*Bulletin of Entomological Research*

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# Coexistence of three specialist predators of the hemlock woolly adelgid in the Pacific Northwest USA

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## Research Paper

**Cite this article:** Rose A, Ross DW, Havill NP, Motley K, Wallin KF (2020). Coexistence of three specialist predators of the hemlock woolly adelgid in the Pacific Northwest USA. *Bulletin of Entomological Research* **110**, 303–308. https://doi.org/10.1017/S0007485319000622

Received: 5 February 2019
Revised: 15 July 2019
Accepted: 11 August 2019
First published online: 27 September 2019

**Key words:**
*Adelges tsugae*; biological control; *Laricobius nigrinus*; *Leucopis* spp.; silver flies

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## Abstract

The hemlock woolly adelgid (Hemiptera: Adelgidae: *Adelges tsugae* Annand) is an invasive insect, introduced from Japan to eastern North America, where it causes decline and death of hemlock trees. There is a closely related lineage of *A. tsugae* native to western North America. To inform classical biological control of *A. tsugae* in the eastern USA, the density and phenology of three native western adelgid specialist predators, *Leucopis argenticollis* (Zetterstedt), *Le. piniperda* (Malloch) (Diptera: Chamaemyiidae), and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), were quantified in the Pacific Northwest. Infested branches were collected from western hemlock (Pinaceae: *Tsuga heterophylla* (Raf.) Sarg.) at four sites around the Puget Sound, Washington and three sites in Oregon. Immature *Leucopis* were identified to species using DNA barcodes. *Leucopis argenticollis* was roughly twice as abundant as *Le. piniperda*. *Laricobius nigrinus* larvae were more abundant than the two species of *Leucopis* during the egg stage of the first adelgid generation, but *Leucopis* were present as feeding larvae during the second adelgid generation when *La. nigrinus* was aestivating in the soil, resulting in *Leucopis* being more abundant than *La. nigrinus* across the entire sampling period. *Adelges tsugae* and *La. nigrinus* densities were not correlated, while *A. tsugae* and *Leucopis* spp. densities were positively correlated. *Leucopis* spp. and *La. nigrinus* densities were negatively correlated. These results support the complementary use of *La. nigrinus* and the two *Leucopis* species for biological control of *A. tsugae* in the eastern USA, and point to the need for further investigation of spatial and temporal niche partitioning among the three predator species.

## Introduction

The hemlock woolly adelgid, *Adelges tsugae* Annand, (Hemiptera: Adelgidae) is a non-native insect pest of hemlock trees (Pinaceae: *Tsuga*) in the eastern USA. *Adelges tsugae* was first collected in the eastern USA in 1951 in Richmond, Virginia (Stoetzel, 2002) and was likely introduced from southern Japan, where it is native (Havill *et al.*, 2006, 2016*a*). Feeding at the base of needles, individuals insert their stylets into xylem ray parenchyma cells, depleting host tree nutrients, and causing water stress and defoliation (Havill *et al.*, 2016*b*). The insect can kill trees of all ages in four to fifteen years after initial infestation (Havill *et al.*, 2016*b*). Mortality may reach 95% in some areas (Havill *et al.*, 2016*b*).

