelges tsugae* | Apr. 15 – June 29, 2013 | 15 | 13 × 10 | 1.83±0.16 |
| | *Adelges piceae* | Apr. 15 – June 29, 2013 | 15 | 17 × 10 | 0.06±0.13 |
| | *Pineus floccus* | Apr. 15 – June 29, 2013 | 15 | 10 × 10 | 0.0±0.0 |

[1] “n” indicates the number of *S. coniferarum* beetles present in each study.

[2] Choice test analyzed with paired t-test (t=2.79, df=4, P=0.493)

[3] No-choice test analyzed with one-way ANOVA. (F= 41.93).

**Table 6.** Larval development time and survivorship at 15°C of *S. coniferarum* on four adelgid hosts.[1]

| Researcher / Study | Host | Dates | Temp (°C) | *S. coniferarum* life stage | n | # Days/Stage (±SE) | % Survival from eggs |
|---|---|---|---|---|---|---|---|
| **Virginia1**<br>Study 8 | *Pineus strobi* | Apr. 7 – Aug. 11, 2011 | 15 | Eggs | 52 | 18.8±1.7 | 100 |
| | | | | Larvae | 19 | 32.7±5.5 | 37 |
| | | | | Pupae | 6 | 23.3±3.8 | 12 |
| | | | | Adults | 3 | **Total =** 82.3.7±9.7 | 6 |
| **Virginia2**<br>Study 9a | *Adelges tsugae* | Apr. 15 – July 9, 2013 | 15 | Eggs | 10 | 21.2±2.6 | 100 |
| | | | | Larvae | 9 | 33.6±1.4 | 90 |
| | | | | Pupae | 4 | 21.3±1.31 | 40 |
| | | | | Adults | 4 | **Total =** 81.0±1.41 | 40 |
| | *Adelges piceae* | Apr. 15 – July 9, 2013 | 15 | Eggs | 17 | 20.5±0.62 | 100 |
| | | | | Larvae | 5 | 25.4±2.38 | 29.4 |
| | | | | Pupae | 1 | 16±0 | 5.9 |
| | | | | Adults | 1 | **Total =**70±0 | 5.9 |
| Study 9b | *Pineus floccus* | Apr. 9 – June 9, 2013 | 15 | Eggs | 11 | 32.2±2.29 | 100 |
| | | | | Larvae | 0 | 0±0 | 36.7 |
| | | | | Pupae | 0 | 0±0 | 0 |
| | | | | Adults | 0 | **Total =** Unknown | 0 |

[1] Chi-square analysis for this 2 x 4 contingency table is 5.16, df = 3, P = 0.16, No significance.

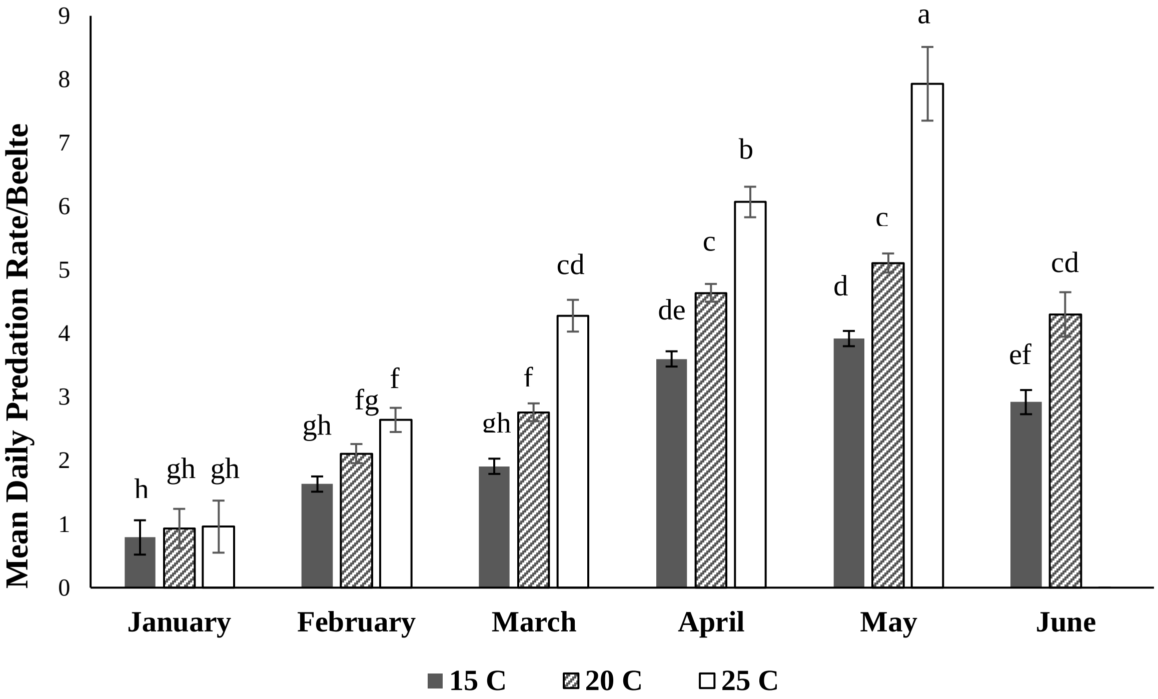

**Figure 1.** Mean (±SE) daily predation rate of *S. coniferarum* at 15, 20 and 25°C from 27 January – 31 May 2014. Different letters above the bars represent a significant difference in predation across all months and treatments at *P*=0.05, Tukey-Kramer honestly significant difference tests. (df = 16, F = 69.05, P < 0.0001).

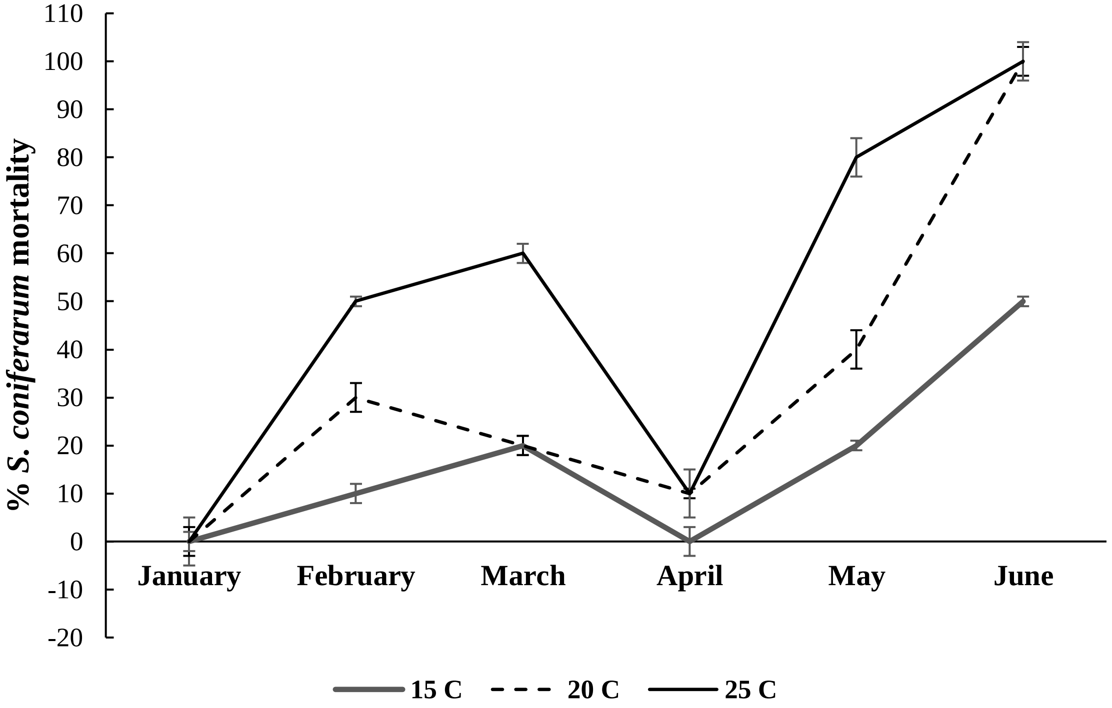

**Figure 2.** Mean **(**±SE**)** percent mortality of *S. coniferarum* at 15, 20 and 25 °C from January- June 2014. Chi-square analysis for this 6×3 contingency table is 871.54, df = 40, P < 0.0001.

