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# Feeding by *Leucopis argenticollis* and *Leucopis piniperda* (Diptera: Chamaemyiidae) from the western USA on *Adelges tsugae* (Hemiptera: Adelgidae) in the eastern USA

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**Abstract**

*Leucopis argenticollis* (Zetterstedt) and *Leucopis piniperda* (Malloch) are known to feed on the lineage of *Adelges tsugae* Annand that is native to western North America, but it is not known if they will survive on the lineage that was introduced from Japan to the eastern USA. In 2014, western *Leucopis* spp. larvae were brought to the laboratory and placed on *A. tsugae* collected in either Washington (North American *A. tsugae* lineage) or Connecticut (Japanese lineage). There were no significant differences in survival or developmental times between flies reared on the two different adelgid lineages. In 2015 and 2016, western *Leucopis* spp. adults were released at two different densities onto enclosed branches of *A. tsugae* infested eastern hemlock (*Tsuga canadensis* (L.) Carr.) in Tennessee and New York. Cages were recovered and their contents examined 4 weeks after release at each location. *Leucopis* spp. larvae and puparia of the F1 generation were recovered at both release locations and adults of the F1 generation were collected at the Tennessee location. The number of *Leucopis* spp. offspring collected increased with increasing adelgid density, but did not differ by the number of adult flies released. Flies recovered from cages and flies collected from the source colony were identified as *L.argenticollis* and *L. piniperda* using DNA barcoding. These results demonstrate that *Leucopis* spp. from the Pacific Northwest are capable of feeding and developing to the adult stage on *A. tsugae* in the eastern USA and they are able to tolerate environmental conditions during late spring and early summer at the southern and northern extent of the area invaded by *A. tsugae* in the eastern USA.

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## Introduction

The hemlock woolly adelgid (*Adelges tsugae* Annand) was introduced to the eastern USA from Japan sometime before 1951 when it was first documented in Virginia (Stoetzel, 2002; Havill *et al.*, 2006). In the 1980s, it began spreading rapidly throughout the range of hemlock causing high levels of tree mortality. It is now present in 19 eastern US states from Georgia to southern Maine where it damages two native hemlock species, eastern hemlock (*Tsuga canadensis* (L.) Carr.) and Carolina hemlock (*Tsuga caroliniana* Engelm.) (Havill *et al.*, 2011). The first efforts to develop and implement classical biological control for *A. tsugae* began in the early 1990s, but increased dramatically in the early 2000s with the formation of the Hemlock Woolly Adelgid Initiative, a cooperative research and development program involving federal and state government agencies and other partners (Onken & Reardon, 2011). To date, the biological control program has focused mostly on two predators, *Sasajiscymnus tsugae* (Sasaji and McClure), a coccinelid imported from Japan, and *Laricobius nigrinus* Fender, a derodontid imported from western North America where *A. tsugae* is also native. Between 1995 and 2010, over 2 million *S. tsugae* were released at more than 400 sites in 16 eastern states (Cheah, 2011). Between 2003 and 2010, several hundred thousand *L. nigrinus* adults and eggs were released at sites in 14 eastern states (Mausel *et al.*, 2011); this predator has become established at numerous eastern US sites, reduces densities of the *A. tsugae* winter generation (Mayfield *et al.*, 2015), and continues to be released. An additional derodontid species from Japan, *Laricobius osakensis*, is also beginning to be released in the eastern USA (Mooneyham *et al.*, 2016). Despite the coordinated effort to control *A. tsugae* with *S. tsugae* and *L. nigrinus*, there is, so far, no indication that they are reducing the rate of hemlock mortality. Consequently, efforts have continued to identify additional biological control agents in Asia and western North America (Onken & Reardon, 2011) where there are endemic lineages of *A. tsugae* (Havill *et al.*, 2016).