In the Pacific Northwest there is a native cluster of clonal lineage of *A. tsugae*, closely related to the Japanese clone that was introduced to the eastern USA (Havill *et al.*, 2006, 2016*a*). Western *A. tsugae* feeds, develops, and reproduces on western hemlocks (*T. heterophylla* (Raf.) Sarg.) and mountain hemlocks (*T. mertensiana* (Bong.) Carriere) with no obvious defoliation or tree mortality (Kohler *et al.*, 2008). It is possible that native predators prevent *A. tsugae* populations from remaining at densities high enough to cause significant impacts to tree health in western North America. Among the 55 species of predators found associated with *A. tsugae* in a survey conducted throughout western Oregon and Washington, there were three species of adelgid-specific predators that were most abundant and frequently encountered (Kohler *et al.*, 2008). Those were two species of silver flies (Diptera: Chamaemyiidae), *Leucopis argenticollis* Zetterstedt, and *Leucopis piniperda* Malloch (misidentified as *Le. atrifacies* Aldrich, see Grubin *et al.*, 2011), and *Laricobius nigrinus* Fender (Coleoptera: Derodontidae). Both of the *Leucopis* species are also present in eastern North America where *A. tsugae* is a pest, but within each species, eastern populations are genetically diverged from western ones and eastern flies feed on pine adelgids (*Pineus* spp.), not *A. tsugae* (Havill *et al.*, 2018). *Laricobius nigrinus* was previously known to be associated with *A. tsugae* in western North America and was introduced to the eastern USA as a biological control of *A. tsugae* beginning in 2003. Several hundred thousand *La. nigrinus* eggs and adults



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have been released and it has become established at over 100 sites in the eastern USA (Mausel *et al.*, 2010; Havill *et al.*, 2016*b*). However, to date, there has been no perceptible slowing of hemlock decline or mortality at sites with *La. nigrinus* compared to sites without *La. nigrinus* (Mausel *et al.*, 2011; Onken and Reardon, 2011; Mayfield *et al.*, 2015). Kohler *et al.* (2008) were the first to report *Le. argenticollis* and *Le. piniperda* associated with *A. tsugae*. More recently, it was reported that the *Leucopis* spp. collectively were 2.3–3.5 times more abundant than *La. nigrinus* on *A. tsugae* infested trees in the western USA, and the larvae were present feeding on *A. tsugae* for a longer period of time than *La. nigrinus* (Kohler *et al.*, 2016). Furthermore, it was determined that the *Leucopis* spp. larvae feed on the egg stage of both *A. tsugae* generations (Kohler *et al.*, 2016), while *La. nigrinus* larvae feed only on the egg stage of the first generation and undergo a period of aestivation when eggs of the second adelgid generation are present (Zilahi-Balogh *et al.*, 2003). Although many details of the life cycle of both *Leucopis* species are unknown, previous research suggests that there are at least two generations per year based on peaks in abundance of different life stages (Kohler *et al.*, 2008; Grubin *et al.*, 2011). Furthermore, F1 adults were collected in as little as 28 days after adult flies were released onto caged hemlock woolly adelgid infested branches in Tennessee and New York indicating that the flies complete a generation in less than 4 weeks (Motley *et al.*, 2017).

The objective of this study was to see if the results of Kohler *et al.* (2016), which were based on data collected at only two sites, one in Oregon and one in Washington, are representative of predator abundance throughout a wider portion of *A. tsugae* range in the Pacific Northwest. Also, in previous studies, it was not possible to determine the species of *Leucopis* immatures and so data on immature abundance were reported as *Leucopis* spp., collectively. Using a recently developed molecular technique to distinguish immature *Leucopis* species (Havill *et al.*, 2018), it was possible for the first time to report *Leucopis* immature abundance by species across the study sites, providing information about the spatial and temporal coexistence of all three predator species.

## Materials and methods

*Adelges tsugae* and associated adelgid-specific predator densities were quantified from samples collected at field sites in western Oregon and Washington USA. Based on previous research, the predators of interest were *La. nigrinus*, *Le. argenticollis*, and *Le. piniperda* (Kohler *et al.*, 2008). Field sites were chosen for their high *A. tsugae* abundance on western hemlock.

