

Biological Control - Parasitoids and Predators

Biological Control of Hemlock Woolly Adelgid: Implications of Adult Emergence Patterns of Two *Leucopis* spp. (Diptera: Chamaemyiidae) and *Laricobius nigrinus* (Coleoptera: Derodontidae) Larval Drop

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Abstract

The hemlock woolly adelgid (Hemiptera: Adelgidae *Adelges tsugae* Annand) poses a serious threat to hemlocks in eastern North America, and ongoing research is focused on the identification and development of biological controls to protect and manage hemlock resources. Three predators native to the Pacific Northwest of North America that have been the focus of much research are *Leucopis argenticollis* (Zetterstedt), *Leucopis piniperda* (Malloch) (Diptera: Chamaemyiidae), and *Laricobius nigrinus* (Fender) (Coleoptera: Derodontidae). This study addresses the knowledge gap of adult *Leucopis* spp. emergence patterns, with comparisons to the timing of larval *La. nigrinus* drop for pupation. Adult *Leucopis* spp. emergence was observed in the lab from field-collected, adelgid-infested foliage from Washington state in 2019 and 2020. Adult *Leucopis* spp. were collected daily as they emerged from foliage collections and identified to species using morphological features; a subset was validated using DNA barcoding. Accumulated heating degree days were calculated to compare a standardized emergence timing across collections made at different locations and temperature regimes. The abundance of the two *Leucopis* spp. and of the combined *Leucopis* spp. and *La. nigrinus* varied among sites and years, and no species was consistently more abundant than the other. Evaluations of seasonal emergence trends of the three species determine the predator complex behaves in a temporally stratified and predictable way. Emergence of adult *Le. argenticollis* was observed first, followed by *La. nigrinus* larval drop, with *Le. piniperda* emerging at the end of larval drop, and finally a second emergence of *Le. argenticollis*.

Key words: biological control, community ecology, niche differentiation

The hemlock woolly adelgid, *Adelges tsugae* (Annand) (Hemiptera: Adelgidae), is an introduced insect that feeds on eastern hemlock (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*Tsuga caroliniana* Engelman) causing tree decline and mortality (McClure 1987). *A. tsugae* was first collected on the east coast of North America in Richmond, VA in 1951 (Stoetzel 2002) and was likely introduced sometime in the first half of the 20th century (Havill et al. 2014). Infestations are now present across a large portion of the eastern hemlocks range and can be found from Georgia to Maine, west to Michigan, and in Ontario and Nova Scotia (USFS

A. tsugae Distribution map, Morgantown Field Office). Molecular evidence suggests that the *A. tsugae* lineage introduced to the east coast is from southern Japan where it can be found on its secondary host *Tsuga sieboldii* (Havill et al. 2006). *A. tsugae* has caused widespread decline and mortality in eastern hemlock throughout the invaded range on the east coast since its arrival (Orwig et al. 2002). Eastern hemlock is a common tree on the landscape, for example, it represents the third most common tree species in New York State (Widmann et al. 2015). Eastern hemlock also serves as a foundation species by creating unique ecosystems that provide important

habitat for plants, vertebrates, and invertebrates. Infestation by the adelgid threatens eastern hemlock, and the species could be functionally removed (Ellison et al. 2005) in some landscapes. Effective management tools are critical to preventing the loss of native hemlocks and the ecological roles they provide in eastern North America.

A. tsugae has a complex, polymorphic life cycle that includes two parthenogenic generations per year; the sistens (overwintering) generation, and the progrediens (spring/summer) generation. The sistens generation hatches in the late spring, then undergoes a summer estivation period as a first instar before developing in the fall, growing through winter, and laying the eggs that will produce the progrediens in the late winter and early spring. Progrediens hatch in early spring as crawlers that settle among their mothers on the previous years foliage and develop into egg-laying adults in the late spring to produce sistens eggs. When the sistens hatch as first instar crawlers, they settle on the newest, or current years hemlock twig growth, and enter summer estivation (McClure 1989). The life cycle of *A. tsugae* also includes a winged morph (sexupara) that develops at the same time as the progrediens generation. In the native range of *A. tsugae* these sexupara fly to the primary host, *Picea torano* (tiger-tail spruce), where they initiate three additional generations that develop on the spruce host (Havill et al. 2014). In North America, there is no known suitable spruce host in the eastern United States (McClure 1989) and thus sexuparae represent a population sink.

Management of the hemlock woolly adelgid in eastern North America has focused on three approaches: the use of chemical insecticides, silvicultural methods, and the development of biological control agents. Chemical control through the use of systemic neonicotinoid insecticides (such as imidacloprid and dinotefuran) is one of the main management tools currently available to control *A. tsugae* in the introduced range, but due to the high cost, challenges with applying the chemicals in remote settings, and potential for nontarget effects, additional tools are needed for a long-term solution in a forest setting (Vose et al. 2013). Silvicultural techniques utilizing thinning to increase light intensity to control *A. tsugae* population growth and boost tree vigor have been an area of research (Fajvan 2008, Brantley et al. 2017), but more data are needed on widescale implementation and efficacy of these techniques. Biological control, using natural enemies from *A. tsugae* adelgid's native range, has been a research focus since the early 1990s (Onken and Reardon 2011). The Pacific Northwest of the United States and western Canada has a native lineage of *A. tsugae* found on western hemlock (*Tsuga heterophylla* Rafinesque) and mountain hemlock (*Tsuga mertensiana* (Bong.) Carr.) that is closely related to the Japanese lineage, and may have resulted from an ancient introduction (Havill et al. 2016). The life cycle of the *A. tsugae* in the West is broadly similar to the introduced eastern *A. tsugae* described above, including the absence of sexual reproduction on a primary host. In the West, predators of *A. tsugae* are abundant; of these *Leucopis argenticollis* (Zetterstedt), *Leucopis pimiperda* (Malloch), and *Laricobius nigrinus* (Fender) are the most common and the only known adelgid specialist, and show promise for biological control on the east coast (Zilahi-Balogh et al. 2002, 2003a, Kohler et al. 2008a).