A beat sampling survey of 116 *A. tsugae* infested western hemlocks (*Tsuga heterophylla* (Raf.) Sarg.) across 16 sites in western Oregon and Washington over 23 months resulted in the collection of over 6000 adult and immature predators representing 55 species from 43 genera, 14 families, and 4 orders (Kohler *et al.*, 2008). *L. nigrinus* was found to be the most abundant comprising 43% of all predators collected. Collectively, two species of *Leucopis* (Diptera: Chamaemyiidae), *L. argenticollis* and *L. piniperda* (misidentified as *L. atrifacies*, see Grubin *et al.*, 2011) were the second most abundant predators comprising 16% of the total. However, the ratio of immatures to adults was over three times higher for the chamaemyiids (9.2:1) compared with the derodontids (2.6:1) or hemerobiids (3.1:1), the third most abundant group, suggesting that beat sampling was less effective at collecting adult chamaemyiids and that their relative abundance may be higher than indicated by counts from beat sampling. *L. nigrinus*, *L. argenticollis*, and *L. piniperda* were the only adelgid-specific predators that were both frequently encountered and abundant in the survey. This was the first record of either *L. argenticollis* or *L. piniperda* collected from *A. tsugae*, although both species have been collected in association with other *Pineus* and *Adelges* species in other parts of North America (Ross *et al.*, 2011). A more recent study in Oregon and Washington found 2.3–3.5 times more *Leucopis* spp. than *L. nigrinus* after sampling and dissecting branches over a year (Kohler *et al.*, 2016). Laboratory, no-choice feeding trials with *Leucopis* spp. larvae collected from *A. tsugae* infested western hemlock indicated that both species can feed, survive, and develop to the adult stage on other adelgid species, although survival was always highest on *A. tsugae* (Grubin *et al.*, 2011).

The objective of the studies reported in this paper was to determine whether *Leucopis* spp. from the Pacific Northwest (PNW) could feed and complete their development on Japanese *A. tsugae* introduced to the eastern USA in the laboratory and under field conditions.

## Materials and methods

### *Laboratory feeding experiment*

In March 2014, western hemlock branches with *A. tsugae* infestations were collected in Olympia and Tacoma, WA. The branches were placed in plastic bags and shipped overnight to the USDA Forest Service, Northern Research Station (USDA-FS-NRS) laboratory in Hamden, CT. Interstate movement of this material was regulated under USDA-APHIS permit number P526P-13-03488 issued to N. Havill. Branches were examined under a dissecting microscope and *Leucopis* spp. larvae were removed, their length measured using an ocular micrometer calibrated with a 2 mm stage micrometer (American Optical Company, Buffalo, New York), and alternately placed into one of two treatment groups. One group received western *A. tsugae* on *T. heterophylla*, and the other received eastern (Japanese) *A. tsugae* on *T. canadensis*. Flies were placed on 5-cm-long branch tips with at least three undisturbed adelgid ovisacs with eggs. Each infested branch and fly larva was placed in a 60-ml plastic cup with a moist filter paper on the bottom. The lid of each cup had a 2-cm diameter hole covered with fine mesh.

Flies were held in a walk-in environmental chamber at 25° C, 60% relative humidity, and a photoperiod of 12:12 (L:D) h. Flies were observed every 1–3 days until they died or pupariated. Puparia were removed from the foliage and placed individually in 5-cm diameter Petri dishes and provided with a 50:50 Wheast (Planet Natural, Bozeman, Montana) and honey paste spotted onto a small square of filter paper to provide nutrition for the adult fly upon emergence. The dates that flies died, pupariated, and/or emerged as adults were recorded. Puparia that did not yield an adult fly or parasitoid were dissected to determine whether a fly or parasitoid died during development.

### *Branch enclosure study*

*T. heterophylla* branches with *A. tsugae* infestations were collected in April and May 2015 and 2016 from several locations in Olympia, Tacoma, Vashon Island, and Whidbey Island, WA. The branches were sealed in plastic bags and shipped

overnight to the USDA-FS-NRS laboratory in Hamden, CT in 2015 and transported directly to the Oregon State University, Department of Forest Ecosystems & Society in Corvallis, OR in 2016.