The number of trees sampled and their locations were: 13 trees at a Sierra Pacific Industries seed orchard on Whidbey Island, Washington (48.206898, −122.635276); six trees in Point Defiance Park in Tacoma, Washington (47.304070, −122.517184); four trees on Vashon Island, Washington (47.462193, −122.438922); and two trees in front of an apartment complex, Tanara Villa Apartments, 6322 N 26th St., Tacoma, Washington (47.271838, −122.523984). Additional sites were opportunistically sampled only once or twice since they supported low *A. tsugae* populations. However, data from collections at these sites were combined with the data from the four main sampling locations for predator totals and some analyses. These additional sites included two trees each sampled once in W.B. Nelson State Park on Highway 34 east of Waldport, Oregon (44.418321, −124.049365), one tree sampled twice in Grant Park in Portland, Oregon (45.540730, −122.628954), and one tree sampled once in a parking lot at NW Walnut St. and 9th St. in Corvallis, Oregon (44.594563, −123.251950). A total of 29 *A. tsugae*-infested western hemlock trees were sampled, of which 25 were at the four main sites in Washington.

Terminal twigs showing *A. tsugae* infestation by the white flocculence or 'wool' made by developing adelgids were collected from sample trees. Two to five haphazardly-chosen, 5–10 cm branch tips at least 0.5 m from each other were collected from each tree on each sampling date. At the four main sites, the same trees were repeatedly sampled at roughly two-week intervals April 3 to July 23, 2016. All twigs from each tree were pooled. Twigs were kept on ice in a cooler for transport and in a refrigerator in the lab prior to processing. In the lab, the numbers of *A. tsugae* nymphs and adults with eggs were counted. Although eggs were not counted, adults were always present with eggs.

*Leucopis* larvae and puparia were identified to species by sequencing DNA barcodes or by the restriction fragment length polymorphism (RFLP) method described in Havill *et al.* (2018). DNA was extracted using the Mag-Bind Blood and Tissue Kit (Omega Bio-Tek, NorCross, Georgia). Insect cuticles were recovered following proteinase digestion, slide mounted as vouchers, and deposited at the Yale Peabody Museum of Natural History with accession numbers YPM#ENT 943594-YPM#ENT943628. For DNA barcode sequencing, the standard 658 base pair portion of the mitochondrial cytochrome oxidase I (COI) gene was amplified using primers LepF1 and LepR1 (Hebert *et al.*, 2004). Sequences were deposited in GenBank with accession numbers MH837098–MH837159. Fly specimens for which amplification of the full DNA barcode fragment was not successful were identified using the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assay described in Havill *et al.* (2018). This assay relies on nucleotide substitutions that are fixed for each species in a shorter fragment of COI that is easier to amplify with primers designed specifically for *Leucopis*. Briefly, a 245 base pair portion of the mitochondrial COI gene was amplified using primers LeucoShortF2 and LeucoShortR. PCR products were digested with restriction enzyme RsaI and run on a 1.5% agarose gel to visualize diagnostic banding patterns for *Le. argenticollis* vs *Le. piniperda*. Adult *Leucopis* were identified based on patterns of postpronotal setae (S. Gaimari, personal communication).

The numbers of *Leucopis* larvae, puparia, and adults of the two species were counted. Empty puparial cases were counted as puparia. However, except for one specimen identified using DNA sequencing, these puparial cases could not be identified to species by morphology or DNA sequencing. The numbers of *La. nigrinus* larvae and adults were counted. *Laricobius nigrinus* were identified based on morphology (Zilahi-Balogh *et al.*, 2002; Havill *et al.*, 2016*b*). As *La. nigrinus* pupates in the soil (Zilahi-Balogh *et al.*, 2002), pupae of this species were not found on branches. The total length of sample twigs, including the length of side branches, was measured to express *A. tsugae* and predator densities per centimeter of hemlock stem.