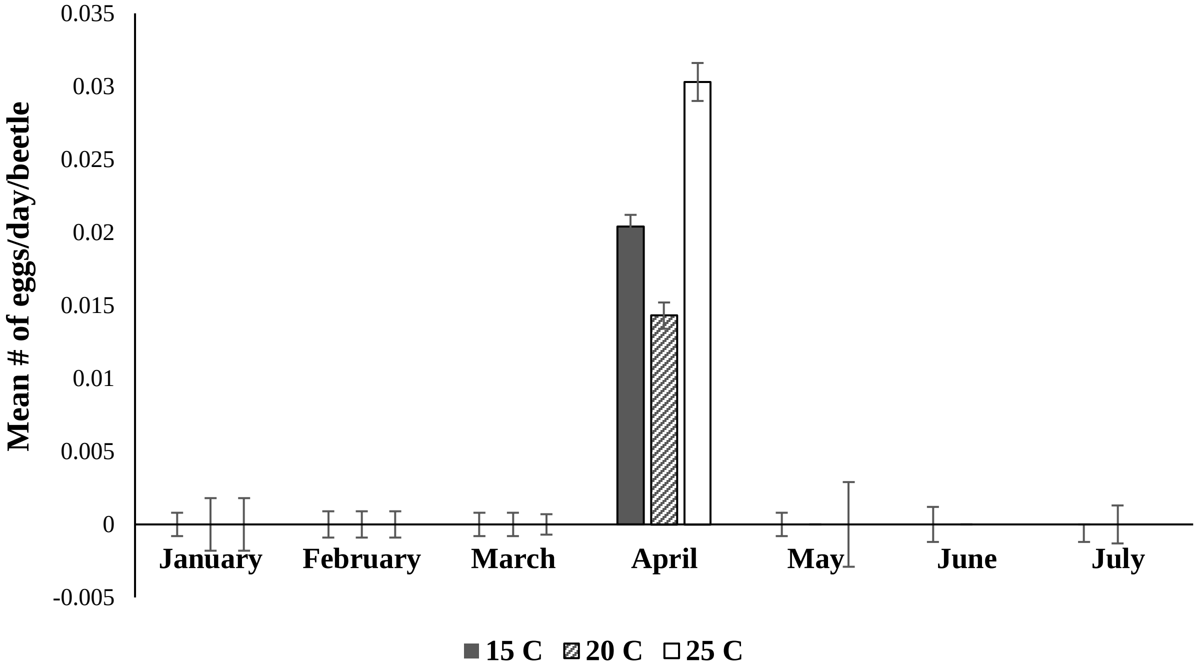

**Figure 3.** Mean (±SE) eggs laid per *S. coniferarum* beetle at 15, 20 and 25°C from January 28 - July 31, 2014. Oviposition at different temperatures was compared using a one-way ANOVA (df = 16, F = 0.69, P = 0.797). No significance.

# CHAPTER FOUR

## Phenology and synchrony of *Scymnus coniferarum* (Coleoptera: Coccinellidae) and its host hemlock woolly adelgid (Hemiptera: Adelgidae) in the Puget Sound, WA.

### Abstract

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is an invasive pest of eastern hemlock, *Tsuga canadensis* (L.) Carrière, in eastern North America. *Scymnus (Pullus) coniferarum* Crotch (Coleoptera: Coccinellidae) is a small lady beetle that preys on HWA in the western United States and is absent from hemlock stands in the eastern United States. This predator was approved for release before a robust host range analysis was completed. It was quickly realized that more information is needed on *S. coniferarum* in its native range, as it relates to phenology and host choices. The objective of the study in this chapter is to investigate the life history of *S. coniferarum* and associated adelgid prey species in the Puget Sound area of Washington state. Seasonal abundance of *S. coniferarum* and adelgid prey were sampled at six sites near Tacoma, WA, twice monthly, for one full year (November 2015 – November 2016). The sites were classified as pure hemlock stands, non-hemlock conifer stands, and mixed stands of hemlock and other native conifers. Host tree species included Douglas-fir (*Pseudotsuga menziesii* (Mirb.)), Shore pine (*Pinus contorta* subsp. *contorta* Dougl.), western white pine (*Pinus monticola* Dougl.), and western hemlock (*Tsuga heterophylla* (Raf.) Sarg.). *S. coniferarum* adults were found on all conifer species sampled. Adults appear to feed on pine adelgid species during its oviposition period (April – May). Each conifer species supports a different adelgid species and presence of *S. coniferarum* on these different species suggest the predator feeds on multiple adelgid species within its native range. Overall, *T. heterophylla* branch sections contained the highest number of adelgids among all host trees sampled. For all of the adelgid species sampled, adults made up the greatest density of life stages present between

November 2015 and 2016. Genetic testing of *S. coniferarum* beetles collected in the Tacoma area found that there is a 6% difference among samples co-habitating the same host tree species. These beetles cannot be separated by morphological characteristics. In light of this information, we have recommended that APHIS halt all shipments of *S. coniferarum* to the eastern United States until further taxonomic clarification can be made.

**Keywords:** *Adelges tsugae*, HWA, *Scymnus coniferarum*, phenology, Washington

## Introduction

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand is an invasive pest insect in the eastern United States. HWA has caused widespread decline and mortality in all age classes of *Tsuga canadensis* (L.) in forests from Maine to the southern Appalachians, with severe impacts on ecological and forest stand dynamics (Orwig et al. 2002, Mayfield et al. 2015). *Scymnus (Pullus) coniferarum* Crotch (Coleoptera: Coccinellidae) is the most recently studied biological control agent under investigation for the control of HWA in the eastern United States. *S. coniferarum* is a native predator of HWA in temperate rainforests of western North America. This predatory lady beetle was discovered in 2006 feeding on HWA along-side the biological control agent *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) (McDonald 2010), which was first released in 2003 to control HWA in the eastern United States (Mausel et al. 2010). Because *S. coniferarum* is considered a native predator of HWA in the western United States, USDA APHIS decided in 2012 to not prevent its shipment and release to the eastern U.S., even though only limited host range testing and biological assessment of this insect in its native range were conducted.

The known host trees for *S. coniferarum* recorded in the literature prior to 2008 were all pine species in British Colombia, Arizona, California, Colorado, Idaho, Nevada, Utah and Wyoming (Gordon 1976). In another publication, *S. coniferarum* was identified as a predator of adelgids collected on adelgid-infested *Pinus contorta* Dougl., and *Pinus radiata* Douglas ex. D.Don. (Whitehead 1967). In collections made in Seattle, WA, *S. coniferarum* was abundant on western hemlock, *Tsuga heterophylla* (Raf.) Sarg. infested with HWA (Montgomery et al. 2009), and to a lesser extent on western white pine, *Pinus monticola* Douglas ex. D.Don., feeding on an unidentified adelgid species (Montgomery and McDonald 2010). These observations indicate that *S. coniferarum* could feed on multiple adelgid species in its native range.

The genus *Scymnus* (Coleoptera: Coccinellidae) contains more than 800 species, with approximately 500 in the subgenus *Pullus*. Three of these species are known to feed on adelgids (Gordon 1985): *Scymnus impexus* Mulsant and *Scymnus suturalis* Thunberg, both introduced to the U.S. from Europe, and *S. coniferarum* (Montgomery and Lyon 1995, Montgomery and Keena 2011). *Scymnus suturalis* have been recovered feeding on adelgid-infested *T. canadensis, Pinus strobus* (L.), and *Pinus sylvestris* (L.), but favors adelgids associated with *Pinus* species (Montgomery and Lyon 1995). In laboratory studies, *S. suturalis* has fed on all stages of HWA. It is possible that these beetles move among these three host tree species, depending on the presence and life stage of adelgids that are seasonally available. The oviposition period of *S. suturalis* occurs in the spring, and eggs have been recovered on *T. canadensis* this time of year (Montgomery and Lyon 1995). *Scymnus impexus* is mostly associated with *A. piceae* and other fir colonizing adelgids, but may feed on other adelgid species (Delucchi 1954). Another *Scymnus* subgenus found only in Asia, *Neopullus* Sasaji, includes more than 30 species, of which

3-5 specialize on adelgids. Most of these species are generalists, and feed on both aphids and adelgids on coniferous tree species (Lu et al. 2001, Yu et al. 2000).