The most studied candidate species for biological control of *A. tsugae* is *La. nigrinus*, a member of the tooth-necked fungus beetles (Coleoptera, Derodontidae). More than 400,000 *La. nigrinus* have been released throughout the eastern United States since 2003 (Jubb et al. 2020). They have become widely established in the eastern United States and have been shown to disperse beyond release locations (Mausel et al. 2010, Foley et al. 2019). The univoltine life cycle of *La. nigrinus* is synchronized with *A. tsugae* adelgid's overwintering generation; adult beetles feed throughout the winter on the sistens

nymphs and lay their eggs in adult *A. tsugae* ovisacs in the spring where the beetle larvae feed on the progrediens eggs (Zilahi-Balogh et al. 2003a,b). As *A. tsugae* progrediens eggs hatch, *La. nigrinus* larvae drop from the foliage of the hemlock to the soil where they pupate. Adult beetles eclose but remain in the soil during the summer when *A. tsugae* is in estivation, they emerge in the fall to feed on sistens nymphs (Zilahi-Balogh et al. 2003a,b; Wiggins et al. 2016). Feeding by *La. nigrinus* adults has been shown to reduce *A. tsugae* sistens density in the field (Mausel et al. 2017, Jubb et al. 2020), and beetle releases are often coupled with chemical treatments in an integrated pest management approach (Mayfield et al. 2015, 2020). However, *La. nigrinus* do not feed on the sistens eggs laid by *A. tsugae* progrediens adults in late spring/early summer, allowing this generation of the *A. tsugae* to escape predation. Population models indicate that, even with the removal of most of the adult sistens (upwards of 90%), *A. tsugae* populations may not be substantially reduced due to an increase in density dependent survival (Elkinton et al. 2011). The removal of *A. tsugae* through predation may decrease competition for settling space (McClure 1991, Sussky and Elkinton 2014), creating a compensatory effect in *A. tsugae* populations. Field experiments have provided support for this finding, with no significant difference in progrediens densities after 80% of sistens ovisac were fed on by *La. nigrinus* (Crandall et al. 2020). These findings highlight the need for a spring–summer predator that feeds on progrediens nymphs and sistens eggs to achieve a reduction in *A. tsugae* densities using biological control.

Two flies in the family Chamaemyiidae, known as silver flies, may offer paths to increase predation rates leading to effective reduction of *A. tsugae* populations in the introduced range. Silver flies are important predators of sternorrhynchous Hemiptera with larvae that feed on various stages of adelgids, scales, white flies, and mealy bugs (Gaimari 2010). *Leucopis argenticollis* and *Le. pimiperda* are holarctic species of silver flies with distinct lineages in eastern and western North America (Havill et al. 2016). In the West, both species feed preferentially on *A. tsugae* (Kohler et al. 2008a), with fly larvae most abundant during the egg-laying stages in both *A. tsugae* generations (Grubin et al. 2011). In many early studies, larvae from these two *Leucopis* spp. have been grouped together due to a lack of characters which distinguish the larvae morphologically, but molecular techniques have made it possible to identify immature silver flies to species (Havill et al. 2018, Rose et al. 2019). These two *Leucopis* spp. collectively represent the second most abundant group of *A. tsugae* predators observed in western North America based on beat sheet sampling, which favors the collection of *La. nigrinus* (Kohler et al. 2008a), and the most abundant group based on dissection of *A. tsugae*-infested foliage, which favors the collection of juvenile predators (Kohler et al. 2016, Rose et al. 2019). In eastern North America, these species feed on *Pineus strobi* Hartig, a native adelgid species found on eastern white pine (*Pinus strobus* L.) (Havill et al. 2018, Wantuch et al. 2019). The eastern and western lineages of *Le. argenticollis* and *Le. pimiperda* are genetically distinct and occur on separate hosts, *P. strobi* in eastern North America and *A. tsugae* in western North America (Havill et al. 2018).

Several lines of evidence support the potential of *Leucopis* spp. as an effective biocontrol agent for *A. tsugae* in the East. First, caged field releases of *Leucopis* spp. have shown successful reproduction on eastern hemlock infested with Japanese *A. tsugae* and indicate a positive correlation between *A. tsugae* abundance and predator reproduction in both species of *Leucopis* (Motley et al. 2017). Second, *Leucopis* spp. reared on western *A. tsugae* on western hemlock and Japanese *A. tsugae* on eastern hemlock showed no difference in survival, larval size, time to pupariation, time as puparia, or time

to adult, all suggesting there is no preference for *A. tsugae* lineage (Motley et al. 2017). Although the western *Le. argenticollis* and *Le. piniperda* can complete development on other species of adelgid in laboratory no-choice experiments, the average life span and survival to adult for these flies is greater when reared on *A. tsugae*, indicating greater suitability of *A. tsugae* as a host (Grubin et al. 2011). Also, adult *Leucopis* have been found to emerge in a sinusoidal pattern, with peak abundance oscillating between the two species over a 29-d study, providing some insight into how these predators coexist in the West (Neidermeier et al. 2020). In sum, these promising results of research on *Le. argenticollis* and *Le. piniperda* provide an incentive to continue exploring the potential of these species for biological control of *A. tsugae* in eastern North America.

Wild-caught immature life stages of *Leucopis* spp. collected in the western range may contain parasitoid species (Kohler et al. 2008b); making them unsuited for direct release in eastern landscapes. To prevent the introduction of parasitoids from western populations into eastern landscapes, larvae are reared to adult for release in the East. The presence of parasitoids makes understanding the dynamics of adult emergence critical to the success of implementing this group as a biological control tool for *A. tsugae* management. We observed the emergence patterns of adult *Le. argenticollis* and *Le. piniperda* from collections made at multiple sites in the Pacific Northwest of North America in 2019 and 2020. Adult emergence patterns have been observed in one season over a 29-d period (Neidermeier et al. 2020), but the goal of the present study was to better understand the emergence patterns over an entire release season when *A. tsugae* eggs are present on the east coast. Releasing at the appropriate developmental stage of the host species is essential to successful establishment of these predators. We selected sites throughout the Puget Sound region of Washington state where foliage infested with western *A. tsugae* could be collected for observation of predator emergence in 2019 and 2020. We quantified differences in the emergence distributions of *Le. argenticollis* and *Le. piniperda*, differences in their sex distributions, and the abundance of predators at each site. The length of our sampling season was designed to overlap with the periods of documented *A. tsugae* oviposition and the availability of *A. tsugae* eggs for predator feeding on the east and west coast. The long sampling period provided an understanding of the number of generations of *Leucopis* spp., their overlap with *La. nigrinus*, and the ideal times to release adults on the east coast.