### Data analyses

Mean densities for each predator species and *A. tsugae* were calculated for all trees at each sample location. Each data point for mean cumulative densities represented the mean density of repeated measures of a species on a particular tree over all sampling events. These mean cumulative densities of the combined *Leucopis* spp. and *La. nigrinus* were found to be normally distributed and nearly normally distributed, respectively, by graphing the frequencies of occurrence of mean cumulative densities

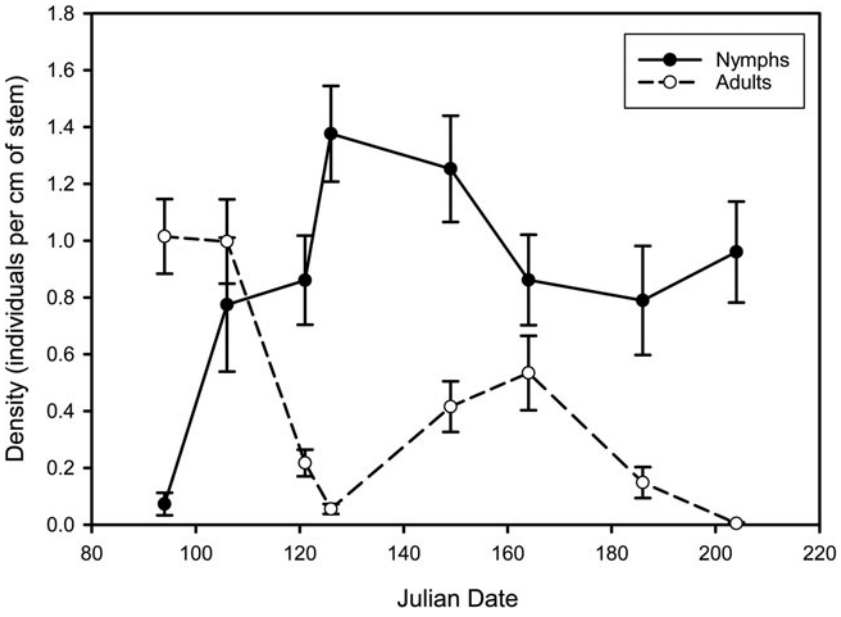


**Fig. 1.** Mean densities of *Adelges tsugae* nymphs and adults across the four main sample sites from April 3 to July 23, 2016. Error bars are standard errors of the mean. Prior to about Julian date 130, adults are sistentes and nymphs are prodgredientes, after Julian date 130, adults are progredientes and nymphs are sistentes.

binned into groups across the range of values in histograms. Mean cumulative densities of each of the *Leucopis* spp., *Le. argenticollis,* and *Le. piniperda*, were found to be highly right-skewed and not normally distributed. Transformations could not consistently normalize these data because they included many zero values. Therefore, tests assuming normality were not applied to the mean cumulative densities of *Le. argenticollis* and *Le. piniperda.* Mean cumulative densities of each of the combined *Leucopis* spp., *La. nigrinus*, and *A. tsugae* were subjected to one-way ANOVA to assess the effect of site as a factor on cumulative density. These analyses informed further analysis by allowing site to be ignored if it was not significant in explaining density. Pearson's product-moment correlation was used to correlate pairwise mean cumulative densities among *A. tsugae*, *La. nigrinus*, and the combined *Leucopis* spp. Spearman's rank correlation coefficient was used to correlate the mean cumulative densities of *Le. argenticollis* and *Le. piniperda*, which were not normally distributed but did satisfy the Spearman's rank correlation coefficient test's assumptions of ordinal or continuous, and monotonically related, data. All statistical analyses were performed using the R-3.3.2 for Windows (32/64 bit) statistical package.

## Results

*Adelges tsugae* adults were most abundant in early April [Julian Date (JD) 93], declining until early May (JD 123) and then increasing to a second peak in mid-June (JD 164) before declining until the end of sampling in late July (JD 204) (fig. 1). *Adelges tsugae* nymphs were at their lowest density in early April (JD 93), increased to a peak in early May (JD 126), and then declined gradually until the end of July (JD 124) (fig. 1).