In order to assess the suitability and safety of *S. coniferarum* as a potential biological control agent of HWA in the eastern U.S., host-range (Chapter 3) and field-cage (Chapter 2) studies were completed, and development of a mass-rearing procedure was attempted (Darr et al. 2016). Overall, *S. coniferarum* was a voracious predator of HWA in both field and laboratory settings (Chapter 2), but oviposition rates for this predator were extremely low (Chapter 3). The inability to rear *S. coniferarum*, and the taxonomic similarity of this species to other generalist predators lead us to develop a study to observe the insect in its native habitat. In this chapter, I report on a phenological study which sampled a variety of host trees native to the Pacific Northwest over the course of one year to determine adelgid and natural enemy species present.

This information will help us understand the timing of life stages and host preferences of *S. coniferarum* and adelgid species present. These are critical data, as we continue to evaluate *S. coniferarum*'s potential as a biological control agent of HWA in the eastern United States. It is possible that *S. conifearum* relies on multiple adelgid prey species in its native range for optimal reproductive and developmental fitness.

The objective of this study is as follows:

Assess seasonal activity (timing of life stages, host preference and associated population levels) of adelgid species, *S. coniferarum,* and other natural enemies present on various conifer tree species present in Tacoma, Washington.

## Materials and Methods

### Location and Timing of Experiments

Six sites were sampled twice monthly for one full year (9 November 2015 – 20 November 2016). The sites used in this study were located in the suburbs of Tacoma, WA (47°16'14.6"N 122°31'16.7"W), in plant hardiness zone 8 (USDA-ARS 2015) (Fig. 1). This location was chosen because *S. coniferarum* has been commonly collected for experimental purposes by Richard McDonald in this region (McDonald 2010).

The sample sites were comprised of two pure hemlock stands (containing only *T. heterophylla*), two stands containing conifers other than *T. heterophylla* (containing *P. contorta* var. *contorta, P. monticola,* and *P. menziesii*), and two mixed stands of western hemlock and native conifers (*T. heterophylla*, *P. contorta*, *P. monticola*, and *P. menziessi*). Each site contained a total of five sample trees in close proximity, in order to discern whether any seasonal movement of *S. coniferarum* occurs among host tree species. A total of 12 *T. heterophylla,* 12 *P. contorta,* 4 *P. menziessi,* and 4 *P. monticola* host trees were tested. Trees at all locations had high densities of adelgids and low-hanging branches that could be accessed easily. The trees selected ranged from 7.4 – 16.2 cm dbh (diameter at breast height). All trees selected were in good health, and able to provide a consistent presence of adelgids for the duration of the study.

Sampling took place every two weeks, allowing me to track adelgid life stage, density, and species present on each sample tree. At the same time, the total number of *S. coniferarum* collected from each host tree was recorded. All life stages (eggs, crawlers, nymphs and adults) of both generations (progrediens and sistens) of HWA and were present during this study. Eggs, crawlers, nymphs and adults of both *Pineus strobi* and *Pineus pini* were also present. Only eggs and adults of *Adelges cooleyi* were recovered over the course of the study.

## Experimental Procedure

At each site, adelgid-infested terminal branches were pruned from each sample tree, with each sample containing at least 100 adelgids per tree/site. The number of branches clipped from each tree varied in order to reach this density. These branch samples were then packaged in 1-L Ziplock™ bags, and shipped overnight to the Beneficial Insects Quarantine Laboratory in Blacksburg, VA. Upon arrival, branch samples were frozen in an industrial freezer to prevent the adelgids present on foliage from developing further. This ensured the associated life stages and measurements were accurately tracked each sample period.

The same trees were also sampled via beat-sheet analysis, to collect *S. coniferarum* and any other predator insects present. Several beats were taken from all accessible branches around the circumference of each tree in all sample sites. Branch clippings and predators collected from beat-sheet sampling were then shipped to Virginia Tech quarantine facility for analysis. Shipping periods coincided with adelgeid sampling shipments. Sample periods at two-week intervals resulted in a total of 26 shipments.

Upon arrival, the branch clippings were examined for adelgid and *S. coniferarum* life stage and density, using a dissecting microscope with a calibrated optical scale. Life stages of adelgids were determined by head-capsule and body-length measurements, as well as the number of skin-castes present. *Scymnus coniferarum* life stages were determined by head-capsule and body-length measurements, as well as visual appearance. Population densities of HWA and other adelgid species present were calculated by dividing the total number of adelgids by the length of each branch, to determine the number of adelgids per $cm^2$. The total number of *S. coniferarum* collected from each sample tree was recorded with the associated tree species and site location. The number of *S. coniferarum* collected from beat-sheet sampling was then compared to adelgid density and life stages documented on all sample trees, in order to identify which adelgid species and life stages were available when *S. coniferarum* populations were present.

## Adelgidae species identification

To determine the species of adelgids present on conifers, representative samples of adelgids and predators collected from each host tree species were sent to Dr. Nathan Havill, Research Entomologist at the Northern Research Station (USFS) for identification through a series of genetic analyses (nphavill@fs.fed.us). Samples were identified by comparing DNA barcodes to those on the Barcode of Life Data System (BOLD; Ratnasingham and Hebert 2007). DNA barcodes were generated using standard methods. DNA was extracted using the Mag-Bind Blood & Tissue Kit (Omega Bio-Tek, Norcross, Georgia). DNA was extracted from adelgids after grinding a single individual with a pestle. Other individuals from the same collection were slide mounted and saved as a voucher. For larvae or pupae of other insects, the cuticle was preserved after proteinase digestion and slide mounted as a voucher. All vouchers were deposited at the Yale Peabody Museum of Natural History (YPM). The 5' end of mitochondrial cytochrome *c* oxidase subunit I (COI) was amplified using primers LepF1 and LepR1 (Herbert et al. 2004), and bi-directional sequencing was performed at the DNA Analysis Facility on Science Hill, Yale University using an ABI 3730 sequencer (Life Technologies, Grand Island, NY). Chromatograms were edited using Geneious v7 (http://www.geneious.com, Kearse et al. 2012). Samples were identified to the most specific taxon possible by searching the BOLD database (www.barcodinglife.org, Ratnasingham and Hebert 2007).

## DNA barcode sequencing for *S. coniferarum*

In spring, 2015, samples of *S. coniferarum, S. suturalis and Scymnus camptodromus* Yu and Liu samples were compared through DNA sequencing. A divergence in the speciation of *S. coniferarum* samples was found that did not match the other *Scymnus* samples tested. This prompted further analysis of *S. coniferarum* samples collected from its native range of Tacoma, WA.

From 29 August – 10 September 10 2016, adult *S. coniferarum* were collected in Tacoma, Washington from *T. heterophylla*, *P. monticola*, *P. contorta,* and *Pseudotsuga menziesii* (Mirb.). A sample of beetles from each host was selected randomly in proportion to the numbers collected from each host, except that all four samples from *P. monticola* were included. Beetles were delivered to the Virginia Tech quarantine facility and immediately placed in 95% ethanol at 15°C. For analysis, the abdomen was removed from each beetle for later examination of genitalia, and the remainder placed in a well of a 95-well microplate. In February 2017, these microplated samples were sent to the Canadian Centre for DNA Barcoding in Guelph, Ontario, Canada for barcoding. Standard protocols (Hajibabaei et al. 2005, DeWaard et al. 2007) were employed for DNA extraction and amplification, sequencing of the COI barcode region, sequence editing and alignment.

Useful data were obtained for 81 beetles. Dr. Nathan Havill combined the barcoding results of these beetles with that from 17 other beetles previously barcoded, as well as barcodes for *Scymnus suturalis* and *Sasajiscymnus tusgae.* A neighbor joining phylogenetic tree, using uncorrected P-distance, with the software Geneious was created (Kearse et al. 2012).