Materials and Methods

Field Collections

Field collection sites were identified in the Puget Sound area of Washington state by visual survey for *A. tsugae*-infested western

hemlock trees in early to mid-February 2019 and 2020. Research sites were selected using three criteria: presence of dense *A. tsugae* populations, presence of *Leucopis* spp., and a rural and/or forested setting. Sites with rural and forested characteristics were chosen as a means of limiting the impacts of the urban heat island effect on insect development (Diamond et al. 2014). Point Defiance Park and Shannon Point Marine Center are near urban areas but were chosen for their 560 acres of forest (500 acres of old growth; Sutalo 2010), and 78 forested acres surrounding the station, respectively. Collections made throughout the Pacific Northwest in February of each year were used to determine the presence of predators, provide information for site selection, and served as the first sample of the season in 2019 and for new sites added in 2020. Samples were collected at multiple times from late winter through early spring (Table 1). The sites sampled in 2019 included two locations on Orcas Island (Lakshmi Road and Point Lawrence Road) and one location on Fidalgo Island at the Western Washington University Shannon Point Marine Center. Sites sampled in 2020 were Point Defiance Park in Tacoma, WA, the Shannon Point Marine Center, and the Lakshmi Road, Orcas Island site. In 2019, each location was sampled six times between 20 February and 31 July. In 2020 four collections were made at Point Defiance and Shannon Point and two collections were made at Lakshmi Road in 2020, between 13 February through 29 May and 14 February through 15 March, respectively. Only two collections were possible at Lakshmi Road in 2020 due to the collapse of the *A. tsugae* population. See Table 1 for site collection dates. A temperature data logger (Onset HOBO U23 Pro V2, Bourne, MA) was placed in the shade on the bole of one collection tree at all collection sites in both years, logging every 15 min.

Collections were made by clipping *T. heterophylla* branches from the lower and mid-canopies (between 1 and 7 m high) of trees heavily infested with *A. tsugae*, using a 5-m pole pruner. Infested branches were double-bagged and sealed using plastic contractor bags (42-gallon, 3 mil) and placed into shipping boxes with all seams sealed. The sealed boxes of infested western hemlock foliage were then shipped overnight to the Sarkaria Arthropod Research Laboratory (SARL) quarantine greenhouse, in Ithaca, NY, for observation (USDA APHIS permit P526P-18-00945). Infested foliage was placed inside custom fabricated acrylic cages (Leigh-Dale Specialties, Syracuse, NY) with 120- μ mesh (Component Supply Co., Sparta, TN) to allow ventilation and prevent the escape of western *A. tsugae* crawlers and other insects. Infested hemlock branches were inserted in saturated floral foam in plastic shoeboxes (Sterilite, 35.6 cm \times 20.3 cm \times 12.4 cm, Townsend, MA) to keep branches hydrated. Cages were checked daily in the afternoon for insect emergence. Adult *Le. argenticollis*, *Le. piniperda*, and dropped larvae of *La. nigrinus* were collected using an aspirator (1135A Aspirator, BioQuip Products, Rancho Domingues, CA). Effort was made to

Table 1. Site collection dates and localities, 2019 and 2020

Site	Collection period	Collection dates	Locality
2019			
Lakshmi	20 Feb. to 30 July	20 Feb., 27 Mar., 29 April, 10 June, 16 July ^a , 30 July ^a	Olga, Orcas Island, WA
Point Lawrence	21 Feb. to 30 July	21 Feb., 27 Mar., 30 April, 10 June, 16 July ^a , 30 July ^a	Olga, Orcas Island, WA
Shannon Point	21 Feb. to 31 July	21 Feb., 24 Mar., 1 May, 11 June, 18 July ^a , 31 July ^a	Anacortes, Fidalgo Island, WA
2020			
Lakshmi	16 Feb. to 15 Mar.	16 Feb., 15 Mar.	Olga, Orcas Island, WA
Shannon Point	17 Feb. to 27 May	17 Feb., 20 Mar., 1 May, 27 May	Anacortes, Fidalgo Island, WA
Point Defiance	13 Feb. to 28 May	13 Feb., 19 Mar., 29 April, 28 May	Tacoma, WA

^aNo predator emergence from these collection dates.

collect all *La. nigrinus* larvae, though a subset of the larvae could have been lost in the water surrounding the saturated floral foam. *Leucopis* spp. were frozen for identification and *La. nigrinus* were collected for rearing to adult (Salom et al. 2012) for future release. The greenhouse had natural lighting and was temperature controlled (55–65°C). A temperature data logger was placed in the quarantine facility to record rearing temperatures. Temperatures were logged every 15 min (Onset HOBO MX1101 Data Logger, Bourne, MA).

Leucopis spp. Identification

Each adult fly was identified to species under a dissecting microscope by observing postpronotal setae, *Le. argenticollis* have three setae present on the postpronotum that are absent in *Le. piniperda* (S. D. Gaimari, personal communication) (see [Supp Fig. S1](#) [online only]). Male genitalia were also used in identification; *Le. piniperda* have a long curved aedeagus that is often protruding surrounded by a smooth hypandrium, and *Le. argenticollis* are characterized by a barbed surstylar lobe and rounded aedeagus (McAlpine and Tanasijtshuk 1972, Tanasijtshuk 2002) (see [Supp Fig. S2](#) [online only]). A subset of 92 flies identified using these morphological criteria were confirmed through DNA barcoding following procedures introduced in Havill et al. (2018). DNA was extracted using the Mag-Bind Blood & Tissue kit (Omega Bio-Tek, Norcross, GA). Adult flies were incubated whole with proteinase K (55°C) for at least 12 h, allowing the internal tissues to digest while leaving the chitinous cuticle intact. Fly cuticles were removed as vouchers and stored in 95% ethanol. Vouchers were deposited in the Cornell University Insect Collection, accession number 1285. Endpoint PCR for the barcoding region of the mitochondrial COI gene was performed following Hebert et al. (2004) and Havill et al. (2018) and Sanger Sequencing was performed by the Biotechnology Resource Center (BRC) Genomics Facility at the Cornell Institute of Biotechnology (<http://www.biotech.cornell.edu/brc/genomics-facility>).