Sixty-two immature *Leucopis* specimens, were identified using full DNA barcode sequences and 14 were identified using PCR-RFLP. A total of 51 *Le. argenticollis* and 25 *Le. piniperda* larvae and puparia were identified (table 1). Overall, *Leucopis argenticollis* was more abundant than *Le. piniperda*. However, this difference was due entirely to the large number of *Le. argenticollis* compared to *Le. piniperda* collected at the Whidbey Island site (table 1). Nearly identical numbers of both species were collected at the Vashon Island site and slightly more *Le. piniperda* were collected compared to *Le. argenticollis* at the Point Defiance and Tacoma sites. Of the five adult *Leucopis* that were collected, or possibly emerged from puparia after collecting and before sample processing, three were *Le. argenticollis* and two were *Le. piniperda* (Table 1). The densities of *Le. argenticollis* and *Le. piniperda* followed similar patterns over time with only slight differences (fig. 2). Larvae of *Le. argenticollis* increased from early April (JD 93) to early May (JD 121), declined briefly in early May (JD 121–126), and then increased to a maximum in mid-June (JD 164) before declining consistently until the end of July (JD 204) (fig. 2a). In contrast to *Le. argenticollis*, *Le. piniperda* declined briefly from early to mid-April (JD 93–106), but then followed a similar pattern for the remainder of the summer (fig. 2a). Considering all life stages together both species followed a nearly identical pattern throughout the spring and summer, declining briefly from early to mid-April (JD 93–106) before increasing to a plateau from early May to mid-June (JD 126–164), and then declining steadily until the end of July (JD 204) (fig. 2b).

Across all sample dates and sites, over twice as many *Leucopis* spp. ($n = 120$) were collected than *La. nigrinus* ($n = 52$) (table 1), although during the first 2–3 weeks of sampling *La. nigrinus* was three times more abundant than the *Leucopis* spp. The total numbers of combined *Leucopis* spp. were consistently greater than *La. nigrinus* at each site, except Point Defiance, where there was one more *La. nigrinus* collected than *Leucopis* spp. (table 1). Also, across all sites, 70 of the 178 total samples had *Leucopis* spp. present, while only 24 samples had *La. nigrinus* present.

*Leucopis* spp. larvae were present in samples over a longer period of time than *La. nigrinus* larvae (fig. 3). Specifically, the overall densities of larval *La. nigrinus* were highest in April (JD 93–106), declined by early May (JD 121), and remained at low densities until sampling ended in late July (JD 204) (fig. 3). The overall densities of larval *Leucopis* spp. increased in May (JD 121), were highest in June (JD 164), and declined in late July (JD 204) (fig. 3). When compared across the four main sampling locations, there was no significant effect of site on *A. tsugae* (combined nymphs and adults) or predator (combined larval, puparial, and adult *Leucopis* spp., and combined larval and adult *La.*

**Table 1.** Numbers of adelgid specific predators collected at four primary sample sites and total for all sample sites in Oregon and Washington, USA, from April 3 to July 23, 2016[a]

| | Site | | | | |
|---|---|---|---|---|---|
| Species/Life stage | Whidbey Island | Vashon Island | Point Defiance | Tacoma | Total |
| *Laricobius nigrinus* | 28 | 5 | 16 | 1 | 52 |
| Larvae | 25 | 3 | 15 | 1 | 46 |
| Adults | 3 | 2 | 1 | 0 | 6 |
| *Leucopis* spp. | 59 | 26 | 15 | 19 | 120 |
| Larvae | 36 | 10 | 7 | 10 | 64 |
| Puparia | 19 | 16 | 7 | 9 | 51 |
| Adults | 4 | 0 | 1 | 0 | 5 |
| *Leucopis argenticollis* | 42 | 8 | 2 | 2 | 54 |
| Larvae | 28 | 2 | 2 | 1 | 33 |
| Puparia | 11 | 6 | 0 | 1 | 18 |
| Adults | 3 | 0 | 0 | 0 | 3 |
| *Leucopis piniperda* | 4 | 7 | 5 | 11 | 27 |
| Larvae | 1 | 4 | 1 | 8 | 13 |
| Puparia | 2 | 3 | 2 | 3 | 10 |
| Adults | 1 | 0 | 1 | 0 | 2 |

[a]Data are presented for all *Leucopis* combined and separately for each species. The sums for the individual species do not equal the values for the two species combined because our methods were unsuccessful at identifying every specimen to species.