**Data Analysis**

All data were tested for normality using goodness-of-fit tests, and for heterogeneity of variance through plots of residuals. All statistical tests were carried out using JMP software (SAS Institute Inc. 2012). The mean and standard error were calculated for the number of *S. coniferarum* collected from each tree species and sample period. The mean totals of *S. coniferarum* collected per host tree species were then compared using a one-way analysis of variance (ANOVA). Adelgid species, host tree species, adelgid life stage present, and associated adelgid density (# of adelgids/cm) were documented from each sample branch. Data were tested for differences for mean densities by host tree species and sample period, using a one-way ANOVA. If significant, the Tukey-Kramer range test was used to determine a significant difference among treatments.

## Results

### Adelgidae and natural enemy species identification

Dr. Nathan Davill completed DNA analyses of 26 adelgid and predator samples collected from the sample sites previously described (nphavill@fs.fed.us). These analyses confirmed that the adelgid species collected from *P. monticola* was pine bark adelgid, *Pineus strobi* Hartig (10 samples). This is the first record of *P. strobi* in the western United States. The adelgids found on *P. contorta* were identified as *Pineus pini* Macquart (5 samples). The adelgids collected from *T. heterophylla* were confirmed as HWA. *Adelges cooleyi* Gillette was identified on *P. menziesii.*

Several predator species were identified on *P. contorta, P. monticola* and *T. heterophylla* host trees sampled. A single Coniopterygidae, or 'dustywing' was identified on a *P. menziesii* branch sample. One *Neoleucopis* spp. and one unknown cecidomyiid fly were identified on two *P. contorta* samples. Several samples collected from *T. heterophylla* were identified as *Leucopis* *piniperda* Malloch, one of two abundant *Leucopis* species known to feed on HWA in the western United States (Kohler et al. 2008).

### DNA barcode sequencing for *S. coniferarum*

Both the previous and the present samples of *S. coniferarum* fell into two distinct clusters with a 6% divergence in DNA among the two clusters (Table 1). The first sample had 53% of the beetles in Cluster 1 (personal observation) whereas the second sample had only 11% in Cluster 1. Although the proportion in each cluster is highly statistically different in both samples (Fisher's exact test, P=0.0005), the reason for this can only be speculative as there are many known and unknown differences in the samples. For example, the first samples were collected from fall through spring, presumably on hemlock, whereas the second round of sampling took place in late summer on several hosts.

The *S. coniferarum* samples fell into two distinct taxonomic clusters with a 6% divergence in DNA among the two clusters. 12.3% of the 81 total samples were identified as "Cluster 1", and 87.7 % were in "Cluster 2". *S. coniferarum* adults were identified on *P. menziesii, P. contorta,* and *T. heterophylla.* The clusters of *S. coniferarum* in this sample did not correspond to host tree species. However, it is not possible to statistically test this, according to the Chi-square value based on the 2 × 4 contingency table, due to the low n size in Cluster 1 (Table 1).

### Predator/Host Density Relationships and Seasonal Patterns

#### Adelgidae

The analysis of branch samples showed a significant difference in the density of adelgids among host tree species among all sample periods (*df* = 3, 6896; *F* = 72.78; *P* < 0.0001).

Overall, *T. heterophylla* branch sections contained the highest number of adlegids/cm (2.39 ± 0.08) among all host tree species sampled, and *P. menziesii* contained the lowest overall density of adelgids/cm (0.04 ± 0.21) (Table 2).

For all of the adelgid species sampled, adults made up the highest density of life stages present between November 2015 and November 2016 (Figs 2a-d). On *T. heterophylla,* the number of adult HWA present was significantly higher at (6.9 ± 0.28 adelgids/cm) than *P. strobi* on *P. monticola* (3.6 ± 0.77 adelgids/cm), *P. pini* on *P. contorta* (1.9 ± 0.41 adelgids/cm) and *A. cooleyi* on *P. menziesii* (0.11 ± 0.41 adelgids/cm) (*df* = 3, 6896; F = 47.61; P < 0.0001).

### ***S. coniferarum***

In beat sheet sampling, *S. coniferarum* adults were collected from *P. contorta, P. monticola,* and *T. heterophylla* host trees. No larvae or eggs were collected. A total of 215 adult *S. coniferarum* were recovered during the year-long sample period. *S. coniferarum* was found on all host tree species, with the exception of *P. menziesii* (*df* = 3, 83; *F* = 2.24; *P* = 0.1130) (Table 3). No *S. coniferarum* were collected off of *P. menziesii*, and *A. cooleyi* densities reflected low population levels (Table 2). The number of *S. coniferarum* collected was highest between February and April for each *P. contorta*, *P. monticola,* and *T. heterophylla* host trees (Fig. 3). The greatest number of *S. coniferarum* recovered among all sample periods was collected from *P. contorta* on 19 June 2016 (32 *S. coniferarum* adults), followed by 25 February 2016 (27 *S. coniferarum* adults) on the same host tree species (Fig. 3). The life stages of *P. pini* recorded on both sample dates were adults and eggs (Fig. 2d). No *S. coniferarum* were recovered between 19 December 2015 and 30 January 2016 on all sample trees. When HWA was in diapause, no *S. coniferarum* were collected from hemlock host trees (Fig 3). While *S. coniferarum* beetles are present on both *P. contorta* and *P. monticola* at this time, populations are present at reduced rates (Fig. 3).

## Discussion

*Scymnus coniferarum* is considered an "adelgid specialist" (Montgomery and McDonald 2010). This beetle is described as a predator of adelgid species primarily associated with pine and hemlock species (Crotch 1874, Whitehead 1967, Gordon 1976, Montgomery and McDonald 2010, Darr Chapter 3). Both Crotch 1847 and Gordon 1976 collected "large numbers" of *S. coniferarum* from *P. contorta* and *Pinus radiata* D. Don infested with adelgids Adult *S. coniferarum* beetles were observed feeding on *T. heterophylla* infested with *A. tsugae* and on *P. monticola* infested with an "unidentified pine adelgid" in the Seattle metropolitan area in 2008 and 2009 (Montgomery et al. 2009, Montgomery and McDonald 2010).

*S. coniferarum* is classified within the genus *Scymnus* and subgenus *Pullus*. A majority of the species within the genus *Scymnus,* and all *Pullus* species, are aphidophagous, feeding on either aphids or adelgids (Gordon 1985, Whitehead 1967). Only three species of adelgid-feeding insects in the subgenus *Pullus* are known to occur in North America: *S. coniferarum, Scymnus (Pullus) suturalis* and *Scymnus (Pullus) impexus* (Montgomery and Keena 2011). Both *S. suturalis* and *S. impexus* are adelgid specialists (Gordon 1985). *S. suturalis* preys on *Pineus spp* in the northeast and central United States, and *S. impexus* feeds on *A. piceae* throughout much of Canada and the pacific northwestern region of the United States (Gordon 1985, Montgomery and Lyon 1995). The *Scymnus (Pullus)* species described have been found feeding on multiple adelgid species on a variety of host trees. It is possible that species within this subgenus rely on a complex of adelgids within their native range.

Chinese *Scymnus* lady beetles belonging to the genus *Scymnus* and subgenus *Neopullus* were first recovered in 1995 in foreign exploration efforts towards the biological control of HWA. Beetles were collected from the Yunnan, Sichuan, and Shaanxi provinces, which contained mixed stands of multiple hemlock and pine species. *Neopullus* is the largest genus in family Coccinellidae, and while there are 22 described species in the subgenus *Scymnus (Neopullus),* the hosts have been identified for only a portion of these species. *Scymnus (Neopullus) hoffmanii* is known to be a predator of aphids in China and Japan (Yang and Zheng 1991). Seven *Scymnus (Neopullus)* species were collected from hemlock tree species in China (Yu et al. 2000). Of these, three species were selected to be evaluated further as potential biological control agents: *Scymnus (Neopullus) camptodromus* Yu & Liu, *S. (N.) sinuanodulus* Yu & Yao and *S. (N.) ningshanensis* Yu & Yao. These beetles were recovered from *Tsuga chinensis, T. dumosa,* and *T. forrestii* (Montgomery and Keena 2011).

