forests

MDPI

Article

Phenology and Synchrony of *Scymnus coniferarum* (Coleoptera: Coccinellidae) with Multiple Adelgid Species in the Puget Sound, WA, USA

Molly N. Darr [1,*], Rachel K. Brooks [2], Nathan P. Havill [3], E. Richard Hoebeke [4] and Scott M. Salom [1]

1 Department of Entomology, Virginia Tech, Blacksburg, VA 24061, USA; salom@vt.edu
2 School of Plant and Environmental Sciences, Virginia Tech, Blacksburg, VA 24061, USA; rkbrooks@vt.edu
3 Northern Research Station, Forest Service, USDA, Hamden, CT 06514, USA; nphavill@fs.fed.us
4 Museum of Natural History and Department of Entomology, University of Georgia, Athens, GA 30602, USA; rhoebeke@uga.edu
* Correspondence: mdarr@vt.edu; Tel.: +1-703-431-9579

Received: 16 August 2018; Accepted: 4 September 2018; Published: 11 September 2018

Abstract: The hemlock woolly adelgid (HWA), *Adelges tsugae*, is an invasive pest of *Tsuga* spp. in eastern North America. *Scymnus coniferarum* is a predaceous beetle that was collected from HWA in the western United States. Limited knowledge of this insect in its native habitat led to studies to evaluate its potential for biological control of HWA. Seasonal abundance was sampled at six sites in Tacoma, WA, twice monthly, for one year on different host trees of potential adelgid prey. Tree species included *Pseudotsuga menziesii*, *Pinus contorta*, *Pinus monticola*, and *Tsuga heterophylla*. *Scymnus coniferarum* adults were found on all conifer species, except *P. menziesii*. Each conifer species supported a different adelgid species, suggesting *S. coniferarum* feeds on multiple adelgid species. More *S. coniferarum* were found on pine than hemlock. DNA barcoding of *S. coniferarum* found two distinct clusters that differed by 6% divergence. Beetles in each cluster were co-habiting the same conifer species, and they could not be distinguished morphologically. Further taxonomic studies are needed to understand the significance of DNA barcode sequence divergence. Because *S. coniferarum* was more frequently associated with pine adelgids than HWA, and because of remaining taxonomic uncertainty, *S. coniferarum* may not be suitable for HWA biological control.

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

1. Introduction

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, is an invasive pest insect in the eastern United States. HWA is a hemlock-specific herbivore, native to all *Tsuga* spp. in western North America and Asia [1]. Since its introduction, 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 [2,3]. This pest insect is extremely prolific and difficult to control due to its complex lifecycle. HWA reproduces parthenogenically, with two overlapping generations per year. These generations are made up of the overwintering "sistens" generation, and the spring "progrediens" generation [4]. *Scymnus* (*Pullus*) *coniferarum* Crotch (Coleoptera: Coccinellidae) is a native predator of HWA in western North America. This predatory lady beetle was discovered in 2006 feeding on HWA alongside the biological control agent *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) [5], which was first released in 2003 to control HWA in the eastern United States [6]. 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 United States, even

though only limited host range testing and biological assessment of this insect in its native range were conducted [7].

The known host trees for *S. coniferarum* recorded in the literature prior to 2008 were exclusively listed as *Pinus* spp., located in British Colombia, Arizona, California, Colorado, Idaho, Nevada, Utah, and Wyoming [8]. 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. [9]. More recently, in collections made in Seattle, WA, *S. coniferarum* was collected on western hemlock, *Tsuga heterophylla* (Raf.) Sarg. infested with HWA [10], and to a lesser extent on western white pine, *Pinus monticola* Douglas ex. D. Don., feeding on an unidentified adelgid species [11]. These observations indicate that *S. coniferarum* can 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* Mulsant [12]. Three of these species are known to feed on adelgids: *Scymnus impexus* Mulsant and *Scymnus suturalis* Thunberg, both intentionally introduced to the United States from Europe, and *S. coniferarum* [13,14]. *Scymnus suturalis* have been recovered feeding on adelgid-infested *T. canadensis*, *Pinus strobus* (L.), and *Pinus sylvestris* (L.), but favors adelgids that are associated with *Pinus* species [13]. 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. *Scymnus impexus* is mostly associated with *Adelges piceae* Ratz. and other adelgids that colonize *Abies* spp. [15]. Another *Scymnus* subgenus that is found only in Asia, *Neopullus* Sasaji, includes more than 30 species, of which three to five species are known to specialize on adelgids. Most other *Scymnus Neopullus* species are generalists, and feed on both aphids and adelgids on conifers [16,17].

In order to assess the suitability and safety of *S. coniferarum* as a potential biological control agent of HWA in the eastern United States, host-range [18] and field-cage studies [19] were completed. Development of a mass-rearing procedure was also attempted [18]. Overall, *S. coniferarum* fed at a similar rate as other HWA predators in both field and laboratory settings, but oviposition rates for this predator on HWA-infested hemlock were extremely low [18,19]. The inability to rear *S. coniferarum* in the laboratory and the taxonomic similarity of this species to other generalist predators meant that we were limited to observing the insect in its native habitat. In this paper, we report on a phenological study that 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. Seasonal activity (timing of life stages, host association, and population density) of multiple adelgid species and *S. coniferarum* were assessed on several conifer tree species located in Tacoma, Washington. These are critical data for evaluating *S. coniferarum*'s potential as a biological control agent of HWA in the eastern United States. It is possible that *S. coniferarum* relies on multiple adelgid prey species in its native range for optimal reproductive and developmental fitness.

2. Materials and Methods

2.1. Location and Timing of Experiments

Six sites were sampled twice monthly for one year (24 October 2015–20 November 2016). The sites that were 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 (Figure 1) [20]. These sites were recommended by Dr. Richard McDonald, independent contractor for Symbiont Biological Pest Management, who had collected *S. coniferarum* in this region (drmcbug@skybest.com) [5].