Heating Degree Day Accumulation

The accumulated number of heating degree day (HDD) was calculated at the time of fly emergence using the modified sine wave method (Allen 1976) on local climate data collected from each field site and from the SARL quarantine facility (Fig. 1). Daily HDD values and accumulated HDD over the field season were calculated in Matlab version 9.7.0.1261785 (R2019b) Update 3, script provided by R. Talbot Trotter, III and Diego Gabriel Huerta. Previous studies by Salom et al. (2002) and Tobin and Turcotte (2018) identified a base threshold temperature of 4°C for prolegens development. No phenological analyses have been conducted to identify a base temperature suitable for development of *Leucopis* spp. In the absence of these data and based on the close ecological association between the *Leucopis* spp. and *A. tsugae*, this temperature was used as a base temperature for calculating HDD accumulation. It is worth noting that as long as both environmental temperature ranges remain above the base temperature, differences among calculations based on different base temperatures will be linear across a range of base temperatures. HDD accumulation is being used as a unit of comparison in this study and not as a predictor of development. Regardless of the base temperature used the patterns will be identical, only causing a change in HDD accumulation rate (Fig. 1). The date of the first foliage collection for each site was used as a starting point at which the accumulation of HDD was calculated for both the laboratory and field populations, as this is the point at which laboratory and field population development would diverge based on temperature-driven changes in phenological development. For the

HDD calculation at the time of emergence in the quarantine facility, the accumulated HDDs were added to the HDD accumulated in the field up to the time of collection. Field local climate data were used to calculate accumulated HDD at each field site which supplied the accumulated HDD at each collection date. *Leucopis* spp. collections were evaluated using HDD accumulation at the time of adult emergence, and *La. nigrinus* at the time of larval drop. All subsequent statistical tests on emergence distributions were based on HDD at time of emergence.

Statistical Analysis

Leucopis spp. emergence trends did not follow a normal distribution, based on the Shapiro–Wilks test for normality; therefore, a two-sided, nonparametric Kolmogorov–Smirnov test (Conover 1971) was performed to evaluate the emergence distributions of *Le. argenticollis* and *Le. piniperda*, and the emergence distributions of the sexes of each species. A chi-square test was used to compare relative species abundance by site, because it was impossible to assure that collecting effort was comparable across all sites. Chi-square tests were also used to evaluate the relative abundance of *Leucopis* spp. and *La. nigrinus* larval drop by site. All sites were evaluated independently. Data analysis was performed in R studio version 4.0.2 (R Core Team 2020). Plots were made using the ggplot2 package (Wickham 2016).

Results

Leucopis spp. Identification and Predator Abundance

In total, 4,029 adult *Leucopis* spp. were collected, 954 in 2019 and 3,075 in 2020. DNA barcoding of 92 flies in 2019 (75 *Le. argenticollis* and 17 *Le. piniperda*) all matched morphological identifications using postpronotal setae and male genitalia. Because identification using morphology was rapid and accurate, the remainder of the samples was identified morphologically.

Flies collected in 2019 included 541 *Le. argenticollis* and 413 *Le. piniperda*. In 2020 there were 2,169 *Le. argenticollis* and 1,860 *Le. piniperda*. The relative abundance of *Leucopis* spp. differed significantly by site in both 2019 and 2020 (Table 2). A higher abundance of *Le. piniperda* were collected at Lakshmi and Point Lawrence, but a higher abundance of *Le. argenticollis* at Shannon Point Marine Center in 2019. In 2020 there was a higher abundance of *Le. argenticollis* at Point Defiance and Shannon Point, and only *Le. piniperda* was present at Lakshmi (Table 2).

Predator collections from the 2019 study sites included a total of 954 *Leucopis* spp. and 2,214 *La. nigrinus* larvae. The relative abundance of *Leucopis* spp. and *La. nigrinus* prepupae differed significantly between sites in 2019 (Table 3). At the Lakshmi Road site there was a higher abundance of *Leucopis* spp. than *La. nigrinus* while the reverse was observed at Shannon Point, and Point Lawrence. Collected predators in 2020 totaled 3,075 *Leucopis* spp. and 1,580 *La. nigrinus* larvae. A statistically significant difference was found in the relative abundance of *Leucopis* spp. and *La. nigrinus* between sites in 2020 (Table 3). *Leucopis* spp. adults were more abundant at Point Defiance and Lakshmi than *La. nigrinus* larvae. At Shannon Point *La. nigrinus* had a higher relative abundance than *Leucopis* spp. (Table 2).

Temporal Distributions of *Leucopis* spp. Emergence

Emergence distributions differed significantly between *Le. piniperda* and *Le. argenticollis*, $\alpha = 0.01$, indicating that there is temporal separation in the two species emergence patterns. In 2019 at Lakshmi ($D = 0.9325$, $P = <0.001$), Point Lawrence ($D = 0.9286$,

