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Biological Control - Parasitoids and Predators

Phenology of *Leucotaraxis argenticollis*, a specialist predator of the invasive hemlock woolly adelgid, in the eastern United States

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In hemlock stands within eastern US forests, classical biological control has been one of the main strategies used to manage the hemlock woolly adelgid, *Adelges tsugae* Annand. Specialist predator species may offer a management solution to help regulate *A. tsugae* populations. In the Pacific Northwest, a suite of specialist predators has been a focus of research and includes 2 species of silver fly, *Leucotaraxis argenticollis* (Zetterstedt) and *Leucotaraxis piniperda* (Malloch) (Diptera: Chamaemyiidae). *Leucotaraxis* spp. phenology has been documented in the Pacific Northwest, but the phenology of either western *Leucotaraxis* species is unknown in the eastern United States. This study sought to document the phenology of *Le. argenticollis* in NY in 2021 and in VA in 2021 and 2022. Nylon mesh cages were applied over eastern hemlock branches infested with *A. tsugae* to contain *Le. argenticollis* adults. Biweekly and monthly branch samples were taken in 2021 and 2022, documenting all life stages of *A. tsugae* and of *Le. argenticollis* that were observed. In 2021 and 2022, *Le. argenticollis* adults and eggs were present during the oviposition stage of the 2 generations of *A. tsugae*. In addition, *Le. argenticollis* larvae were present when *A. tsugae* ovisacs had eggs and while *A. tsugae* nymphs of both generations were present. These observations indicate that *Le. argenticollis* phenology is well synchronized with *A. tsugae* in the eastern United States.

Key words: biological control, *Leucotaraxis*, phenology, hemlock woolly adelgid, silver fly

Introduction

The hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), is an invasive species in eastern North America and threatens the existence of eastern hemlock, *Tsuga canadensis* (L.) Carrière (Pinales: Pinaceae) and Carolina hemlock, *Tsuga caroliniana* Engelm. (Pinales: Pinaceae) (Havill et al. 2014). It was first described by Annand in 1924 and was first collected in the eastern United States in Richmond, VA in 1951 (Stoetzel et al. 2002). *Adelges tsugae* is a small aphid like insect with a polymorphic life cycle (McClure 1989). The life cycle of *A. tsugae* in the eastern United States consists of the sistens, the progrediens, and sexupara (McClure 1989). Each generation goes through the following life stages: egg, 1st instar crawler, 2nd–4th instar nymphs, and adult. Sistens produce progrediens and occasionally sexupara, and progrediens produce the sistens. The sistens generations are present from July through April and progrediens are present from April through June, but phenology of *A. tsugae* varies depending on its geographic location (McClure 1989, Mausel et al. 2008, Joseph et al. 2011, Tobin and Turcotte 2018). Both sistens and progrediens remain on hemlock, reproducing parthenogenically, requiring only 1 individual to survive and infest a hemlock (Tobin et al. 2013). Whereas the sexupara fly away to seek out its primary host the tigertail spruce, *Picea torano* (K. Koch) Koehne (Pinales: Pinaceae), where sexual reproduction occurs (Havill et al. 2006). However, since no known suitable spruce host species are present in the eastern United States, sexupara represent a population sink for the species (McClure 1987, 1989).

In its invasive range, *A. tsugae* lacks specialized predators, consisting of only generalist predators that are unable to regulate *A. tsugae* populations (Wallace and Hain 2000, Onken and

Reardon 2011). Therefore, other strategies such as chemical control and classical biological control have become the main methods used for management. Imidacloprid, a widely used insecticide for *A. tsugae* management, is used mostly in urban settings or high valued targeted areas (Cowels et al. 2006, Benton et al. 2015). However, it is only a short-term treatment that is not practical on its own, and poses challenges in the forest setting, due to labor costs, access challenges, and nontarget effects (Dilling et al. 2009, Falcone and DeWald 2010, Kung et al. 2015). There are no known parasitoids of *A. tsugae* and other species in the family Adelgidae (Onken and Reardon 2011). Therefore, classical biological control has focused on specialist predators of *A. tsugae*. Since 2018, 8 predatory species have been released in the classical biological program of *A. tsugae* (Limbu et al. 2018). One species of beetle, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), native to northwestern North America (Zilahi-Balogh et al. 2002), was first released in 2003 and has established throughout the eastern United States (Mausel et al. 2010, Jubb et al. 2021, Crandall et al. 2023). Jubb et al. (2020) determined that *La. nigrinus* predation impacts the *A. tsugae* sistens generation, and Preston et al. (2023) showed that *La. nigrinus* predation can improve the health of hemlock trees in the short-term. However, due to high reproductive potential of the spring-summer progrediens generation, Crandall et al. (2020) showed that without additional predator pressure, *A. tsugae* populations rebound. This suggests additional predators are needed to target the progrediens generation.