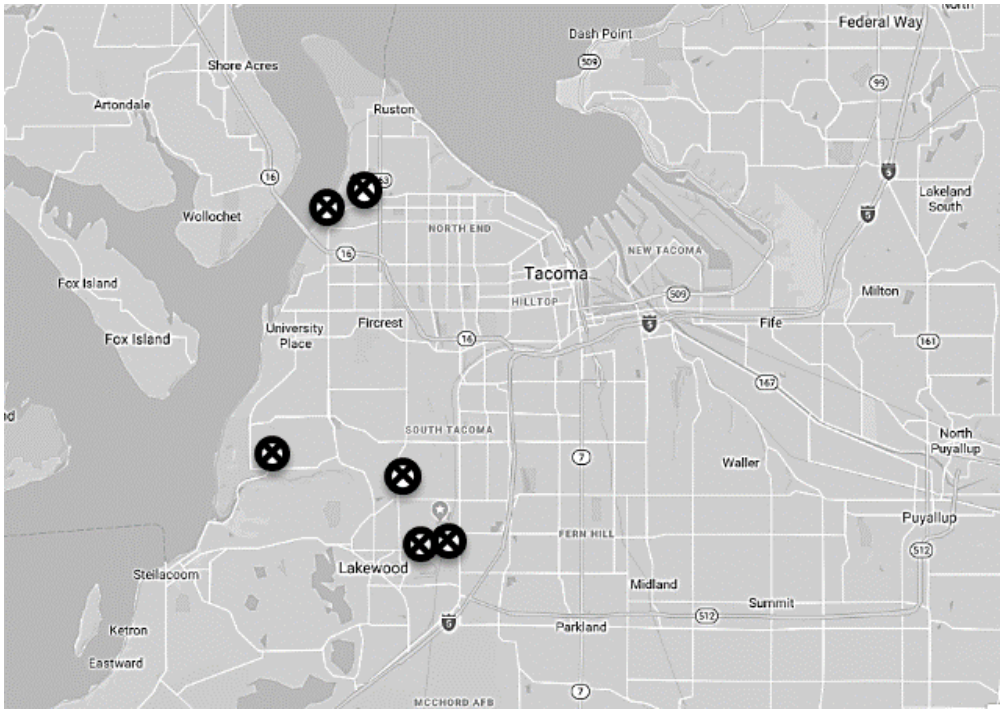

Figure 1. Six sites (two pure hemlock stands, two stands containing conifers other than hemlock, and two mixed stands of western hemlock and native conifers) were sampled in Tacoma, WA twice monthly for one full year (October 2015–November 2016) to determine seasonal abundance of *S. coniferarum* and adelgid species present.

The sample sites were comprised of two pure hemlock stands (containing only *T. heterophylla*), two stands containing only *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. menziesii*). Each site contained a total of five sample trees in close proximity, to discern whether any seasonal movement of *S. coniferarum* occurs among host tree species. A total of 12 *T. heterophylla*, 12 *P. contorta*, four *P. menziesii*, and four *P. monticola* host trees were sampled. 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 were in good health, and they provided a consistent presence of adelgids for the duration of the study.

Sampling took place every two weeks, allowing for us to track adelgid species, their life stage, and their density on each sample tree. At the same time, the total number of *S. coniferarum* collected from each host tree was recorded.

2.2. 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, USA. Upon arrival, the branch samples were stored at −20 °C to prevent the adelgids from developing further. This ensured the associated life stages and measurements were accurately recorded for each sample period.

The same trees were also sampled via beat-sheet analysis, to collect *S. coniferarum* and any other predaceous insects present. Ten beats were taken from all accessible branches around the circumference of each tree in all sample sites. Any recovered insects were packaged and included in

the aforementioned branch sample shipments at the end of each sample period. Sample periods at two-week intervals resulted in a total of 29 shipments.

Branch clippings were examined for adelgid and *S. coniferarum* life stage and density, while 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 cast skins 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 were calculated by dividing the total number of adelgids by the length of each branch, to determine the number of adelgids per cm. 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 that were documented on all sample trees, in order to identify which adelgid species and life stages were available when *S. coniferarum* populations were present.

2.3. Data Analysis

Adelgid species, host tree species, adelgid life stage present, and associated adelgid density (# of adelgids/cm) were documented from each sample branch. Statistical tests were carried out while using JMP software [21]. Insect density data were tested for normality using goodness-of-fit tests, and for heterogeneity of variance through plots of residuals. Data were tested for differences for mean densities by host tree species and sample period, using a one-way ANOVA. If significant ($P < 0.05$), the Tukey-Kramer range test was used to determine a significant difference among treatments.

In a separate analysis, the mean and standard error were calculated for the number of *S. coniferarum* collected from each tree species and sample period. Sample trees which produced no *S. coniferarum* were removed for analysis. The mean totals of *S. coniferarum* collected per host tree species were then compared while using a one-way analysis of variance (ANOVA) [21]. Tree species and sample date were the main effects tested, with tree species as a fixed variable, and sample date included as a random variable. Based on fit statistics and normal and quantile-quantile plots, analyses were performed assuming a Poisson distribution while using DIST-P option of the MODEL statement. Where differences among species were detected ($P < 0.05$), mean comparison tests were performed using the adjust = simulate option in the least means statement.

2.4. Adelgid Species Identification

Representative samples of adelgids collected from each host tree species were identified while using DNA barcodes and/or morphology. Pine adelgids (*Pineus* spp.) are difficult to identify with morphology alone, so DNA barcodes were generated for adelgids that were collected from *Pinus* spp. For adelgids collected from *Tsuga* and *Pseudotsuga*, morphological identification was sufficient. DNA barcodes were generated using standard methods [22]. DNA was extracted using the Mag-Bind Blood & Tissue Kit (Omega Bio-Tek, Norcross, GA, USA). 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 voucher specimens and deposited at the Yale Peabody Museum of Natural History with accession numbers ENT857029, ENT905367-ENT905382, and ENT94317-ENT943146. The 5′ end of mitochondrial cytochrome *c* oxidase subunit I (COI) was amplified using primers LepF1 and LepR1, 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, USA). Chromatograms were edited using Geneious v7 [23,24]. Sequences were deposited to GenBank with accession numbers MF572805-MF572808, and MH721202-MH721206. Samples were identified by comparing DNA barcodes to those on the Barcode of Life Data System [25], and using keys of morphological characters [26].

2.5. DNA Barcode Sequencing and Morphological Examination of S. coniferarum

From 29 August to 10 September 2016, adult *S. coniferarum* were collected by Dr. Richard McDonald in Tacoma, Washington from *T. heterophylla*, *P. monticola*, *P. contorta*, and *P. menziesii.* These beetles were collected independently from the sampling procedure described above, and the collection sites were not restricted to those presented in Figure 1. A sample of 87 beetles was selected in proportions from each host similar to those recovered in the study described above, except that all four samples from *P. monticola* were included. Beetles were delivered to the Beneficial Insects Quarantine Laboratory in Blacksburg, VA, USA and immediately placed in 95% ethanol at 15 °C. The abdomen was removed from each beetle for later examination of genitalia, and the remainder of the insects were sent to the Canadian Centre for DNA Barcoding in Guelph, Ontario, Canada for barcoding. Standard protocols [27,28] were employed for DNA extraction and amplification, sequencing of the COI barcode region, sequence editing, and alignment. Sequences were deposited to GenBank with accession numbers MH747746-MH747834. Uncorrected P-distances among DNA barcode sequences were calculated with PAUP* v.4.0a [29].