*nigrinus*) mean cumulative densities in three one-way ANOVAs (*A. tsugae*: $F$-value = 1.88, df = 3, 21, $P$-value = 0.17; *Leucopis*: $F$-value = 1.95, df = 3, 21, $P$-value = 0.15; *La. nigrinus*: $F$-value = 0.4, df = 3, 21, $P$-value = 0.75).

The Pearson's product-moment correlation of *A. tsugae* and combined larval and adult *La. nigrinus* densities was found to be non-significant, with an alternative hypothesis of positive correlation ($t$-value = −0.34; df = 23; $P$-value = 0.63; 95% CI = −0.40 to 1; estimated correlation = −0.07). The Pearson's product-moment correlation of *A. tsugae* and larval *La. nigrinus* densities was likewise found to be non-significant, with an alternative hypothesis of positive correlation ($t$-value = −0.63; df = 23; $P$-value = 0.73; 95% CI = −0.45 to 1; estimated correlation = −0.13). Combined larval and adult *La. nigrinus* and *Leucopis* spp. densities were significantly, moderately, negatively correlated, with an alternative hypothesis of negative correlation ($t$-value = −1.81; df = 23; $P$-value = 0.04; 95% CI = −1 to −0.02; estimated correlation = −0.35). Larval *La. nigrinus* and *Leucopis* spp. densities were significantly, moderately, negatively correlated, with an alternative hypothesis of negative correlation ($t$-value = −1.92; df = 23; $P$-value = 0.03; 95% CI = −1 to −0.04; estimated correlation = −0.37). *Adelges tsugae* density correlated significantly, moderately, and positively with both larval and combined larval, puparial, and adult *Leucopis* spp. densities, tests with an alternative hypothesis of positive correlation (*A. tsugae* and larval *Leucopis*: $t$-value = 2.64; df = 23; $P$-value < 0.01; 95% CI = 0.17 to 1; estimated correlation = 0.48; and *A. tsugae* and combined *Leucopis*: $t$-value = 1.98; df = 23; $P$-value = 0.03; 95% CI = 0.05 to 1; estimated correlation = 0.38). *Leucopis argenticollis* and *Le. piniperda* were to some degree significantly, negatively correlated (at $P \leq 0.10$) using a non-parametric test, Spearman's rank correlation coefficient, with an alternative hypothesis of negative correlation ($\rho$ = −0.28; df = 23; $P$-value = 0.09).

## Discussion

Across all sample trees and dates, *Leucopis* spp. collectively were 2.3 times more abundant than *La. nigrinus*. This is consistent with a previous study that found *Leucopis* spp. to be 2.3–3.5 times more abundant than *La. nigrinus* (Kohler *et al.*, 2016). However, the earlier study reported data collected at only two sites, one in Oregon and one in Washington. The present study includes data from four primary sample sites in the Puget Sound area of Washington and three less intensively sampled sites in Oregon. These new data suggest that collectively *Leucopis* spp. are more abundant than *La. nigrinus* in the Pacific Northwest USA. The numbers above may be somewhat misleading regarding the relative abundance of the two genera. Since *La. nigrinus* pupates in the soil, this life stage would have been excluded from the clipped branch samples. In contrast, *Leucopis* spp. puparia are located on twigs near *A. tsugae*. Excluding puparia, a total of 69 *Leucopis* spp. were collected, about 1.3 times the number of *La. nigrinus* ($n$ = 52). The results of the present study also confirm the previous finding that *Leucopis* spp. larvae are present on *A. tsugae* infested twigs for a much longer period of time than larvae of *La. nigrinus* (fig. 3) and that *Leucopis* spp. larvae feed on the egg stage of both *A. tsugae* generations while *La. nigrinus* larvae feed only on the egg stage of the first adelgid generation (Kohler *et al.*, 2016).