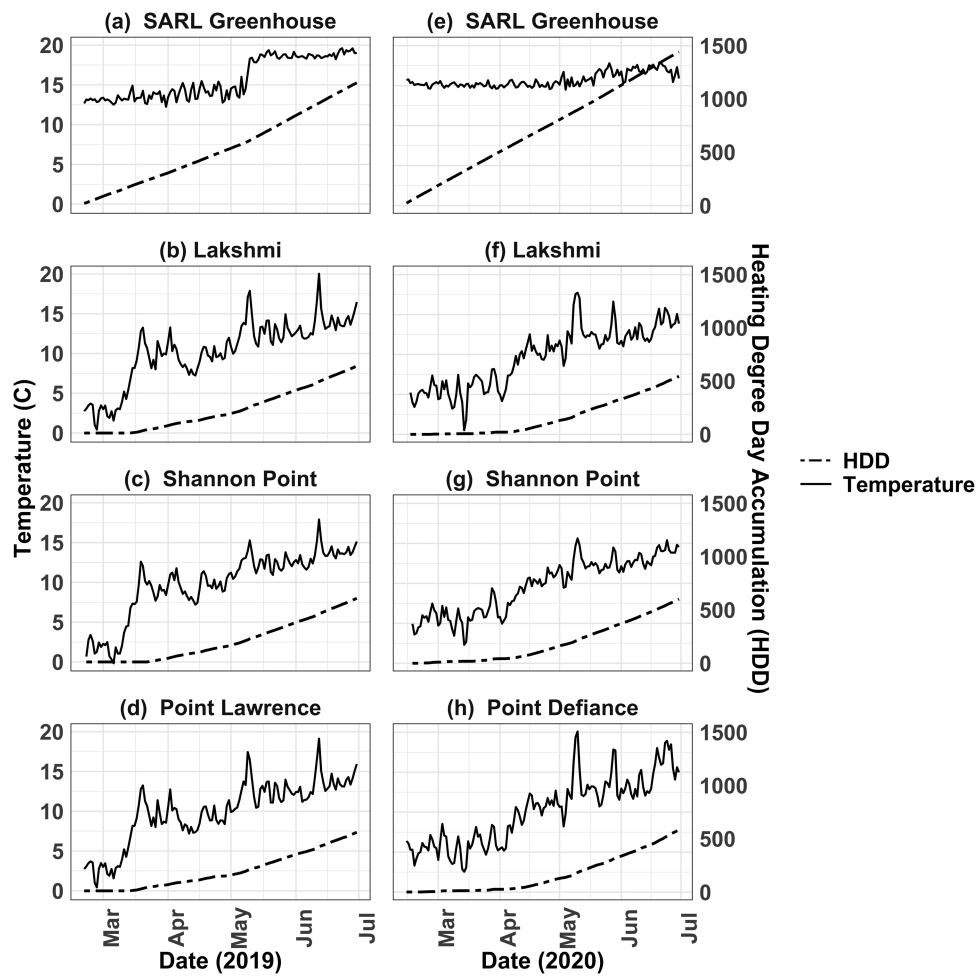


Fig. 1. Comparison of temperature and HDD accumulation between field sites and the SARL greenhouse in 2019 (a-d) and 2020 (e-h).

Table 2. Chi-square results and abundance of adult *Le. argenticollis* and *Le. piniperda*, 2019 and 2020

Collection site	<i>Leucopis</i> spp.	<i>Le. argenticollis</i>	<i>Le. piniperda</i>	Percent (%) <i>Le. argenticollis</i>	Percent (%) <i>Le. piniperda</i>	Test statistics and P-value ($\alpha = 0.01$)
2019						
Lakshmi	560	241	319	43.04	56.96	$\chi^2 = 314.05$, df = 2, $P = <0.001$
Point Lawrence	100	14	86	14.0	86.0	
Shannon Point	294	286	8	97.28	2.72	
2020						
Lakshmi	18	0	18	0.0	100.0	$\chi^2 = 112.88$, df = 2, $P = <0.001$
Point Defiance	2,948	1,521	1,427	51.59	48.41	
Shannon Point	109	107	2	98.17	1.83	

$P = <0.001$), and Shannon Point ($D = 0.7177$, $P = <0.001$) *Leucopis* spp. emergence distributions were significantly different. In 2020 at Point Defiance ($D = 0.3824$, $P = <0.001$) emergence distributions were significantly different. The only exception to this pattern was in 2020 at Shannon Point where there was not a significant difference in emergence distributions ($D = 0.4439$, $P = 0.444$). Lakshmi 2020 was left out of distribution analysis because *Le. argenticollis* was not present.

Distributions of male and female *Leucopis* spp. were evaluated by species at each site to see if there was a difference in the timing and pattern of emergence between the sexes. The patterns of male and female emergence did not differ for either species at any of the sites in both 2019 and 2020, $\alpha = 0.01$. Statistics were as follows:

in 2019 Lakshmi (*Le. argenticollis* $D = 0.0925$, $P = 0.691$; *Le. piniperda* $D = 0.0952$, $P = 0.4696$), Point Lawrence (*Le. argenticollis* $D = 0.2917$, $P = 0.932$; *Le. piniperda* $D = 0.0761$, $P = 0.999$), and Shannon Point (*Le. argenticollis* $D = 0.2458$, $P = 0.2458$; *Le. piniperda* $D = 0.2$, $P = 0.9999$); and in 2020, Point Defiance (*Le. argenticollis* $D = 0.0747$, $P = 0.03$; *Le. piniperda* $D = 0.0445$, $P = 0.480$), and Shannon Point (*Le. argenticollis* $D = 0.1103$, $P = 0.902$; *Le. piniperda* $D = 0$, $P = 0.1$).

Seasonal Emergence Patterns

Adult *Leucopis* spp. emerged from infested foliage collected from the field in mid- to late February through June in both years. The first date of adult emergence was recorded on 3rd March in 2019 and 24th

Table 3. Chi-square results of species abundance of *Leucopis* spp. and *La. nigrinus*, 2019 and 2020

Collection Site	All predators	<i>Leucopis</i> spp. adults	<i>La. nigrinus</i> larvae	Percent (%) <i>Leucopis</i> spp.	Percent (%) <i>La. nigrinus</i>	Test statistics and P-value ($\alpha = 0.01$)
2019						
Lakshmi	763	560	203	73.39	26.61	$\chi^2 = 942.28$, df = 2, P = <0.001
Point Lawrence	298	100	198	13.95	86.05	
Shannon Point	2,107	294	1,813	33.56	66.44	
2020						
Lakshmi	19	18	1	94.74	5.26	$\chi^2 = 2,047.8$, df = 2, P = <0.001
Point Defiance	3,529	2,948	581	83.54	16.46	
Shannon Point	1,107	109	998	9.85	90.15	

February in 2020. Collections were made from late February through late July in 2019, with no emergence in the last two collections (Table 3), indicating the sampling period captured the end of the emergence season. In 2020 collections were made from mid-February through late May at Shannon Point and Point Defiance, with emergence in all four collections. Only two collections were made at Lakshmi in 2020, due to rapidly decreasing *A. tsugae* populations, with emergence observed in both 2020 collections (Table 3).