To confirm the identity of each of the 87 beetles from which DNA was extracted and sequenced, the genitalia (males and females both) were extracted from the abdomens by microdissection (by ERH). Once removed, the genitalia were examined in glycerin on microscope well slides with the optics of a Zeiss Stemi-2000C stereo microscope. Male (phallobase, including parameres and basal lobe, and sipho) and female (spermathecal capsule) structures were meticulously compared with detailed line drawings of *S. coniferarum* genitalia found in Gordon (1985: 152–153).

3. Results

3.1. Adelgid Species Identification

The DNA barcoding and morphological examination of adelgids that were collected from the sample sites showed *P. monticola* hosted pine bark adelgid, *Pineus strobi* (Hartig) (n = 5 individuals sequenced). This is the first record of *P. strobi* in the western United States [30]. The adelgids found on *P. contorta* were identified as *Pineus pini* (Macquart) (n = 5 individuals sequenced). The adelgids collected from *T. heterophylla* were confirmed as HWA and *Adelges cooleyi* (Gillette) was identified on *P. menziesii*. All the life stages of HWA (eggs, crawlers, nymphs, and adults) of both generations were present during this study. Eggs, crawlers, nymphs, and adults of both *P. strobi* and *P. pini* were also present. Only eggs and adults of A. cooleyi were recovered over the course of the study.

3.2. Predator/Host Density Relationships and Seasonal Patterns

3.2.1. Adelgids

The analysis of branch samples showed a difference in the density of adelgids among host tree species across all the sample periods (df = 3, 6896; F = 72.78; P = 0.0001). Overall, *T. heterophylla* branch sections contained the greatest number of adelgids/cm (2.39 ± 0.08) among all host tree species sampled, and *P. menziesii* contained the lowest density of adelgids/cm (0.04 ± 0.21) (Table 1).

Each host tree species showed seasonal differences in the adelgid population densities. On *T. heterophylla*, the mean number (±SE) of sistens present per sample period (8.0 ± 0.41 adelgids/cm) was greater from October–April than May–July (1.8 ± 0.15 adelgids/cm), when progrediens were present (Figure 2). This pattern was also evident in *P. strobi* populations on *P. monticola* host trees. Between October and April, the number of adelgids/cm was 7.16 ± 0.48, and 1.2 ± 0.33 for the rest of the year (Figure 3). *Adelges cooleyi* populations were present between March and May, and they were largely absent for the rest of the year (Figure 4). *Pineus pini* densities were greater in the fall and spring, but they were consistently low in the winter and summer (Figure 5).

Table 1. Mean number of adelgids (±SE) per host tree species, sampled biweekly in Tacoma, WA, USA from October 2015 to November 2016 [1].

Host Tree Species	Adelgid Species	Mean # Adelgids/cm ± SE [2]
Tsuga heterophylla	*Adelges tsugae*	2.39 ± 0.08 A
Pinus contorta	*Pineus pini*	1.23 ± 0.20 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 collected from each sample tree species (October 2015–November 2016). All adelgid life stages with the exception of eggs were included in these calculations; [2] Adelgid density / host tree species were compared using a one-way ANOVA. Different letters represent a significant difference in adelgid density (Mean # adelgids/cm) among host tree species ($P < 0.05$), Tukey-Kramer significant different tests (df = 3, 6896; $F = 72.78$; $P = 0.0001$).

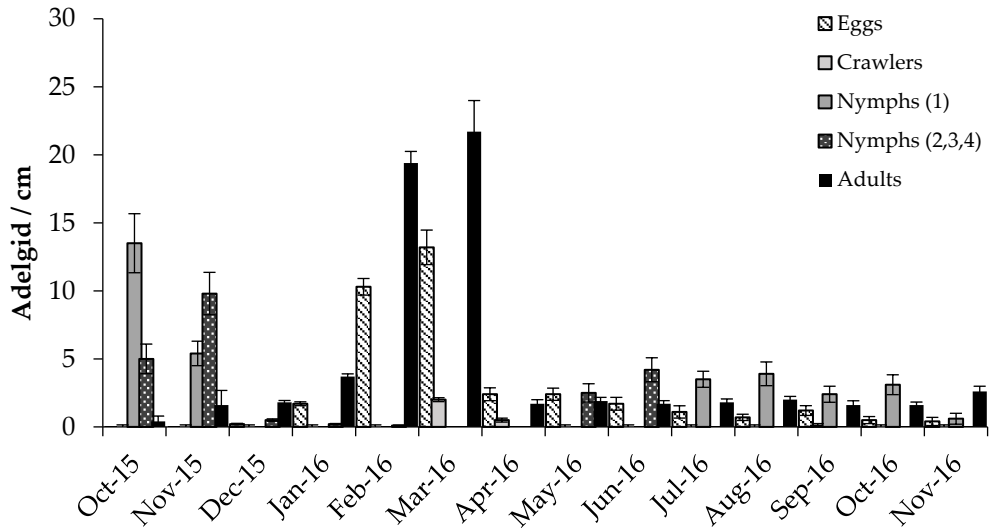

Figure 2. Mean number (±SE) of *Adelges tsugae* by lifestage collected from *Tsuga heterophylla* in Tacoma, WA, USA (October 2015–November 2016).