Foliage was held in cages to monitor for adult fly eclosion. Two types of cages were used: $60 \times 60 \times 60$ cm$^3$ tent-style fine mesh bugdorms (Item number BD2120, MegaView Science, Taiwan), or custom built $50 \times 45 \times 45$ cm$^3$ plexiglass cages with mesh insets on the top and side. Upon arrival, foliage was clipped into pieces that would fit in the cages and the stems were inserted in $22.5 \times 10.5 \times 8.0$ cm$^3$ floral foam blocks held in Sterilite® plastic shoeboxes ($31 \times 19 \times 10$ mm$^3$). The floral foam was saturated with deionized water, with additional water left standing in the bottom of the shoe box to compensate for evaporation. Two shoeboxes with foliage were placed into each cage. A paste of honey and Wheast was spread on strips of yellow paper, which were taped to the inside wall of each cage. A combination of vials containing deionized water, dilute honey water, and dilute honey-Wheast water were stopped with a cotton wick and placed in each cage as well (based on Gaimari & Turner, 1996). Water in the shoeboxes and the food and water vials were replenished 1–2 times per week as needed. Once cages were prepared, they were held in two walk-in environmental chambers with a photoperiod of 16:8 (L:D) h at either 15 or 17°C in 2015 and in a laboratory at room temperature in 2016.

Cages were checked every 1 or 2 days. During these checks, any arthropods other than *Leucopis* spp. (especially predators that might prey on emerging flies) were removed. Adult *Leucopis* spp. found in the cages were collected with an aspirator and moved to a collective adult cage, which was similar to the rearing cages, except a small amount of uninfested *T. canadensis* foliage was used instead of infested *T. heterophylla* foliage. Also, the foliage was placed into a 1000 ml flask filled with water and covered with parafilm® instead of floral foam in a shoe box to prevent flies from becoming trapped in the water. Consequently, flies had foliage to alight on, but they did not have a prey source on which to lay their eggs. Adult cages were held at 15°C with a photoperiod of 16:8 (L:D) h.

For two nights prior to a field release in 2015, cages with adults were removed from the environmental chambers. They were placed in a room (~23°C) near a window in case the dawn and/or dusk periods were required to stimulate mating behavior. Each day, they were removed from the chamber at approximately 04:15 p.m. and returned to the chamber at approximately 07:15 a.m. the next morning. No attempts were made to expose the adult flies to dawn or dusk lighting in 2016.

On the day of shipment to field sites, adult flies were sorted by sex based on dimorphism of the abdomens, viewed under a dissecting microscope with individual flies in 5 cm Petri dishes. Flies were then placed in separate female and male cages with vials of water, honey water, and honey-Wheast water, but no foliage. The required number of females and males for field experiments onto caged branches could then be drawn from each cage. It was not possible to determine the species of the flies prior to release because the character used to distinguish them could not be seen on live flies. *L. argenticollis* have several long setulae on the postpronotum, medial from the postpronotal seta, while *L. piniperda* have no such setulae (S. Gaimari, 2015, personal communication).

In preparation for shipment to experimental field sites, plastic aspirator vials were prepared similarly to Gaimari & Turner (1996). Adult flies were aspirated into the vials in specific sex ratios according to the experimental design. Flies were in transport to experimental field sites in insulated boxes with ice for <24 h.

Enclosed branch experiments were performed at two locations, near Grandview, TN (35.74853, −84.82871) and Skaneateles Lake, Niles, NY (42.80186, −76.30139), located near the southern and northern edges, respectively, of the invasive range of *A. tsugae* in the eastern USA. Flies were placed on enclosed branches in TN on 12 May 2015 and 10 May 2016. Flies were placed on enclosed branches in NY on 5 June 2015 and 27 May 2016. Ambient conditions during releases in TN and NY were 20–23°C and sunny and 22–24°C and partly cloudy, respectively.

The date for each experimental field release was timed to coincide with *A. tsugae* entering the progrediens nymph stage, so that if flies reproduced, the larvae could feed on eggs of the next generation (sistentes). All live *A. tsugae* progrediens nymphs (evidenced by fresh woolly ovisac production) 50 cm from the terminal end on each branch were counted and recorded. Prior to enclosure, treatments were assigned at random to infested *T. canadensis* branches. There were four treatments, each replicated on six branches in 2015 and seven branches in 2016. The treatments were: (1) enclosed branch with 2F:2M *Leucopis* spp., (2) enclosed branch with 6F:4M *Leucopis* spp. in 2015 and 5F:5M *Leucopis* spp. in 2016, (3) enclosed control branch without *Leucopis* spp. and (4) non-enclosed control branch without *Leucopis* spp. All branches were tapped along their length 20 times to dislodge predators prior to enclosing.