In addition to hemlock, other conifers were sampled to determine the extent that alternate hosts may be used by the three *Scymnus (Neopullus)* species. Only *S. (N.) sinuanodulus* was found on host trees other than hemlock. Consistent populations of *S. (N.) sinuanodulus* were found on *Pinus armandii* var. *amamiana* (Koidz.) Hatus., infested with *Pineus armandicola* Zhang, Zhong, and Zhang. When HWA was in diapause in September, *S. (N.) sinuanodulus* was more numerous on *P. armandi* than on hemlock (Montgomery and Keena 2011). During these collections, larvae of two non-target *Scymnus (Pullus)* species were recovered in large numbers (*Scymnus (Pullus) yunshanpingensis* Yu and *S. (P.) geminus* Yu and Montgomery). These findings indicated that the species in the subgenus *Pullus* may have pine adelgids as primary hosts, but will still feed and oviposit on HWA. The *Neopullus* species studied may have HWA as a primary host, but feed on pine adelgids as well. Subsequently, none of the Chinese *Scymnus*

*(Neopullus)* were successfully reared, established, or provided control. With the habitat range and behavioral characteristics of taxonomically similar species in mind, further investigation was conducted to understand the life cycle and feeding behavior of *S. coniferarum* in its native environment.

**Adelgidae and natural enemy species identification**

DNA analyses of 26 adelgid and predator samples collected from the sample sites used in this study was completed. Adult adelgids of *P. strobi* were identified on samples of *P. monticola; P. pini* on *P. contorta; A. tsuage* (HWA) on *T. heterophylla;* and *A. cooleyi* on *P. menziesii.* The natural enemies identified included one Conioptergidae "dustywing" larvae on *P. menziesii;* an unidentified *Neoleucopis* spp. and Cecidomyiid on *P. contorta,* and *Leucopis piniperda* on *T. heterophylla.*

**Predator/Host Density Relationships and Seasonal Patterns**

Branch analysis of *T. heterophylla* samples showed HWA to have the highest overall density of all adelgid species tested, and *P. menziesii* had the lowest amount of adelgids present. Of all adelgid life stages recorded (egg, crawler, aestivating nymph, nymph (2, 3, 4) and adult), adult adelgids were the most common stage documented.

In beat sheet sampling, *S. coniferarum* adults were collected from *P. contorta, P. monticola,* and *T. heterophylla* host trees. According to Dr. Havill's identification analyses, these results indicate that *S. coniferarum* is likely a predator of *P. pini, P. strobi,* and *A. tsugae* in the western United States. No *S. coniferarum* eggs or larvae were found, which may be attributed to the aggressive nature of beat-sheet sampling. However, it should be noted that no *S. coniferarum* eggs or larvae were found in the analysis of branch clippings. I am unsure of the reason behind the lack of immature *S. coniferarum* stages in sampling efforts. The number of *S.*

*coniferarum* adults collected did not significantly vary among host trees, except for *P. menziesii*, where no *S. coniferarum* were collected. The number of *S. coniferarum* adults collected peaked between February and May on *P. contorta, P. monticola,* and *T. heterophylla* host trees. This time of year corresponds with the oviposition period of *S. coniferarum* adults tested in host-range analyses (Chapter 3). The greatest total number of *S. coniferarum* beetles collected across all sample periods occurred on 26 March, 2016 on *P. monticola*. Moderate densities of *P. strobi* adults and eggs were present on samples collected on each of these dates. Similar to *P. monticola*, the number *S. coniferarum* collected from *P. contorta* was highest when HWA was in aestivation, and *P. pini* adults and eggs were available (Fig. 3). In Tacoma WA, HWA begins aestivation in June, and it is possible *S. coniferaum* beetles rely on pine adelgids for sustenance this time of year.

Seasonal activity and populations of adelgid prey species and *S. coniferarum* indicate that *S. coniferarum* feeds on multiple adelgid species throughout the year within its native range. *S. coniferarum* adults were collected at similar rates on pine and hemlock sample trees. Each host tree species supports a different adelgid species, and *S. coniferarum* populations fluctuated among different adelgid hosts depending on what life stages were available. Conifer sample trees are often nearby western hemlock stands, and it is possible that *S. coniferarum* seasonally moves between host tree species.

The feeding behavior and seasonal activity of *S. (Pullus) coniferarum* appears to be similar to observations of Chinese *Pullus* species described in Hagen et al. (1999). This publication states that species in the subgenus *Pullus* may rely on pine adelgids as primary hosts, but will oviposit and feed on both pine and hemlock adelgids. *S. coniferarum* may rely on both pine and hemlock adelgid species for optimal fitness, and this could help explain why *S.*

*coniferarum* would not lay eggs on a sustained basis when provided *A. tsugae* in laboratory studies at Virginia Tech.

### DNA barcode sequencing for *S. coniferarum*

From 29 August – 10 September 2016, adult *S. coniferarum* were collected in Tacoma, Washington from *T. heterophylla*, *P. monticola*, *P. contorta*, and *Pseudotsuga menziesii* (Mirb.). In February 2017, a sample of beetles from each host tree were sent to the Canadian Centre for DNA Barcoding in Guelph, Ontario, Canada for barcoding. Dr. Nathan Havill combined the barcoding results from these with that from 17 other examples previously barcoded as well as barcodes for *Scymnus suturalis* and *Sasajiscymnus tusgae* and created a neighbor joining phylogenetic tree, using uncorrected P-distance, with the software Geneious (Kearse et al. 2012).

Genetic testing of *S. coniferarum* beetles collected in the Tacoma found that the samples fell into two distinct taxonomic clusters with a 6% divergence in DNA among the two clusters. This could mean that the *S. coniferarum* beetles collected in Tacoma, WA may in fact be two cryptic species of beetles. Because these species cannot be separated by morphological characteristics and are co-inhabiting the same host trees, we have recommended that APHIS halt all shipments of *S. coniferarum* to the eastern United States until further taxonomic clarification can be made.