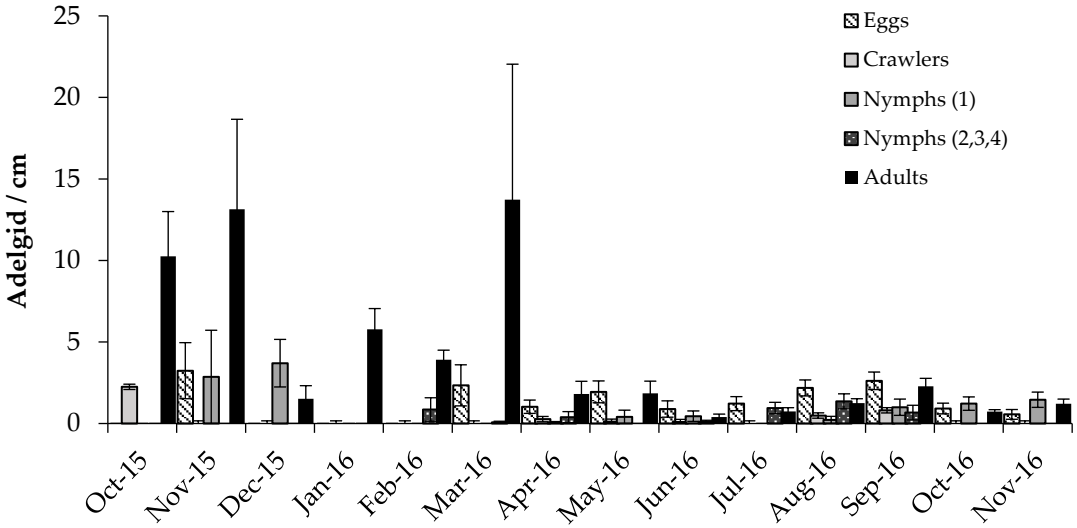

Figure 3. Mean number (±SE) of *Pineus strobi* by lifestage collected from *Pinus monticola* in Tacoma, WA, USA (October 2015–November 2016).

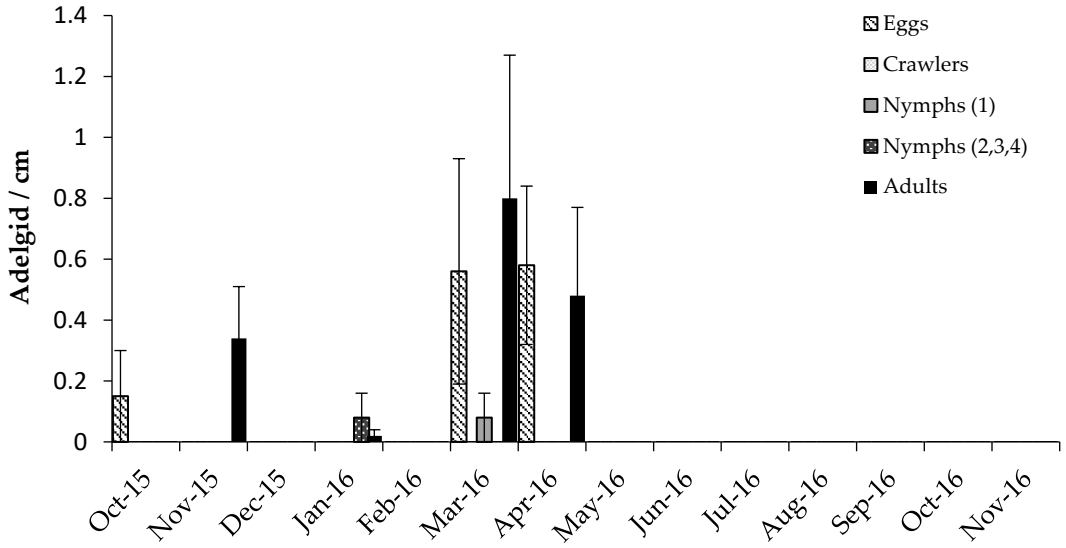

Figure 4. Mean number (±SE) of *Adelges cooleyi* collected by lifestage from *Pseudotsuga menziesii* in Tacoma, WA, USA (October 2015–November 2016).

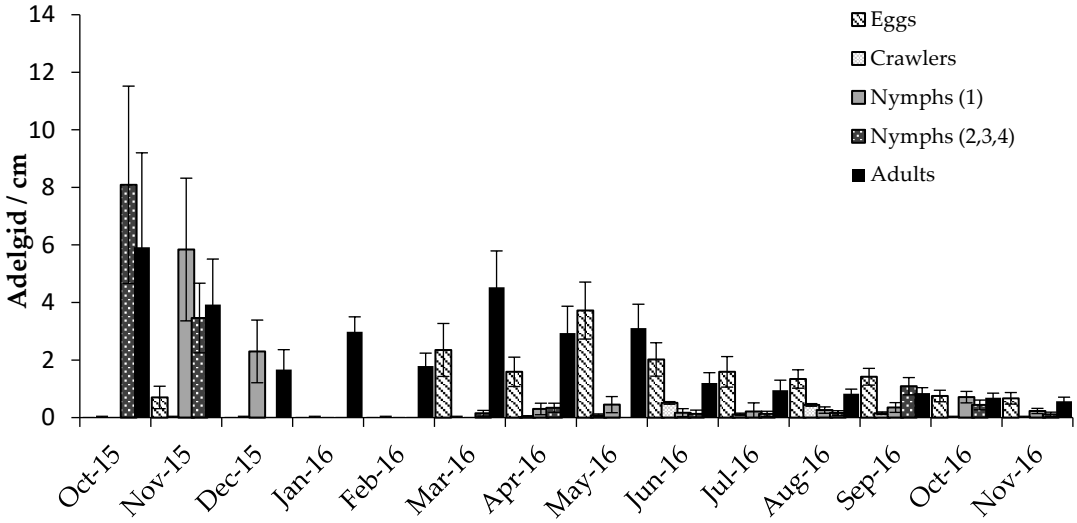

Figure 5. Mean number (±SE) of *Pineus pini* collected by lifestage from *Pinus contorta* in Tacoma, WA, USA (October 2015–November 2016).

HWA sistens adults were present on *T. heterophylla* on 24 October 2015. It was difficult to discern when progrediens adults first appeared, as HWA adults were present for the duration of the study (October 2015–November 2016). Peak occurrence of sistens adults was observed on 12 March 2016, averaging 21.7 ± 2.29 adults/cm. During this time, it was common to see multiple HWA adults that were settled at the base of each needle. The earliest eggs laid by sistens adults were observed on 2 January 2016. HWA eggs remained present through November 2016, and it is uncertain when sistens adults stopped laying eggs and progrediens adults began. This could possibly indicate overlapping sistens and progrediens generations in this region of North America (Figure 2).