Branch enclosures were $71 \times 48$ cm$^2$ bags made of fine mesh nylon netting (Item number DC3148, MegaView Science Co., Taiwan). To secure enclosures to branches, a piece of foam pipe insulation was wrapped onto the branch 50 cm from the end. The open end of the enclosure was secured around the pipe insulation with two zip ties. Flies were added to the enclosures through the zipper.

Branches from the 2015 study were collected 28 days (9 June) after experimental release in TN, and 33 days (8 July) after release in NY. Branches from the 2016 study were collected 29 days (7 June) after experimental release in TN, and 26 days (22 June) after release in NY. Branches were collected by placing a large plastic bag around each branch, clipping the branch, and sealing the plastic bag. In 2015, branches were shipped overnight with ice packs to the USDA Forest Service laboratory in Hamden, CT, where they were kept at 7°C until processed. Branches collected in 2016 were shipped overnight with ice packs to the USDA Forest Service George D. Aiken Forestry Sciences Laboratory in Burlington, VT and stored at 7°C until processed. Branches were clipped into small pieces approximately 10 cm long. All *A. tsugae* ovisacs, settled adults, and new *T. canadensis* growth were recorded. Each branch was thoroughly searched for fly offspring under a dissecting microscope. The entire contents of each mesh enclosure was thoroughly searched for fly offspring and the number of offspring in each life stage was recorded. Larvae and adults were collected into 95% ethanol and stored at −20°C. Puparia were held in individual 5 cm Petri dishes until eclosion of adults, which were then placed into 95% ethanol and stored at −20°C.

Up to 20 larvae and/or adult flies per enclosure were identified using DNA barcoding. DNA was extracted using the Mag-Bind Blood & Tissue Kit (Omega Bio-Tek, Norcross, Georgia). DNA was extracted from adults after grinding three legs removed from one side of the specimen with the

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remainder saved as a voucher. Larvae underwent non-destructive extraction by cutting a small slit in the side of the specimen, incubating with proteinase for at least 1 h in a microcentrifuge tube, and then spinning at 14,000 rpm to squeeze the body contents into solution. The cuticle was removed before resuming extraction and was later slide mounted as a voucher. All vouchers are deposited at the Yale Peabody Museum of Natural History (Voucher Nos. XXXX). The 658 bp portion of the mitochondrial cytochrome oxidase I gene used for DNA barcoding animals was amplified and sequenced using standard protocols (deWaard *et al.*, 2008).

### *Statistical analyses*

Differences in mean initial larval length, time to pupariation, and puparial duration between eastern (Japanese) versus western *A. tsugae* were tested using unpaired *t*-tests. Differences in percent survival to pupariation, percent survival to adult, and percent parasitism of *Leucopis* spp. were compared using chi-square tests to compare the equality of proportions between treatments. Total number of *Leucopis* spp. offspring per enclosure verses initial *A. tsugae* ovisac populations were tested using a linear regression. Differences in the total number of *Leucopis* spp. offspring between enclosed densities were analyzed using a one-way analysis of variance. Statistical analyses were performed using R version 3.1.1 and RStudio v2.1 (R Core Team, 2014).

## Results

### *Laboratory feeding experiment*

Of the 102 *Leucopis* spp. larvae that were collected from the infested foliage and used in the experiment, 53 were reared on eastern *A. tsugae* and 49 were reared on western *A. tsugae*. There were no significant differences in initial larval size, time to pupariation, puparial duration, time to adult, percent survival to adult, or percent parasitism among the two groups reared on different populations of *A. tsugae* (Table 1). All parasitoids emerged during the puparial stage. These flies would have been parasitized during the egg or larval stage in the field.

### *Branch enclosure study*

For both years at the TN site, the mean number of *Leucopis* spp. offspring was 9.1 in 2F:2M treatments and 15.2 in 6F:4M/5F:5M treatments. These values were not statistically different ($F = 1.36$; $P = 0.251$). The mean numbers of larvae, puparia, and adult offspring recovered per enclosure were 3.9, 5.0, and 0.15, respectively, for the 2F:2M treatment and 5.2, 8.3, and 0.5, respectively, for the 6F:4M/5F:5M density treatment.