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# Tables and Figures

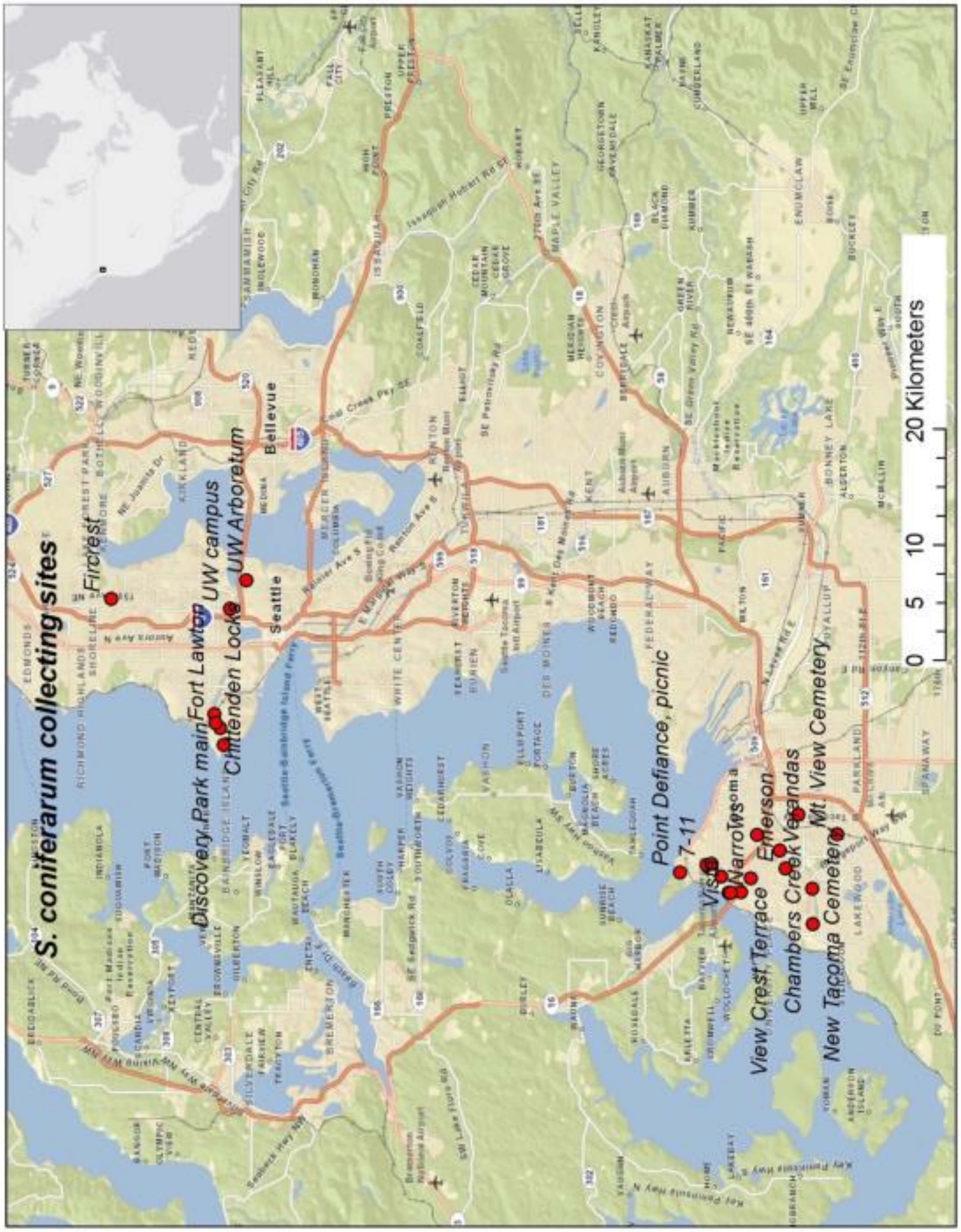

**Figure 1.** Sample sites in Tacoma, WA.

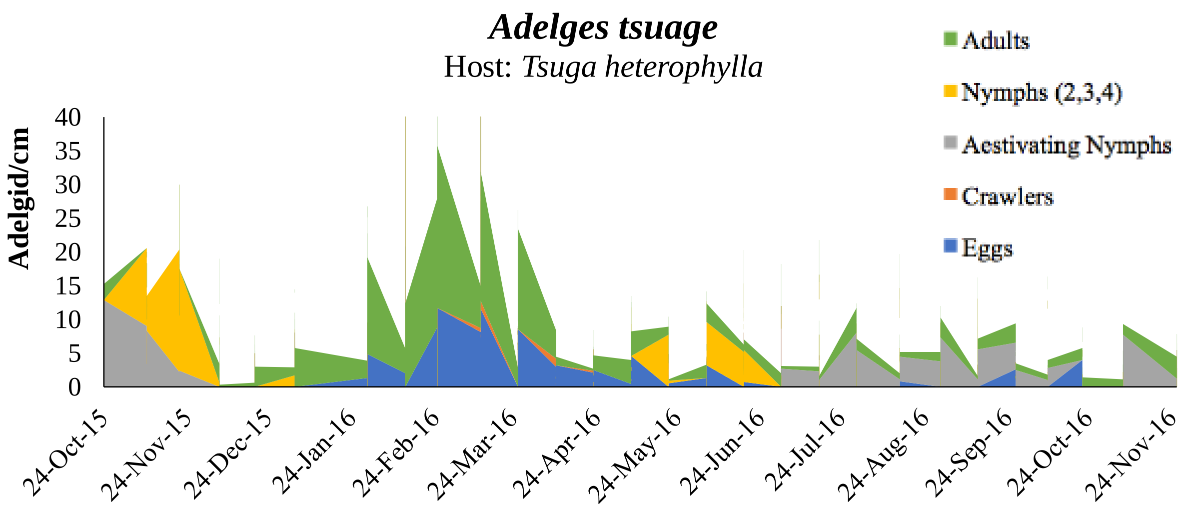

**Figure 2a.** Life stage and densities (Mean # adelgids/cm/sample date) of *Adelges tsugae* collected from *Tsuga heterophylla* in Tacoma, WA.

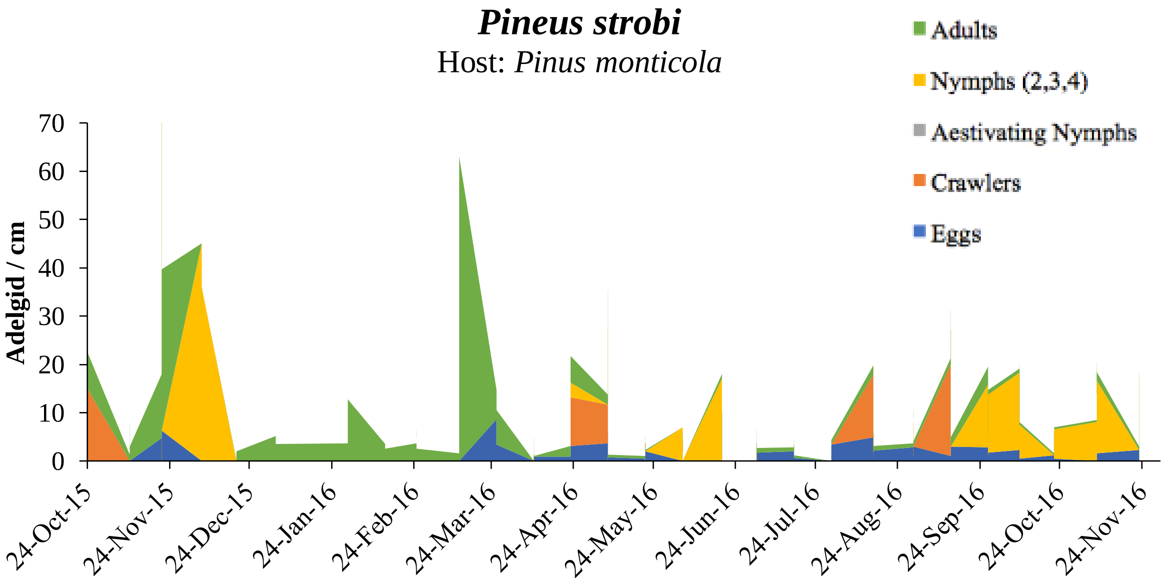

**Figure 2b**. Life stage and densities (Mean # adelgids/cm/sample date) of *Pineus strobi* collected from *Pinus monticola* in Tacoma, WA.

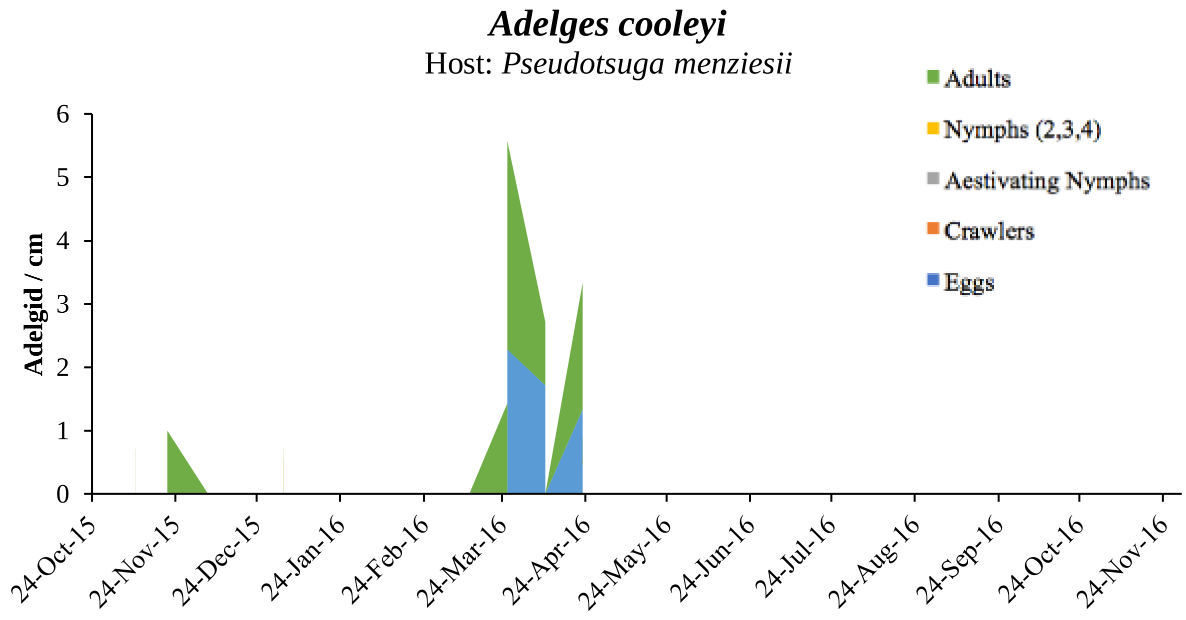

**Figure 2c.** Life stage and densities (# adelgids/cm/sample date) of *Adelges cooleyi* collected from *Pseudotusga menziesii* in Tacoma, WA.