3.2.2. *Scymnus coniferarum*

In beat sheet sampling, a total of 215 adult *S. coniferarum* were recovered during the year-long sample period. *Scymnus coniferarum* was found on all host tree species, with the exception of *P. menziesii*. *Adelges cooleyi* densities on *P. menziesii* reflected low population levels. The scarcity of adelgid prey

on this host species may have contributed to the absence of *S. coniferarum* (Table 1). Recovery of *S. coniferarum* adults was significantly greater from *P. contorta* and *P. monticola* host trees than in *T. heterophylla* (df = 2, 539; $F = 8.20$, $P = 0.0003$) (Table 2). When HWA was in diapause (July–October), *S. coniferarum* was primarily collected from *P. monticola* and *P. contorta* (Figures 2 and 6). This suggests that *S. coniferarum* fed on *P. pini* and *P. strobi* populations when HWA was unavailable (Figures 2 and 6). Overall, *S. coniferarum* adults were most active from early March through June, and were present on *P. monticola*, *P. contorta,* and *T. heterophylla* host trees during this time. *Scymnus coniferarum* recovery was especially high in March and April on *P. monticola*, *P. contorta,* and *T. heterophylla* host trees when adult adelgid populations were present. *Scymnus coniferarum* adults were recovered in lower numbers the rest of the year, and they were not present between 19 December 2015 and 30 January 2016 (Figure 6).

Table 2. Mean *S. coniferarum* recovery (±SE) via beat-sheet sampling on different host tree species in Tacoma, WA, USA from October 2015–November 2016 [1]. Values not sharing a similar letter are significantly different ($P < 0.05$).

Host Tree Species	*n* [2]	Mean # *S. coniferarum* ± SE [3]
Tsuga heterophylla	6	0.22 ± 0.07 B
Pinus contorta	9	0.46 ± 0.25 A
Pinus monticola	4	0.44 ± 0.19 A
Pseudotsuga menziesii [4]	4	0.0 ± 0.0

[1] Mean and standard error were calculated for the number of *S. coniferarum* collected from each sample tree species over all sample dates. Trees with zero *S. coniferarum* recovery over the course of the sampling period were removed from analysis; [2] The number of trees sampled each sample period; [3] Mean # *S. coniferarum* collected/host tree species were compared using a one-way ANOVA (df = 2,539; $F = 8.20$, $P = 0.0003$). SE uses a pooled estimate of error variance; [4] Mean *S. coniferarum* recovery from *P. menziesii* was excluded from analysis because no *S. coniferarum* were recovered from this tree species.

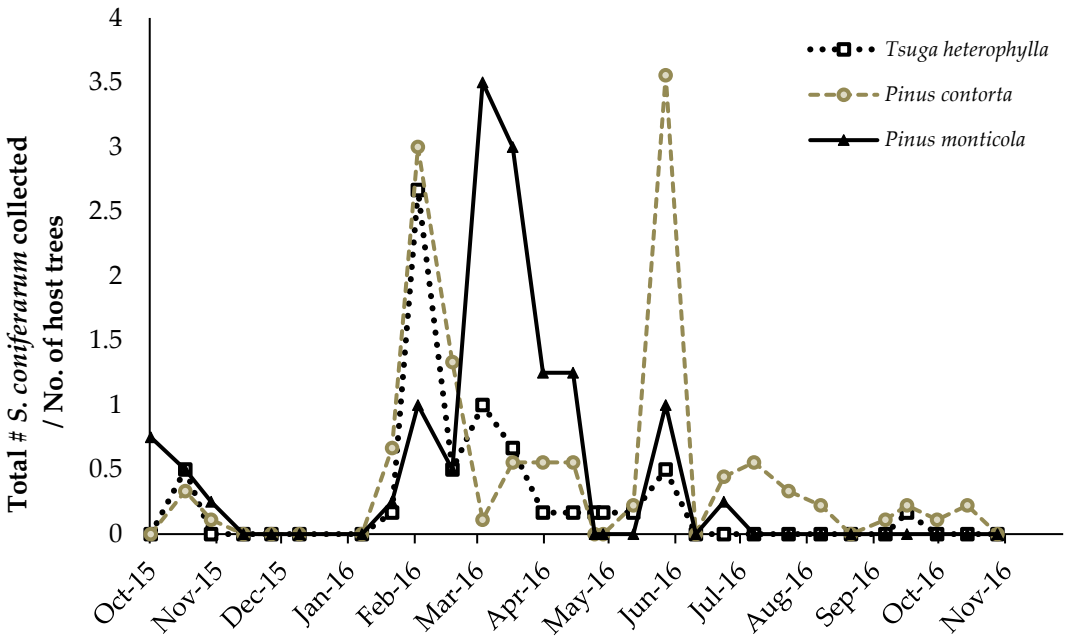

Figure 6. Total *Scymnus coniferarum* adults collected from each host tree species through beat-sheet analysis (October 2015–November 2016). No beetles were recovered from *Pseudotsuga menziesii*.

3.3. DNA Barcode Sequencing and Morphological Examination of S. coniferarum

The COI DNA barcode sequences generated here (658-bp alignment, 81 individuals) had the characteristics of true arthropod mtDNA, as opposed to nuclear pseudogenes, which are recognized by the presence of indels or stop codons when translated to amino acids. Sequences from *S. coniferarum* samples fell into two distinct clusters that diverged by 6%. 12.3% of the samples were identified as

Cluster 1 and 87.7% were in Cluster 2. *Scymnus coniferarum* adults from each cluster were collected from *P. contorta*, *P. menziesii*, and *T. heterophylla*. *Pinus monticola* yielded beetles from Cluster 2 but not Cluster 1. There was no apparent relationship between sequence cluster and host tree species (Table 3).

Table 3. Number and proportion (%) of *S. coniferarum* from each tree species in each cluster collected in Tacoma, WA, USA from 29 August–10 September 2016.

	Species of Host Tree				
Group	***Pinus contorta***	***Pinus monticola***	***Pseudotsuga menziesii***	***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

Notwithstanding the 6% genetic divergence that was observed between two distinct sequence clusters of *S. coniferarum* samples studied, no discernible morphological differences could be found among them. Male and female genitalia were essentially identical for specimens representing the two sequence clusters.

4. Discussion

4.1. Background

Scymnus coniferarum is considered an adelgid specialist [11]. This beetle is described as a predator of adelgid species that is primarily associated with pine and hemlock species [8,9,11,18,31]. Both Crotch (1874) and Gordon (1976) collected "large numbers" of *S. coniferarum* from *P. contorta* and *Pinus radiata* D. Don infested with adelgids [8,31]. Adult *S. coniferarum* beetles were observed feeding on *T. heterophylla* infested with HWA and on *P. monticola* infested with an "unidentified pine adelgid" in the Seattle metropolitan area in 2008 and 2009 [10,11].