Quarantine lab temperatures were different than field temperatures (Fig. 1); therefore, emergence time was evaluated using HDD accumulation (Fig. 1) at time of emergence based on the accumulation of HDD in the lab and field environments experienced by the insects. This approach provided a means of standardization for comparison across the collection periods. Emergence patterns followed a similar pattern between collection years and sites, with *Le. argenticollis* adults emerging first, then *La. nigrinus* larvae dropping from the foliage, followed by an emergence of *Le. piniperda* adults, and finally a second emergence of *Le. argenticollis* adults (Fig. 2).

Discussion

Leucopis spp. Identification and Predator Abundance

Identification of *Le. argenticollis* and *Le. piniperda* to species has been a major challenge in working with this group and in early studies led to the need for rearing immatures or grouping species (Kohler et al. 2008a, 2016; Grubin et al. 2011), and requiring DNA barcoding in later studies (Motley et al. 2017, Rose et al. 2019, Neidermeier et al. 2020). The present study used DNA barcoding evidence to demonstrate the reliable identification of *Leucopis* spp. adults using morphological features, i.e., postpronotal setae and male genitalia. The identification of adult flies using morphological features is faster and more cost-effective than molecular techniques and enables efficient separation of the species for both monitoring and release efforts. Male genitalia can be observed on living flies under magnification and postpronotal setae can be observed on chilled flies using magnification. Prior to the present study the preferred method to identify *Leucopis* spp. adults was through destructive sampling using DNA barcoding (Havill et al. 2018, Rose et al. 2019, Neidermeier et al. 2020). Understanding that each species emerges independently, with little overlap, allows for destructive identification of just a few individuals, which then can be extrapolated to the entire emergence period (Fig. 2). However, this approach does not offer a solution to the difficulty of distinguishing between immatures of each species, and for these, molecular techniques are still needed.

Previous studies of adult emergence for *Leucopis* spp. in the Pacific Northwest have reported conflicting results regarding the

abundance of the two species; some studies found *Le. argenticollis* to be more abundant (Kohler et al. 2008a, Grubin et al. 2011, Rose et al. 2019), whereas a recent study reported higher abundance of *Le. piniperda* (Neidermeier et al. 2020). In both 2019 and 2020 we observed statistically significant differences in the proportional abundance of the two fly species across the sites. *Leucopis argenticollis* was more abundant at Shannon Point (2019: 97.28%, 2020: 98.17%) and Point Defiance (51.59%), whereas *Le. piniperda* was more abundant at Lakshmi (2019: 56.96%, 2020: 100%) and Point Lawrence (86.0%). These diverse findings from earlier studies and between our sites in this study indicate that differences in abundance between the two *Leucopis* spp. are variable by site and year, and that there is not a consistent pattern of abundance across space or time. Previous studies of *Leucopis* spp. adults have found a significant difference in *Le. piniperda* abundance by site but not with *Le. argenticollis* (Neidermeier et al. 2020). It has also been reported that *Leucopis* spp. are sometimes more abundant than *La. nigrinus* (Kohler et al. 2016, Rose et al. 2019) while the reverse can also be true (Kohler et al. 2008a), through branch dissection and beat sampling, respectively. While the relative abundance of *La. nigrinus* at each site in this study has potential to be an underestimate due to sampling limitations, this does offer a biologically significant comparison since all samples were treated identically. Thus, differences in abundance between these predator groups vary by site and year, and it is unclear what drives this variation. This remains a critical issue, as *Leucopis* spp. and *La. nigrinus* are the two most abundant groups of specialist *A. tsugae* predators in the West (Kohler et al. 2008a), and therefore show the most promise for managing *A. tsugae* in the East. Determining the conditions that favor one species or another is likely key to understanding how *A. tsugae* is regulated under natural conditions in the Pacific Northwest, and understanding the conditions under which biological control may be most effective in eastern North America. Community dynamics of dispersal and niche differentiation are influenced by many factors including habitat heterogeneity, spatial scale, and dispersal type (Cottenie 2005) and could explain differences in predator species composition. Diverse findings in predator abundance in this study and previous work (Kohler et al. 2008a, Grubin et al. 2011, Rose et al. 2019, Neidermeier et al. 2020) collectively provide insight into the predator dynamics of the system over time. Lakshmi offers a vivid example of predator population fluctuation between years, with all three species present in abundance in 2019 (*Le. argenticollis*: $n = 241$, *Le. piniperda*: $n = 319$, *La. nigrinus*: $n = 203$), and only a small number of two species present in 2020 (*Le. piniperda*: $n = 18$, *La. nigrinus*: $n = 1$). This site provides an indication of how predator composition can change as *A. tsugae* populations decline, and the potential of *Leucopis* spp. to be drivers of the community dynamics. These data highlight the importance of understanding the community ecology of predator complexes, for the purposes implementing biological control.