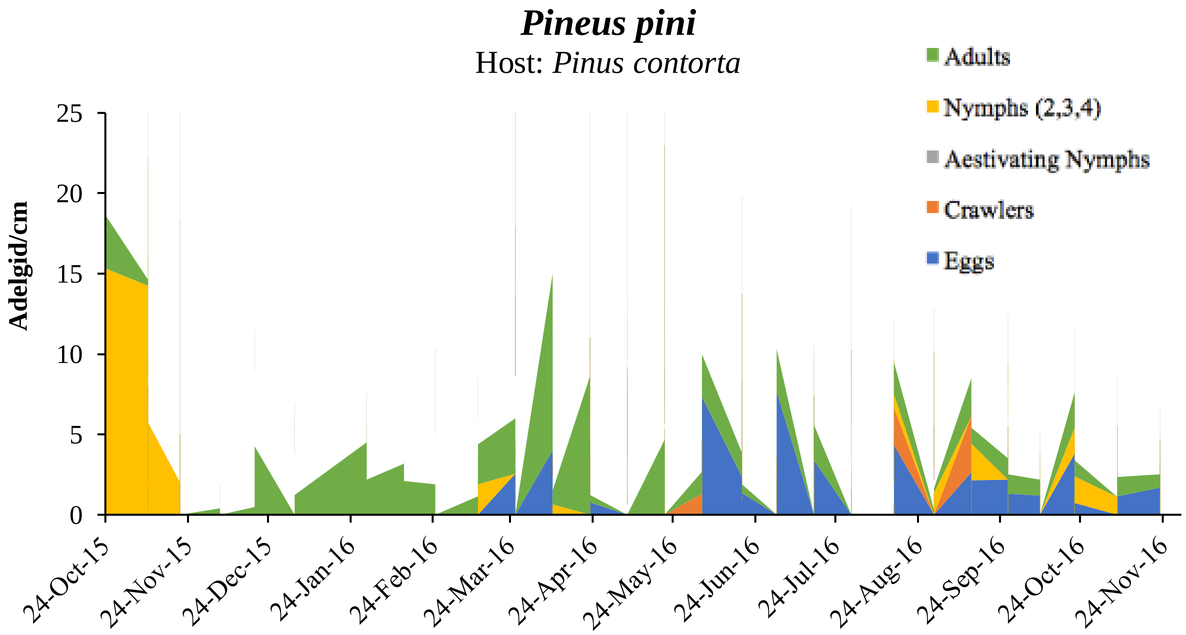

**Figure 2d.** Life stage and densities (# adelgids/cm/sample date) of *Pineus pini* collected from *Pinus contorta* in Tacoma, WA.

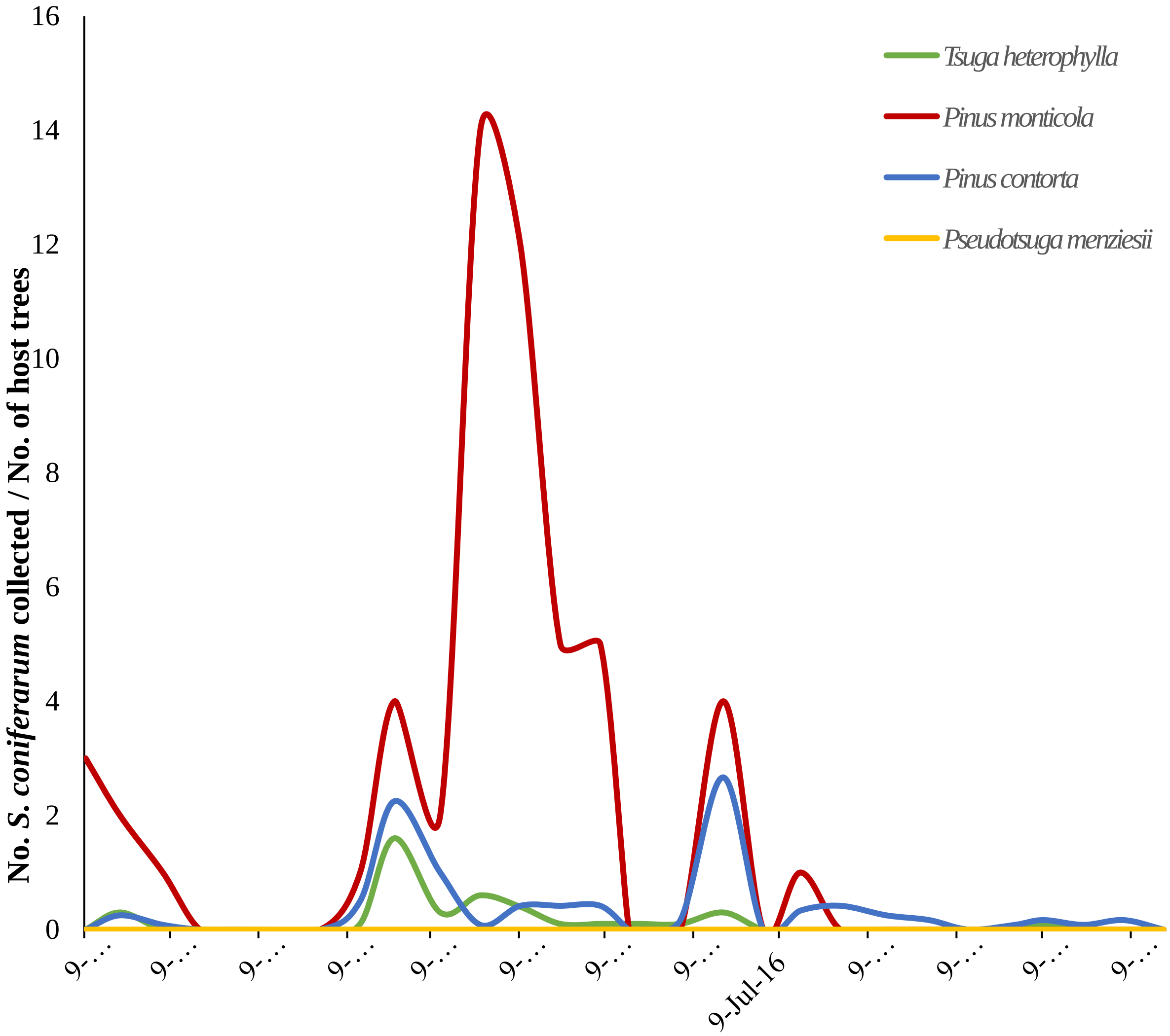

**Figure 3.** Total *S. coniferarum* adults collected from each host tree species through beat-sheet analysis (November 2015 – November 2016).

**Table 1.** Number and proportion (%) of *S. coniferarum* from each tree species in each cluster.[1]

| | **Species of Host Tree** | | | | |
|---|---|---|---|---|---|
| Group | *Pinus contorta* | *Pinus monticola* | *Pseudotsuga menzeisii* | *Tsuga heterophylla* | Total |
| Cluster 1 | 1 (10.0%) | 0 (0.0%) | 3 (30.0%) | 6 (60.0%) | 10 |
| Cluster 2 | 29 (40.1%) | 4 (5.6%) | 16 (22.5%) | 22 (31.0%) | 71 |

[1] Chi-square analysis for this 2x4 contingency table is 5.16; *df* = 3, 77; *P* = 0.160, No significance.