In all previous studies of these *Leucopis* spp., it was not possible to distinguish the species of immatures based on morphological characters. Using a recently developed DNA sequencing technique, 86% of the *Leucopis* spp. immatures collected in the present study were identified to species. Combining the immature and adult totals for each species, *Le. argenticollis* was more abundant ($n$ = 51) than *Le. piniperda* ($n$ = 25). In a previous study of predators on 116 *A. tsugae* infested western hemlock across 16 sites in western Oregon and Washington, USA, a total of 99

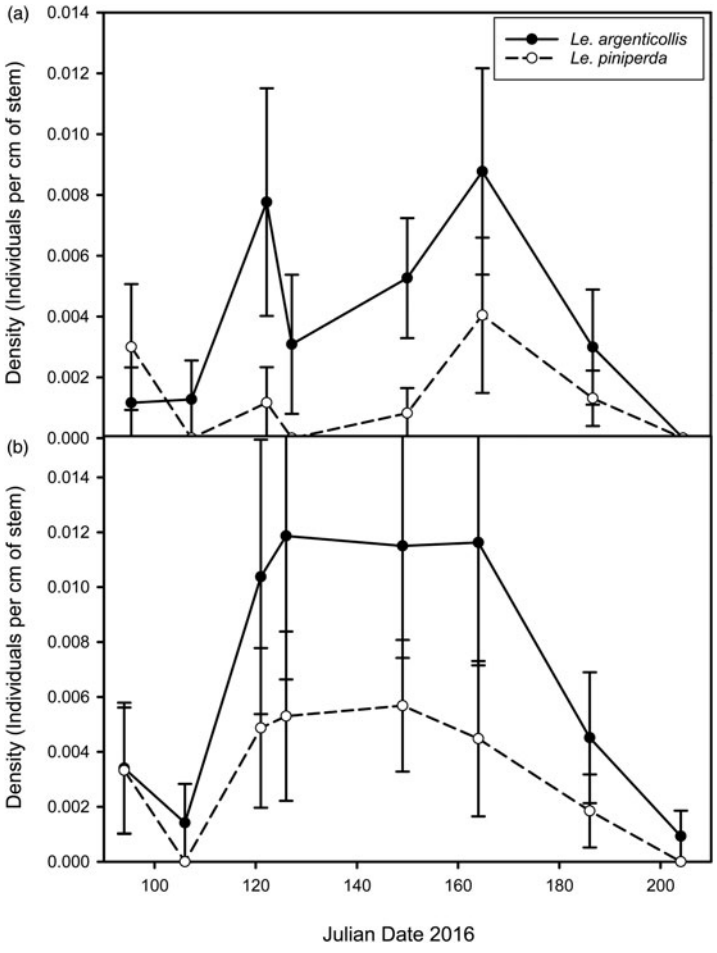


**Fig. 2.** Mean densities of all identified *Le. argenticollis* and *Le. piniperda* larvae (a) and combined larvae, puparia, and adults (b) across the four main sample sites from April 3 to July 23, 2016. Error bars are standard errors of the mean.