For both years at the NY site, the mean number of *Leucopis* spp. offspring was 4.2 in 2F:2M treatments and 7.7 in 6F:4M/5F:5M treatments. These values were also not statistically different ($F = 1.022$; $P = 0.322$). The mean numbers of larvae, puparia and adult offspring recovered per enclosure were 0.9, 3.2, and 0.1, respectively, for the 2F:2M treatment and 2.2, 5.4, and 0.2, respectively, for the 6F:4M/5F:5M treatment.

At both the TN and NY sites, the number of *Leucopis* spp. offspring were linearly correlated to the number of initial *A. tsugae* ovisacs ($R^2 = 0.22$ and 0.54, respectively) (fig. 1).

Both *L. argenticollis* and *L. piniperda* were recovered from the TN site, with only *L. argenticollis* found in 36.8% of the enclosed branches, only *L. piniperda* found in 47.3% and both species found in 15.7% of the enclosed branches. Only *L. argenticollis* was recovered from the NY site (Table 2).

## Discussion

The results of these experiments demonstrate that, under both laboratory and field conditions, *Leucopis* spp. from the PNW are capable of feeding and developing on a diet of the Japanese lineage of *A. tsugae* that was introduced to the eastern USA. There were no significant differences in survival or developmental times in the laboratory experiment between *Leucopis* spp. reared on *A. tsugae* from the two different geographic regions. This suggests that *Leucopis* spp. from the PNW would have suitable prey if released in the eastern USA as biological control agents for *A. tsugae*.

Propagule pressure, defined as the number of individuals released and the number of releases, is a key component of establishment success (Lockwood *et al.*, 2005). Because the number of *Leucopis* spp. offspring collected did not differ significantly by the number of adult flies released, we could not draw conclusions about an optimal release density based on these experiments. This lack of significance could be explained by the relatively small difference in number of individuals between the treatments. Future work should increase the difference between the number of individuals released in each treatment, and increase the number of treatments, to better understand this relationship.

It is important for biological control agents to be able to establish on both high and low densities of their prey (DeBach & Rosen, 1991). The linear relationship between *Leucopis* spp. offspring and initial *A. tsugae* populations (fig. 1) indicates that *Leucopis* spp. exhibit this characteristic. *Leucopis* spp. were able to survive and reproduce on the relatively low number of live ovisacs per enclosed branch (an average of six ovisacs per branch) found in NY during the 2016 field release.

The difference in species recovered from enclosures between TN and NY suggests that there is a temporal difference in life cycles of *L. argenticollis* and *L. piniperda*. Since both species were recovered at the TN site, but only *L. argenticollis* was recovered at the NY site, *L. piniperda* may complete its development earlier than *L. argenticollis* in the PNW (adults released in TN were collected earlier than the adults released in NY). This difference could be a function of niche partitioning, but more work is needed to understand phenological differences between the species in the PNW.

Because *A. tsugae* has two generations per year in its invaded range, it is critical that biological control efforts address both. Kohler *et al.* (2016) found that *Leucopis* spp. exhibit peak abundances coinciding with both *A. tsugae* progrediens and sistens egg stages in the PNW. While this study shows that *Leucopis* spp. can establish and reproduce during the progrediens egg stage in the invaded range of *A. tsugae*, it is yet unknown whether *Leucopis* spp. can survive and reproduce during both generations of *A. tsugae* in the eastern USA. Future work will focus on feeding, reproduction, and survival of *Leucopis* spp. during the different generations of *A. tsugae* in the eastern USA, particularly during *A. tsugae* aestivation and sistens egg stages.

For biological control to be effective, biological control agents must be able to establish and spread under conditions throughout the year and geographical extent of their target's

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Table 1. Survival parameters for *Leucopis* spp. larvae reared on the eastern and western US populations of *Adelges tsugae* under laboratory conditions.

| Growth and survival parameters | Larvae reared on the eastern USA (Japanese) *A. tsugae* ($N = 53$) | Larvae reared on the western USA *A. tsugae* ($N = 49$) | Test statistics and *P*-value | | |
|---|---|---|---|---|---|
| Initial larval length (mm) | 2.37 | 2.30 | $t = -0.46$ | df = 1, 90 | $P = 0.652$ |
| Time to pupariation (days) | 4.48 | 4.21 | $t = 0.53$ | df = 1, 50 | $P = 0.597$ |
| Puparial duration (days) | 10.53 | 9.53 | $t = 1.1$ | df = 1, 29 | $P = 0.279$ |
| % Survival to pupariation | 50.0 | 63.0 | $X^2 = 1.59$ | df = 1 | $P = 0.21$ |
| % Survival to adult | 30.4 | 37.0 | $X^2 = 0.40$ | df = 1 | $P = 0.51$ |
| % Parasitized puparia | 2.86 | 2.24 | $X^2 = 0.03$ | df = 1 | $P = 0.75$ |