Scymnus coniferarum belongs to the subgenus *Pullus.* A majority of the species within the genus *Scymnus*, and all *Pullus* species, feed on multiple aphid or adelgid species on a variety of host trees [9,12]. Only three adelgid-feeding species in the subgenus *Pullus* are known to occur in North America: *S. coniferarum*, *S. suturalis,* and *S. impexus* [14]. Both *S. suturalis* and *S. impexus* are adelgid specialists [12]. *Scymnus 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 [12,13]. Gordon (1976) states that most *Scymnus* and *Pullus* species are extremely active, fly well, and are not restricted to a particular prey species. Because of this, their establishment does not depend on the availability of a specific host [8].

The feeding behavior and seasonal activity of *S.* (*Pullus*) *coniferarum* also appears to be similar to observations of Chinese *Pullus* species that are described in Hagen et al. (1999) [32]. 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. *Scymnus 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 HWA in laboratory studies [18]. These findings suggest that adelgid specialists in the genus *Scymnus* rely on a complex of adelgids within their native range, and would support the evidence that *S. coniferarum* occupies a variety of host tree species and feed on multiple adelgid species throughout the year. The lack of host specificity implies that *S. coniferarum* is not a viable biological control agent of HWA in eastern North America.

4.2. Predator/Host Density Relationships and Seasonal Patterns

In beat sheet sampling, *S. coniferarum* adults were collected from *P. contorta*, *P. monticola*, and *T. heterophylla*, indicating that *S. coniferarum* likely feeds on *P. pini*, *P. strobi*, and HWA in the western United States. HWA is native to western North America [1], but both *Pineus* species that were recovered

in this study are not. *Pineus strobi* is native to eastern North America [30,33] and *P. pini* is native to Europe [26], so these are not likely to be ancestral prey species of *S. coniferarum*. Two other native western pine adelgids, *P. coloradensis* (Gillette) and *P. pinifoliae* (Fitch), use one or both of these pine host species [26], so these could be the adelgid prey that *S. coniferarum* evolved to feed on.

The number of *S. coniferarum* adults collected varied significantly among host trees. No *S. coniferarum* were collected from *P. menziesii*. This suggests that *A. cooleyi* on *P. menziesii* is not a preferred prey species. However, it should also be noted that low adelgid populations were present, and they may have been a contributing factor to the absence of *S. coniferarum*. Significantly less *S. coniferarum* beetles were recovered from *T. heterophylla* than from *P. contorta* and *P. monticola. Scymnus coniferarum* populations were consistently sampled from *P. contorta* and *P. monticola* in July, August, and September of 2016, but were not found on *T. heterophylla*. In western North America, HWA begins aestivation in July, and while HWA adults were present, they were only available in limited quantities [34]. It is likely that *S. coniferaum* relies on pine adelgids for sustenance during this time of year. The greatest number of *S. coniferarum* collected per host tree was recovered on 26 March 2016 from *P. monticola*, where high densities of *P. strobi* adults were present. This time of year corresponds with the oviposition period of *S. coniferarum* adults tested in host-range analyses [18]. It is possible that *S. coniferarum* beetles target host tree species with high prey populations during their oviposition period to increase rates of reproductive success.

No *S. coniferarum* eggs or larvae were found from beat sheet sampling or branch clippings. The reason for the lack of immature *S. coniferarum* in our samples remains unknown when considering that larvae are known to feed and develop on HWA in the laboratory and in field cages [18,19].

HWA phenology in Tacoma, WA was anomalous when compared to the life cycle timing of HWA in Virginia [35] and Connecticut [36], but strikingly similar when compared to HWA phenology in British Columbia [34]. A study conducted in 2003 reported sistens adults first appearing on 25 October 1999 in British Columbia [34], when the earliest sistens matured between January and February in Virginia and Connecticut [35,36]. The first appearance of sistens adults with progrediens eggs occurred at the end of January in British Columbia [34], and in early January in this study. Furthermore, the peak occurrence of sistens adults was observed on 12 March 2000 in British Columbia [34], and 12 March 2016 at our field sites in Tacoma, WA. Similar to this study, the population density of the sistens generation was drastically higher than that of the progrediens generation in British Columbia [34]. In both of these cases, it is possible that after a heavy sistens generation, progrediens populations were lower due to the competition from limited colonization sites for settling nymphs. The earlier maturation of the sistens generation in British Columbia and Tacoma when compared with reports by McClure (1989) [36] in Connecticut and Gray and Salom (1996) [35] in Virginia is likely attributed to the differing ecosystem and temperate climate of the coastal Pacific Northwest, where average temperatures rarely drop below freezing or exceed 24 °C, and rainfall is greater [37].

Seasonal activity and populations of adelgid prey species and *S. coniferarum* suggest that this predator feeds on multiple adelgid species throughout the year within its native range. 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. This could explain why *S. coniferarum* adults were collected at similar rates on pine and hemlock trees when adult adelgids were present. Conifer sample trees are often nearby western hemlock stands, and it is possible that *S. coniferarum* seasonally moves between host tree species.

4.3. DNA Barcode Sequencing and Morphological Examination of S. coniferarum

Generating of DNA barcode for *S. coniferarum* beetles found that the sequences fell into two distinct clusters that diverged by 6%. Genetic distance of this magnitude is typically correlated with the presence of different species in insects [38]. For *S. coniferarum*, DNA barcode clustering did not correlate with differential prey association, or differences in the morphology of the genitalia. This could mean that the *S. coniferarum* beetles that were collected in Tacoma, WA may in fact be two

cryptic species of beetles that could not be readily distinguished with morphology. Alternatively, one of the sequence clusters could represent mitochondrial DNA sequences that have been transferred into the nucleus, but indels or stop codons were not present in the portion of COI that was sequenced. A third explanation could be that *S. coniferarum* contains deeply divergent COI sequences as a result of mitochondrial capture from a hybridization event, or as a result of other demographic factors [39]. Distinguishing among these different explanations will require additional information from nuclear loci and additional morphological analyses. Because of the remaining taxonomic uncertainty, we have recommended that USDA-APHIS halt all shipments of *S. coniferarum* to the eastern United States until further clarification can be made.

These studies show that *S. coniferarum* likely relies on multiple host tree and prey species for establishment. This, coupled with the possibility that *S. coniferarum* could be two cryptic species, leads the authors to conclude that *S. coniferarum* is not an ideal candidate for the biological control of HWA.