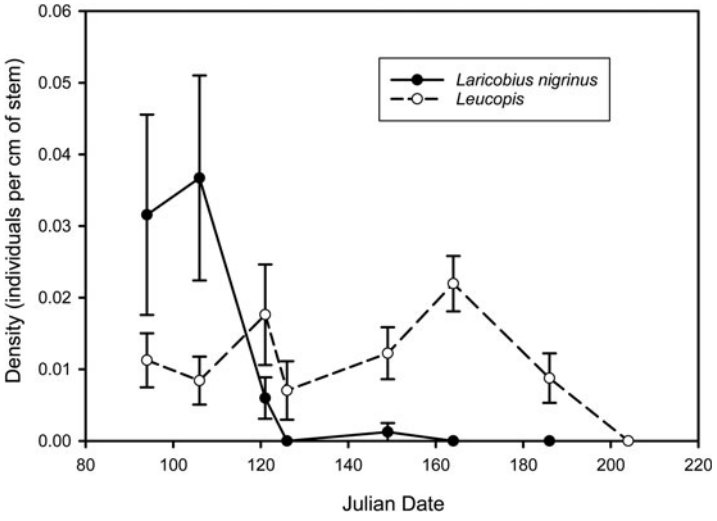


**Fig. 3.** Mean densities of larval *Leucopis* spp. combined and *La. nigrinus* across all sites, from April 3 to July 23, 2016. Error bars are standard errors of the mean.

adult *Leucopis* spp. were collected. Eighty-seven percent of those adults were *Le. argenticollis* and 13% were *Le. piniperda* (misidentified as *Le. atrifacies*, see Grubin *et al*., 2011) (Kohler *et al*., 2008). The results reported here indicate that *Le. argenticollis* may be significantly more abundant than *Le. piniperda* at some sites in the Pacific Northwest, but the two species are present at similar population densities at others. Although *Le. argenticollis* is more abundant than *Le. piniperda* at some sites, there appears to be no difference in phenology between the two species (fig. 2). Since there was no significant effect of site on combined *Leucopis* spp., *La. nigrinus*, or *A. tsugae* cumulative densities, linear correlations were used to test interspecific associations across all sites. *Laricobius nigrinus* densities were not correlated with *A. tsugae* densities, while *Leucopis* spp. densities were positively

correlated with *A. tsugae* densities. Grubin *et al.* (2011) and Motley *et al.* (2017) also found positive correlations between *Leucopis* spp. and *A. tsugae* densities. Kohler *et al.* (2008) found that the abundances of larval *Leucopis* spp. and *La. nigrinus* were positively correlated with *A. tsugae* abundance score. Collectively, these results suggest that *Leucopis* spp. populations respond positively to increasing *A. tsugae* populations and *Laricobius nigrinus* populations also respond positively to increasing *A. tsugae* populations but less consistently. The negative interspecific correlations between the intraguild predators may indicate possible competition or avoidance among the *Leucopis* spp. and *La. nigrinus*.

The data reported here add to the growing body of evidence that suggests the *Leucopis* spp. may be responsible for regulating *A. tsugae* populations below levels that cause any significant impact to western hemlock in the Pacific Northwest USA either independently or in combination with *La. nigrinus*. As reported here and in Kohler *et al.* (2016), the *Leucopis* spp. are more abundant than *La. nigrinus* and feed on both generations of *A. tsugae* while *La. nigrinus* feeds only on the first adelgid generation in their native ranges of the Pacific Northwest USA. Furthermore, both *Leucopis* species appear to be restricted to *A. tsugae* as prey in the Pacific Northwest USA, although both species have been reported from other adelgid hosts across North America and *Le. argenticollis* has been reported from other adelgid hosts in Europe, Japan, India and Russia (Ross *et al.*, 2011, 2017; Havill *et al.*, 2018). Recently, it was demonstrated that *Leucopis* species from the Pacific Northwest USA are able to feed and develop to adults on *A. tsugae* populations from the eastern USA both in the laboratory and under field conditions (Motley *et al.*, 2017). Collectively, these results support the continuing study of the *Leucopis* species as potential biological control agents for *A. tsugae* in the eastern USA, and further study of all three predators in the Pacific Northwest to clarify niche partitioning that may explain their coexistence on a common prey.

**Acknowledgements.** We thank Doug Sand and Sierra Pacific Industries for providing access to one of the field sites. Dan Cress helped facilitate this access and we thank him as well. We gratefully acknowledge the role of Ariel Muldoon in the Department of Forest Ecosystems and Society, College of Forestry, Oregon State University for her advice to the lead author on statistical design and computing. Thanks also to the USDA Forest Service Hemlock Woolly Adelgid Initiative for funding this work.

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