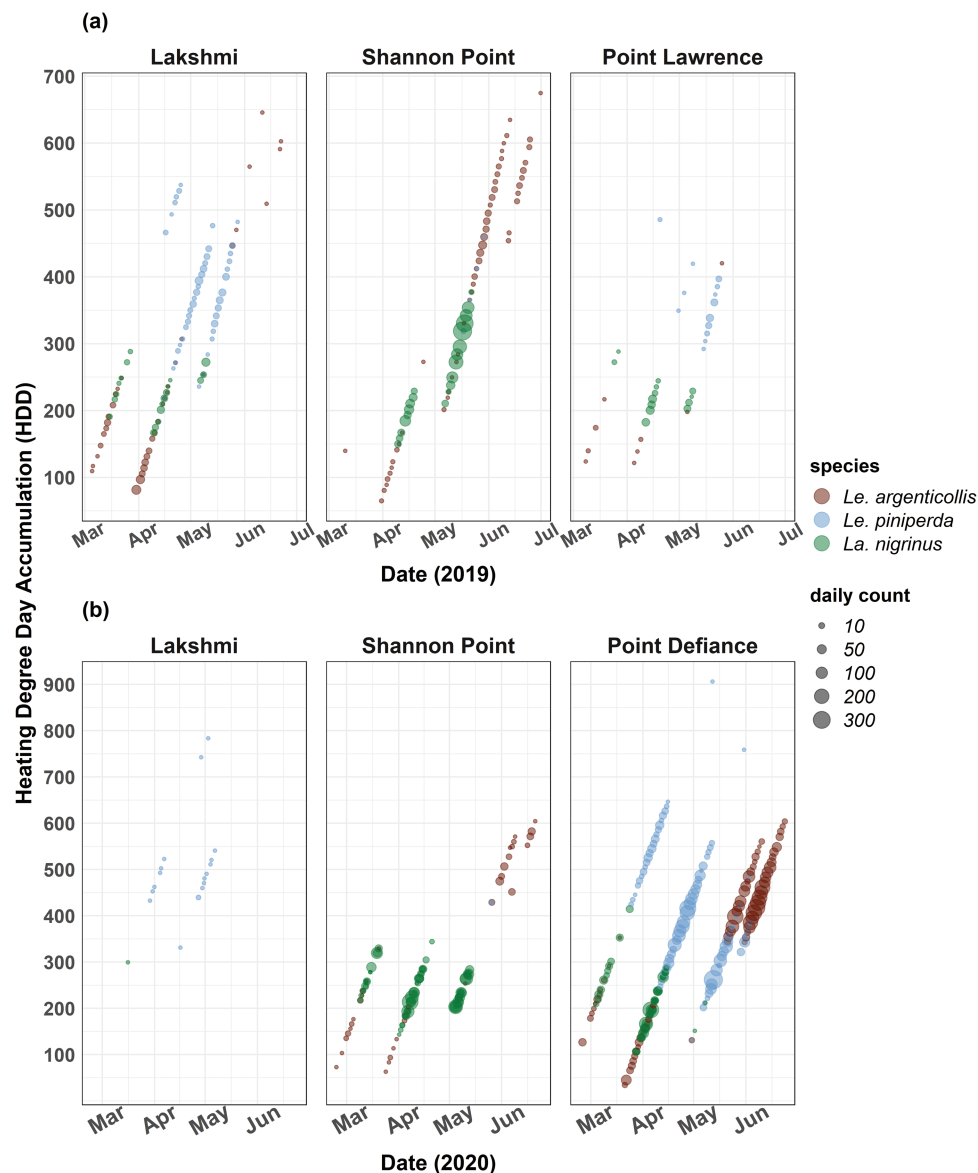


Fig. 2. Emergence trends of *Le. argenticollis* and *Le. piniperda* adults and *La. nigrinus* larval drop by HDD and date in 2019 (a) and 2020 (b).

Temporal Distribution of *Leucopis* spp. Emergence

Adult *Leucopis* spp. emergence distributions for the two species were significantly different at all sites, except for Shannon Point in 2020. However, patterns of emergence for Shannon Point in 2020 are based on a small sample size of *Le. piniperda*, with the only two individuals emerging on the same day and overlapping with *Le. argenticollis*. Our findings that adult emergence distributions differ between the species confirm the sinusoidal pattern of emergence between species reported by Neidermeier et al. (2020). Emergence distributions were not analyzed at the Lakshmi site in 2020, due to the presence of only *Le. piniperda*.

Emergence distributions of males and females were evaluated to determine if the sexes of each *Leucopis* spp. emerge in tandem. In all instances emergence distributions for each species did not differ significantly by sex. Therefore, in practice, if living males are identified to species using genitalia, then females collected at the same time and location can likely be assumed to be the same species, provided only one species of male is detected to avoid the brief times of population overlap that we observed. In addition,

this indicates each species emerges with Fisherian sex ratios (Fisher 1930) and time-consuming attention to sex ratio may not be an important consideration when preparing for releases. Since adult flies are fragile, reducing handling time in preparing releases is an important step to reduce prerelease mortality. Understanding that species emergence distributions are different and emergence by sex does not differ allows greatly reduced handling to lower mortality when preparing field releases.

Seasonal Emergence Patterns

HDD accumulation is a common way to measure physiological time for insects and to track insect development (Salom et al. 2002, Keena and Moore 2010, Limbu et al. 2015). Understanding the relationship of HDD accumulation to developmental stages of organisms allows for the development of phenological models to predict insect activity (Trotter and Keena 2016, Tobin and Turcotte 2018, Crimmins et al. 2020). While this study does not attempt to develop a phenological model or life history tables, it does provide a preliminary way to estimate predator emergence timing using HDD as a developmental

metric. Accumulated HDD at adult emergence also allows direct comparison of emergence patterns across collection periods. This is useful when patterns of predator emergence are key, and field-collected samples must be moved into a controlled environment for observation or monitoring for adult emergence.

In addition to *Leucopis* spp., accumulated HDD were also calculated for *La. nigrinus* larval drop. Larvae of *La. nigrinus* feed almost exclusively on progrediens eggs prior to dropping to the soil to pupate, where they will estivate until fall (Zilahi-Balogh et al. 2003a,b). Collection of infested hemlock foliage for laboratory observation is an effective way to monitor for prepupal larvae as they drop from the foliage (Mausel et al. 2010, Jubb et al. 2020). Observation of *La. nigrinus* provide information on *A. tsugae* development due to their univoltine life cycle and documented synchrony with *A. tsugae*, which reinforces assumptions made using HDD accumulation since prepupal larval drop only happens during the progrediens egg stage. Thus, data on *La. nigrinus* larval drop allow for inference on number of generations for each species of *Leucopis*, based on the number of adult *Leucopis* spp. emergence peaks observed in relationship to larval drop.