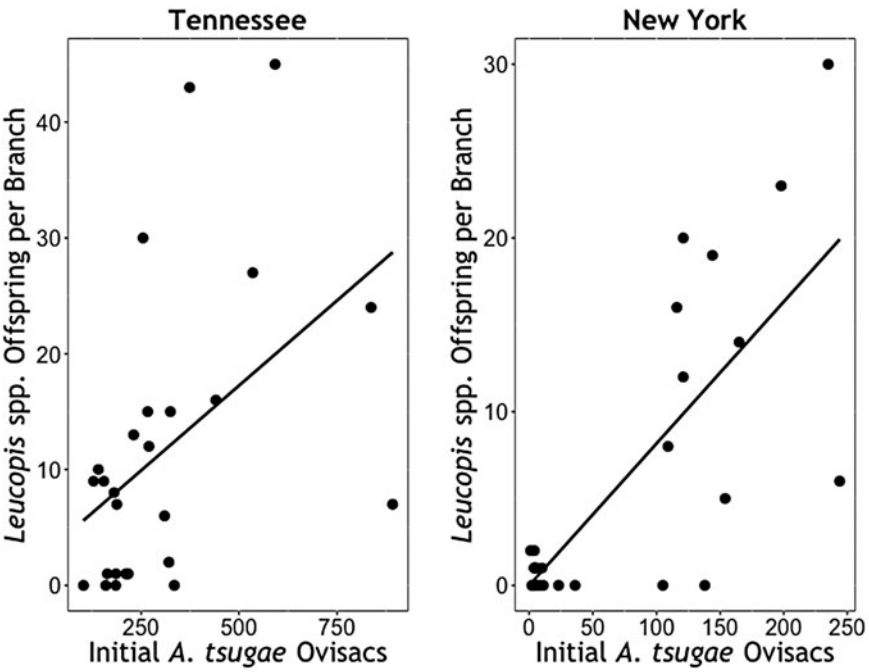


Fig. 1. Number of *Leucopis* offspring collected verses the initial *A. tsugae* populations in each enclosure in TN and NY. Data are pooled for 2015 and 2016.

Table 2. Number of branches with *Leucopis* spp. offspring from enclosed branch studies in Tennessee (TN) and New York (NY) in 2015.

| | *L. argenticollis* | *L. piniperda* | Both |
|---|---|---|---|
| TN | 7 | 9 | 3 |
| NY | 13 | 0 | 0 |

invaded range. Results from the branch enclosure study in the field indicate that environmental conditions at both the northern and southern extremes of the area invaded by *A. tsugae* are within the environmental thresholds of *Leucopis* spp. from the PNW during the late spring and early summer. It remains to be seen whether western *Leucopis* spp. can tolerate environmental conditions throughout the year in the eastern USA. However, the fact that different populations of both species are already present in the eastern USA (McAlpine & Tanasijtshuk, 1972) suggests that the *Leucopis* spp. from the PNW might also be able to tolerate conditions in the eastern USA throughout the year.

There is a growing body of evidence that *Leucopis* spp. have a high potential for impacting *A. tsugae* populations in their invaded range. *Leucopis* spp. are the only examples of successful biological control of adelgids worldwide and have been used effectively in Hawaii, New Zealand, and Chile (Rawlings, 1958; Francke-Grossman, 1963; Zúñiga, 1985; Culliney *et al.*, 1988; Zondag & Nuttall, 1989). A recent publication of data from the PNW demonstrates that *Leucopis* spp. larvae are more abundant and present for a much longer period of time than *L. nigrinus* larvae in their native ranges (Kohler *et al.*, 2016). The data reported here add to the evidence that *Leucopis* spp. warrant increased and continued study as potential biological control agents of *A. tsugae* in the eastern USA.

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