5. Conclusions

Scymnus coniferarum were collected from three of the four conifer species sampled, *T. heterophylla, P. monticola* and *P. contorta*. Each host tree contained a different adelgid prey species, HWA, *P. strobi*, and *P. pini*, respectively. Interestingly, this is the first record of *P. strobi* in the western United States. More *S. coniferarum* were collected from *P. contorta* and *P. monticola* than *T. heterophylla*, particularly when HWA was in diapause. This indicates that *S. coniferarum* feeds on multiple adelgid species in its native range. It is possible that *S. coniferarum* move among these host tree species, and rely on multiple adelgid prey species for optimal reproductive and developmental fitness. DNA barcoding of *S. coniferarum* collected from *T. heterophylla, P. monticola, P. contorta* and *P. menziesii* trees in Tacoma, WA found two distinct clusters that differed by 6% divergence. Beetles in each cluster were co-habitating the same conifer species, and they could not be distinguished morphologically. Further taxonomic studies are needed to understand the significance of DNA barcode sequence divergence. *S. coniferarum* occupies a variety of host tree species and feed on multiple adelgid species throughout the year. The lack of prey specificity and remaining taxonomic uncertainty implies that *S. coniferarum* may not be a suitable biological control agent of HWA in the eastern United States.

Author Contributions: Conceptualization, M.N.D., R.K.B., N.P.H. and S.M.S.; Data curation, M.N.D.; Formal analysis, M.N.D., N.P.H. and E.R.H.; Funding acquisition, S.M.S.; Investigation, M.N.D. and R.K.B.; Methodology, M.N.D., N.P.H. and S.M.S.; Project administration, M.N.D. and S.M.S.; Resources, N.P.H., E.R.H. and S.M.S.; Software, N.P.H.; Supervision, S.M.S.; Visualization, S.M.S.; Writing—original draft, M.N.D.; Writing—review & editing, R.K.B., N.P.H., E.R.H. and S.M.S.

Funding: This research was funded by USDA Forest Service, Grant # 12-CA-11420004-069.

Acknowledgments: The authors thank Lisa Hannon, Michelle Dowling and Kari Stanley for their assistance in field sampling and laboratory analyses. Thanks to Carlyle Brewster for advising in statistical analysis, and Richard McDonald for assistance in experimental design.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Havill, N.P.; Shiyake, S.; Lamb, A.G.; Foottit, R.G.; Yu, G.; Paradis, A.; Elkinton, J.; Montgomery, M.E.; Sano, M.; Caccone, A. Ancient and modern colonization of North America by hemlock woolly adelgid, Adelges tsugae (Hemiptera: Adelgidae), an invasive insect from East Asia. *J. Mol. Ecol.* **2016**, *25*, 2065–2080. [CrossRef] [PubMed]
2. Orwig, D.A.; Foster, D.R.; Mausel, D.L. Landscape patterns of hemlock decline in New England due to the introduced hemlock woolly adelgid. *J. Biogeogr.* **2002**, *29*, 1475–1487. [CrossRef]
3. Mayfield, A.E.; Reynolds, B.C.; Coots, C.I.; Havill, N.P.; Brownie, C.; Tait, A.R.; Hanula, J.L.; Joseph, S.V.; Galloway, A.B. Establishment, hybridization and impact of *Laricobius* predators on insecticide-treated hemlocks: Exploring integrated management of the hemlock woolly adelgid. *For. Ecol. Manag.* **2015**, *335*, 1–10. [CrossRef]

4. McClure, M.S. Density-dependent feedback and population cycles in *Adelges tsugae* (Homoptera: Adelgidae) on *Tsuga canadensis*. *Environ. Entomol.* **1991**, *20*, 258–264. [CrossRef]
5. McDonald, R. Key cues and factors for improving HWA predator recovery efforts. In *Fifth Symposium on Hemlock Woolly Adelgid in the Eastern United States*, 1st ed.; FHTET, 2010-07; Onken, B., Reardon, R., Eds.; USDA Forest Service: Asheville, NC, USA, 2010; pp. 7–16.
6. Mausel, D.L.; Salom, S.M.; Kok, L.T.; Davis, G.A. Establishment of the hemlock woolly adelgid predator, *Laricobius nigrinus* (Coleoptera: Derodontidae), in the eastern United States. *Environ. Entomol.* **2010**, *39*, 440–448. [CrossRef] [PubMed]
7. Federal Register. Available online: https://www.federalregister.gov/documents/2013/03/06/2013-05141/availability-of-an-environmental-assessment-and-finding-of-no-significant-impact-for-a-biological (accessed on 24 July 2018).
8. Gordon, R.D. The Scymnini (Coleoptera: Coccinellidae) of the United States and Canada: Key to genera and revision of *Scymnus*, *Nephus* and *Diomus*. *Bull. Buffalo Soc. Nat. Sci.* **1976**, *28*, 66–68.
9. Whitehead, V.B. The Validity of the Higher Taxonomic Categories of the Tribe Scymnini (Coleoptera: Coccinellidae). Ph.D. Thesis, University of California, Berkeley, CA, USA, 1967.
10. Montgomery, M.; McDonald, R.; Schwartzberg, L. *Scymnus (Pullus) coniferarum* (Coleoptera: Coccinellidae): An adelgid predator native to the western United States. In *Proceedings: 20th US Department of Agriculture Interagency Research Forum on Invasive Species*, 1st ed.; McManus, K., Gottschalk, K., Eds.; USDA Forest Service: Annapolis, MD, USA, 2009; p. 89.
11. Montgomery, M.; McDonald, R. Host preferences of *Scymnus (Pullus) coniferarum*: An adelgid predator. In *Fifth Symposium on Hemlock Woolly Adelgid in the Eastern United States*, 1st ed.; FHTET, 2010-07; Onken, B., Reardon, R., Eds.; USDA Forest Service: Asheville, NC, USA, 2010; pp. 173–176.
12. Gordon, R.D. The Coccinellidae (Coleoptera) of America North of Mexico. *J. N. Y. Entomol. Soc.* **1985**, *93*, 359–362.
13. Montgomery, M.; Lyon, S. Natural enemies of adelgids in North America: Their prospect for biological control of *Adelges tsugae* (Homoptera: Adelgidae). In *Proceedings of the First Hemlock Woolly Adelgid Review*, 1st ed.; FHTET 96-10; Salom, S., Tigner, T., Reardon, R., Eds.; USDA Forest Service: Charlottesville, VA, USA, 1995; pp. 89–102.
14. Montgomery, M.; Keena, M. Chapter 5: *Scymnus (Neopullus)* lady beetles from China. In *Implementation and Status of Biological Control of the Hemlock Woolly Adelgid*, 1st ed.; FHTET, 2011-04; Onken, B., Reardon, R., Eds.; USDA Forest Service: Morgantown, WV, USA, 2011; pp. 176–199.
15. Delucchi, V. *Pullus impexus* (Muls.) (Coleoptera, Coccinellidae), a predator of *Adelges piceae* (Ratz.) (Hemiptera, Adelgidae), with notes on its parasites. *Bull. Entomol. Res.* **1954**, *45*, 243–278. [CrossRef]
16. Lu, W.; Montgomery, M.E. Oviposition, development, and feeding of *Scymnus (Neopullus) sinuanodulus* (Coleoptera: Coccinellidae): A predator of *Adelges tsugae* (Homoptera: Adelgidae). *Ann. Entomol. Soc. Am.* **2001**, *94*, 64–70. [CrossRef]
17. Yu, G.; Montgomery, M.E.; Yao, D. Lady beetles (Coleoptera: Coccinellidae) from Chinese hemlocks infested with the hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae). *Coleopts. Bull.* **2000**, *54*, 154–199. [CrossRef]
18. Darr, M.N. Biological Studies and Evaluation of *Scymnus coniferarum* Crotch, a Predator of Hemlock Woolly Adelgid from Western North America. Ph.D. Thesis, Virginia Tech, Blacksburg, VA, USA, 2017.
19. Darr, M.N.; McAvoy, T.J.; Brewster, C.C.; Salom, S.M. Field-Cage Evaluation of Survival, Reproduction, and Feeding Behavior of Adult *Scymnus coniferarum* (Coleoptera: Coccinellidae), a Predator of *Adelges tsugae* (Hemiptera: Adelgidae). *Environ. Entomol.* **2016**, *45*, 1527–1535. [CrossRef] [PubMed]
20. U.S. Department of Agriculture-Agricultural Research Service. Available online: http://planthardiness.ars.usda.gov (accessed on 25 April 2017).
21. Statistical Analysis Institute. *SAS/Stat User's Guide*; Release v 9.4 Edition; SAS Institute, Inc.: Cary, NC, USA, 2017.
22. Ratnasingham, S.; Hebert, P. The Barcode of Life Data System. *Mol. Ecol. Resour.* **2007**, *7*, 355–364. [CrossRef] [PubMed]
23. Geneious. Available online: http://www.geneious.com (accessed on 15 June 2016).

24. Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock, S.; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C.; et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **2012**, *28*, 1647–1649. [CrossRef] [PubMed]
25. Barcode of Life Data System. Available online: www.barcodinglife.org (accessed on 15 June 2016).
26. Blackman, R.L.; Eastop, V.F. *Aphids on the World's Trees: An Identification and Information Guide*; CABI: Wallingford, UK, 1994; p. 987.
27. Hajibabaei, M.; Dewaard, J.R.; Ivanova, N.V.; Ratnasingham, S.; Dooh, R.T.; Kirk, S.L.; Mackie, P.M.; Hebert, P.D. Critical factors for assembling a high volume of DNA barcodes. *Proc. R. Soc. Lond. Biol.* **2005**, *360*, 1959–1967. [CrossRef] [PubMed]
28. DeWaard, J.; Ivanova, N.; Hajibabaei, M.; Hebert, P. Assembling DNA barcodes: Analytical protocols. In *Environmental Genomics, Methods in Molecular Biology*, 1st ed.; Martin, C., Ed.; Humana Press: Totowa, NJ, USA, 2008; Volume 410, pp. 275–293.
29. Swofford, D.L. *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*, version 4; Sinauer Associates: Sunderland, MA, USA, 2003.
30. Darr, M.N.; Salom, S.M.; Brooks, R.K.; Foottit, R.G.; Miller, G.L.; Havill, N.P. First report of *Pineus strobi* (Hartig, 1839) (Hemiptera: Adelgidae) in western North America. *Pan-Pac. Entomol.* **2018**, *94*, 40–42. [CrossRef]
31. Crotch, G.R. Revision of Coccinellidae of the United States. *Trans. Am. Entomol. Soc.* **1874**, *4*, 363–382. [CrossRef]
32. Hagen, K.S.; Mills, N.J.; Gordh, G.; McMurtry, J.A. Terrestrial arthropod predators of insect and mite pests. In *Handbook of Biological Control: Principles and Applications of Biological Control*, 1st ed.; Bellows, T.S., Fisher, T.W., Eds.; Academic Press: San Diego, CA, USA, 1999; pp. 385–503.
33. Drooz, A. *Insects of Eastern Forests*; USDA Forest Service Miscellaneous Publication: Washington, DC, USA, 1985; pp. 84–85.
34. Zilahi-Balogh, G.M.; Humble, L.M.; Lamb, A.; Salom, S.M.; Kok, L.T. Seasonal abundance and synchrony between *Laricobius nigrinus* (Coleoptera: Derodontidae) and its prey, the hemlock woolly adelgid (Homoptera: Adelgidae) in British Columbia. *Can. Entomol.* **2003**, *135*, 103–115. [CrossRef]
35. Gray, D.R.; Salom, S.M. Biology of hemlock woolly adelgid in the southern Appalachians. In *Proceedings of the First Hemlock Woolly Adelgid Review*, 1st ed.; Salom, S.M., Tigner, T.C., Reardon, R.C., Eds.; USDA, Forest Service, Forest Health Technology Enterprise Team: Charlottesville, VA, USA, 1996; pp. 26–35.
36. McClure, M.S. Evidence of a polymorphic life cycle in the hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae). *Ann. Entomol. Soc. Am.* **1989**, *82*, 50–54. [CrossRef]
37. NOAA. 2018. National Oceanic and Atmospheric Administration Website Database (10 Year Average 2006–2016 for Tacoma, WA). Available online: http://www.ncdc.noaa.gov/ (accessed on 4 September 2018).
38. Hebert, P.D.N.; Cywinska, A.; Ball, S.L.; DeWaard, J.R. Biological identifications through DNA barcodes. *Proc. Biol. Sci.* **2003**, *270*, 313–321. [CrossRef] [PubMed]
39. Morgan-Richards, M.; Bulgarella, M.; Sivyer, L.; Dowle, E.J.; Hale, M.; McKean., N.E.; Trewick, S.A. Explaining large mitochondrial sequence differences within a population sample. *R. Soc. Open Sci.* **2017**, *4*, 170730. [CrossRef] [PubMed]

© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).