Our observations of the predator community over time indicate it is temporally stratified in a predictable way among the three focal species. A clear sequence of predator emergence was observed at all collection sites in both 2019 and 2020. Adult *Le. argenticollis* were the first to emerge; this result is contrary to what has been reported in a prior study that suggested *Le. piniperda* is the first to emerge (Neidermeier et al. 2020). However, our sampling season began earlier and Neidermeier et al. (2020) could have missed the first emergence of *Le. argenticollis* in that study. In the present study *La. nigrinus* began dropping from the foliage toward the end of the first *Le. argenticollis* emergence period and continued dropping until *Le. piniperda* adults began to emerge. *Leucopis piniperda* only had one emergence period at all sites in both years, which always began near the end of *La. nigrinus* larval drop. As daily emergence of *Le. piniperda* began to decrease there was a second emergence of *Le. argenticollis* (Fig. 2).

The number of emergence periods observed in relation to HDD accumulation may indicate the number of generations each species has per year. We found two distinct emergence periods of *Le. argenticollis*, one prior to *La. nigrinus* larval drop and one after, which suggests two generations per year. Alternatively, they could be using a strategy of stratified emergence or multiple emergence periods, to increase the chances of eclosing in favorable conditions (Tamaru et al. 1999). Previous research found two emergence peaks of each species occurring in a 29-d period (Neidermeier et al. 2020) but did not take temperature differences between the field and laboratory into consideration. We found *Le. piniperda* displayed only one distinct emergence period per season, indicating there may only be one generation per year. While our study provided a season-long look at adult emergence adjusted for temperature, it did not consider *Leucopis* spp. immatures, which would provide a more complete picture of the number of generations and phenological differences between the two species. Larval *Leucopis* spp. while present throughout the year (Grubin et al. 2011) have higher abundance in two peaks during *A. tsugae* egg-laying stages (Kohler et al. 2016, Rose et al. 2019). Rose et al. (2019) was the first study to identify immature *Leucopis* to species and reported no difference in phenology between species. Even though immatures of both species of *Leucopis* were found to overlap in earlier studies, sampling began later in the season (April 3) and could have missed earlier occurrences. A longitudinal study that includes all life stages of *Leucopis* spp. is needed

to fully understand the phenological interactions and voltinism of *Leucopis* species.

Implications for Biological Control

Detailed knowledge of *La. nigrinus* biology and its predictable overlap with *A. tsugae* phenology indicates the best timing for each *Leucopis* spp. release. The univoltine life cycle of *La. nigrinus* is well documented to be in synchrony with the *A. tsugae* sistens generation (Zilahi-Balogh et al. 2003a,b), with predictability that enables sampling larval drop during the sistens egg-laying stage (Mausel et al. 2010, Mayfield et al. 2015), and the development of predictive models to guide field sampling efforts (Virginia Tech 2020). The relationship between *La. nigrinus* larval drop and *Leucopis* spp. adult emergence will enable land managers to track *A. tsugae* and *La. nigrinus* development in the field to better time *Leucopis* spp. releases. The phenological synchrony of *La. nigrinus* larvae, *Leucopis* spp. larvae, and *A. tsugae* has shown that *La. nigrinus* larvae are most abundant during the sistens egg-laying stage, and *Leucopis* spp. are most abundant during the egg-laying stage of both *A. tsugae* generations (Kohler et al. 2016, Rose et al. 2019). Our data indicate that *Le. argenticollis* adults emerge both before and after *La. nigrinus* larval drop, suggesting that *Le. argenticollis* could have flexibility and be released during either *A. tsugae* generation. However, we do not yet understand the differences between the two emergence peaks; therefore, optimal release of *Le. argenticollis* requires further study, especially given the wide range of *A. tsugae* phenology on the east coast. Emergence of *Le. piniperda* takes place after *La. nigrinus* larval drop, providing evidence that this species should be released during the progrediens generation (Fig. 2).

The variability in *A. tsugae* phenology on the east coast could make matching predator phenology from the west coast difficult. It is still unknown how early in the spring *Leucopis* spp. collections could begin on the west coast. We were able to successfully collect in mid- to late February in 2019 and 2020, representing the earliest reported collection date of *Leucopis* spp. leading to adult emergence. *A. tsugae* phenology varies throughout its range from northern Georgia to Nova Scotia and is generally different from that in the Pacific Northwest. Emergence in the lab occurred for an extended period of time based on our field collections in 2019 and 2020 (Fig. 2). The ability to collect predators at multiple times in winter and spring provides an opportunity to release and research *Leucopis* spp. throughout *A. tsugae* adelgid's introduced range. Sistens adults of *A. tsugae* lay eggs from early February to mid-May (peaking in mid-March) in northern Georgia (Joseph et al. 2011); from mid-February to mid-May (peak the third week in March) in Virginia (Gray and Salom 1996, Mausel et al. 2008); from mid-February to early June (peak in early April) in Connecticut (McClure 1987); and in February through June in the Pacific Northwest (Zilahi-Balogh et al. 2003a). Progrediens adults were laying eggs from mid-May to the first week in June (peak in late May/early June) in Georgia (Joseph et al. 2011); early June to early July in Virginia (Gray and Salom 1996, Mausel et al. 2008); and late May to late June in Connecticut (McClure 1987). In New York the onset of sistens adult egg laying has been reported to begin on 2nd April in 2018, 11th April in 2019, and 11th March in 2020, with progrediens crawlers hatching on 5th May in 2018, 1st May in 2019, and 17th May in 2020 (USA National Phenology Network 2021). These findings demonstrate year-to-year variation in key *A. tsugae* phenological stages important to the life cycle of these

predators. Collecting predators from a single environment has the potential to create challenges with matching the phenology of the predator in the source environment, with the phenology necessary for successful establishment in landscapes with different seasonality. However, our study offers another demonstration that *Leucopis* spp. can be collected in the Pacific Northwest and reared to adult in the lab (Motley et al. 2017, Neidermeier et al. 2020) and provides information on emergence timing and voltinism critical to success with a prey that has a wide introduced range and varying phenology.

Leucopis spp. show great potential for *A. tsugae* biological control in eastern North America and this study provides guidance about release timing necessary for effective establishment of these species. Mass-rearing protocols for *Leucopis* spp. in the laboratory have yet to be developed; therefore, wild collection is the only available method of obtaining these predators for release. We have demonstrated that an understanding of *Leucopis* spp. emergence trends in their western range is important to implementing *A. tsugae* biological control in the East.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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