**Table 2.** Adelgid density (Mean # adelgids/$cm^2$) per host tree species, sampled biweekly in Tacoma, WA from November 2015 – November 2016.[1]

| **Host tree species** | **Adelgid species** | **Mean # adelgids/cm ± SE[2]** | **Sig. Level[3]** |
|---|---|---|---|
| *Tsuga heterophylla* | *Adelges tsugae* | 2.39 ± 0.08 | A |
| *Pinus contorta* | *Pineus pini* | 1.23 ± 0.2 | B |
| *Pinus monticola* | *Pineus strobi* | 0.82 ± 0.11 | B |
| *Pseudotsuga menziesii* | *Adelges cooleyi* | 0.04 ± 0.21 | C |

---

[1] Mean and standard error were calculated for the number of adelgids/$cm^2$ collected from each sample tree species (November 2015 – November 2016).

[2] Adelgid density / host tree species were compared using a one-way ANOVA.

[3] Different letters represent a significant difference in adelgid density (Mean # adelgids/$cm^2$) among host tree species, *P* = 0.05, Tukey-Kramer honestly significant different tests (*df* = 3, 6896; *F* = 72.78; *P* < 0.0001)

**Table 3.** Mean *S. coniferarum* recovery via beat-sheet sampling (November 2015 – November 2016) per host tree species.[3]

| **Host tree species** | **n**[1] | **Mean # *S. conifearum* ± SE**[2] |
|---|---|---|
| *Tsuga heterophylla* | 12 | 1.45 ± 0.96 |
| *Pinus contorta* | 12 | 4.10 ± 0.96 |
| *Pinus monticola* | 4 | 1.86 ± 0.96 |
| *Pseudotsuga menziesii* | 4 | 0.0 ± 0.0 |

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[1] "n" indicates the number each tree species sampled each sample period.

[2] Mean # *S. coniferarum* collected/host tree species were compared using a one-way ANOVA (df = 3, 83; *F* = 2.24, *P* = 0.113; Not significant). SE uses a pooled estimate of error variance.

[3] Mean and standard error were calculated for the number of *S. coniferarum* collected from each sample tree species, for each sample date.

# CHAPTER FIVE

## Conclusions

In the summer of 2012, I was introduced to *Scymnus (Pullus) coniferarum* Crotch in the quarantine facility at Virginia Tech. This beetle was the latest biological control agent under investigation for the control of *Adelges tsugae* (HWA) Annand infestations of *Tsuga canadensis* (L.) Carriére in the eastern United States. This predator had been approved for release before any host-range studies had been performed to assess the potential impact of *S. coniferarum* introduction. In accepting *S. coniferarum* as the subject of my research, it was my responsibility to learn everything there is to know about this predatory beetle.

*S. coniferarum* is native to the western United States, and had previously been collected in the Tacoma, WA area feeding alongside *Laricobius nigrinus* Rosenhauer on HWA-infested *Tsuga heterophylla* (Raf. Sarg.). My first objective was designed to observe *S. coniferarum* feeding and reproductive behavior in its projected release range of southwestern VA. Field-cage studies were conducted, and we observed that *S. coniferarum* was a voracious predator of all life stages and both generations of HWA when confined to field cages in a closed-release setting. This feeding behavior indicated that *S. coniferarum* could feed on HWA when *L. nigrinus* is aestivating in the soil, fulfilling its role as a summer predator (Salom et al. 2008, Zilahi-Balogh et al. 2002). In both field (Chapter 2) and laboratory (Chapter 3) studies, recovery of *S. conifearum* eggs was low and unsustainable. Predation and mortality were affected by temperature in both settings as well. *S. coniferarum* beetles consumed more adelgids/day at higher temperatures, but also experienced higher mortality under these conditions (Chapter 3).

My second objective was to monitor feeding behavior, oviposition timing, and development of *S. coniferarum* on multiple test prey species. In laboratory studies (Chapter 3), *S.*

*coniferarum* fed and developed on multiple species of Adelgidae including HWA, and did not prefer or develop on Aphidae test species. In my opinion, this confirms *S. coniferarum* as an adelgid specialist, which is not specific to HWA.

In the final objective, I studied the phenology and synchrony of *S. coniferarum* and its adelgid prey in Tacoma, WA, part of *S. coniferarum's* native range. DNA analyses of *S. coniferarum* samples collected off of several native conifer species in the Pacific Northwest were completed by Dr. Nathan Havill. These *S. coniferarum* specimens showed two genetic clusters separated by 6% divergence in DNA. These results suggested that *S. coniferarum* beetles collected in this region could be cryptic species, or make up a species complex. This genetic divergence may contribute to the challenges I experienced in seeing *S. coniferarum* fecundity in the lab and field (Chapter 2). *S. coniferarum* adults were collected from *Pinus contorta* Dougl., *Pinus monticola* D. Don., and *Tsuga heterophylla* (Raf.) Sarg. host trees. According to Dr. Havill's analyses, associated adelgid prey species present were *Pineus pini* L., *P. strobi* Range., and HWA. The total number of *S. coniferarum* beetles collected in beat-sheet analyses, as well as "Cluster" identification (1 or 2) did not correspond with any specific host tree species, with the exception of *Pseudotsuga menziesii* Mirb., from which no *S. coniferarum* were collected. The number of *S. coniferarum* adults collected in beat-sheet sampling was highest between February and April on *P. contorta*, *P. monticola* and *T. heterophylla.* This time of year corresponded with the oviposition period of *S. coniferarum* (Chapter 3). Specifically, the number of *S. coniferarum* collected from *P. monticola* and *P. contorta* was highest when HWA was in aestivation. I believe this is further evidence that *S. coniferarum* beetles rely on pine adelgids when HWA populations are low or unpalatable.

In the literature, *S. coniferarum* is proposed to be an adelgid specialist, primarily associated with pine and hemlock host tree species (Crotch 1874, Whitehead 1967, Gordon 1976, Montgomery and McDonald 2010). A majority of the species within the genus *Scymnus,* and all *Pullus* species, are aphidophagous. Besides *S. coniferarum,* the only other two *Scymnus (Pullus)* species known to be established in North America are adelgid specialists (Gordon 1985). *Scymnus (Pullus) suturalis* Thunberg feeds on both HWA and *Pineus* spp., and *Scymnus (Pullus) impexus* Mulsant has been collected on hemlocks, and is known to feed on *Adelges piceae* Ratzeburg (Gordon 1985, Montgomery and Lyon 1995). In China, *Scymnus (Neopullus) sinuanodulus* was found on hemlock and *Pinus armandii* (Koidz.) Hatus. The larvae of two other *Scymnus (Pullus)* species were found on *P. armandii* as well. The *Neopullus* spp. studied have HWA as a primary host, but feed on pine adelgids as well. Therefore, it is likely that like other species within the subgenus *Pullus, S. (P.) coniferarum* rely on a complex of adelgid species for optimal success. Based on the literature, it is possible that *Scymnus (Pullus)* species rely on pine adelgids as a primary host, but will oviposit and feed on both pine and hemlock adelgid. If *S. coniferarum* were introduced in the eastern U.S., native pines should be present on release sites to maximize establishment potential.

I do not believe the release of *S. coniferarum* in the eastern U.S. would be a danger to native adelgid species present. I also don’t expect this beetle would be able to successfully establish in our target release range. The reasoning for this asserition lies behind the poor performance of *S. coniferarum* in the lab, and the lack of egg or larvae recovery in field-cage studies in southwestern VA. This lady beetle appears to have a complex of prey and temperature-related requirements necessary for optimal success and establishment that have yet to be understood for optimal success and establishment. Further studies should be completed to identify the specific diversity in samples collected from the Pacific Northwestern region of the U.S. Additional host-range tests should also be completed to identify the adegid species necessary for *S. coniferarum* establishment in the eastern U.S.

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