In the Pacific Northwest, 2 species of predatory silver fly *Leucotaraxis argenticollis* (Zetterstedt) and *Leucotaraxis piniperda* (Malloch) (Diptera: Chamaemyiidae) (Gaimari and Havill 2021) were found in high abundance specializing on *A. tsugae* (Kohler et al. 2008, 2016, Grubin et al. 2011, Dietschler et al. 2021). Both are Holarctic species with genetically distinct lineages feeding on *A. tsugae* in the Pacific Northwest (Havill et al. 2023). Both *Leucotaraxis* species are known to feed on eggs and nymphs of both generations of *A. tsugae* during the larval stage (Kohler et al. 2008, Mayfield et al. 2023). In the Pacific Northwest, *Leucotaraxis* spp. have been observed feeding on *A. tsugae* while *La. nigrinus* is present and after *La. nigrinus* drops to the soil for pupation and aestivation (Grubin et al. 2011, Kohler et al. 2016, Dietschler et al. 2021). Current evidence supports that *A. tsugae* populations are regulated by top-down effects in their native Northwestern range suggesting that the combined effect of summer-active and winter-active predators could prevent *A. tsugae* populations from reaching damaging levels (Crandall et al. 2022). This supports the continued approach currently being used to build a predator complex in the eastern United States. In the eastern United States, there are eastern lineages of *Le. argenticollis* and *Le. piniperda* present, which are found feeding mostly on *Pinus strobus* L. (Havill et al. 2018). In addition, the eastern lineages of these species have been found to be genetically distinct from the western lineages (Havill et al. 2023). First releases of the western lineages of *Le. argenticollis* and *Le. piniperda* in the eastern United States occurred in 2015 (Motley et al. 2017). Currently, there is limited evidence of establishment in the eastern United States.

Understanding the phenology of *Le. argenticollis* and *Le. piniperda* and how it relates to *A. tsugae* in eastern North America is essential to increasing establishment success. Our knowledge of the phenology of both species is restricted to what has been observed in the Pacific Northwest. Studies in the Pacific Northwest determined that *Leucotaraxis* spp. were present on both generations of *A. tsugae* (Kohler et al. 2008, Grubin et al. 2011). *Leucotaraxis* spp. larvae were present year-round, but were most abundant when *A. tsugae* sistens and progrediens were producing eggs, from March to mid-May and then from early June to mid-July (Grubin et al. 2011, Kohler et al. 2016). These studies did not separate the 2 *Leucotaraxis* species, therefore it is unclear if both species were present for both generations of *A. tsugae* or if these species target different generations. A recent study separating adults by species determined that *Le. argenticollis* adults emerged when *A. tsugae* progrediens eggs were present in sistens ovisacs, followed by *La. nigrinus* prepupal drop, then the first adult emergence of *Le. piniperda* occurred when *A. tsugae* progrediens nymphs were present (Dietschler et al. 2021). A second emergence of *Le. argenticollis* adults occurred as the emergence of *Le. piniperda* adults decreased (Dietschler et al. 2021). This pattern was observed in the lab, from *A. tsugae* infested foliage collected from field sites in the Pacific Northwest, making phenological observations in the East essential to put western research into context and improve management efficacy.

In order to successfully establish the western lineage of *Le. argenticollis* and *Le. piniperda* in the eastern United States, it is important to understand their phenology and synchrony with prey in the introduced eastern range. This study investigated the phenology and overwintering ability of *Le. argenticollis* in confined releases at field sites in New York and Virginia in 2021 and 2022.

Materials and Methods

Field Site Set up 2021

Two field sites were selected for this study; 1 in Ithaca, NY (GPS coordinates: 42°26′18.5″N 76°24′35.0″W), and the other was in Bland, VA (GPS Coordinates: N37°11.659′ W80°53.404′). Thirty eastern hemlock branches that contained medium–high *A. tsugae* densities (1–3 *A. tsugae*/cm) were selected at each site. Eastern hemlocks selected at the NY field site ranged in size from 6 to 72 cm diameter at breast height (DBH) with a mean of 36.1 ± 4.4 cm. Eastern hemlocks selected at the VA field site ranged in size from 3 to 10.7 cm DBH with a mean of 6.7 ± 0.7 cm. In fall 2020 at the VA field site, a wooden dowel was used to dislodge *A. tsugae* predators that might be present, such as *Laricobius* spp., by hitting each branch approximately 10 times. Nylon cages, 100 cm long × 66 cm wide (MegaView Science Co., Ltd., Taichung, Taiwan) were then secured over each branch to prevent predators from approaching the branches and feeding on *A. tsugae*. In January 2021, nylon cages were secured over selected branches at the New York field site, branches were not beaten because specialist predators of *A. tsugae* had not established at the site. All cages were secured to treatment branches using a piece of foam pipe insulation 7.5 cm long by 1.25 cm wide and zip ties. Three pairs of Onset HOBO data loggers (Onset Computer Corporation, Bourne, MA) were dispersed throughout each field site recording the temperature every 60 min. For each pair of data loggers, 1 was placed inside of the nylon cage and the other was placed on a nearby branch outside of the cage. Relative humidity was also recorded at the NY site, but not at the VA site.

Field Site Set up 2022

Due to high *A. tsugae* winter mortality (99.97%), the field site in Ithaca, NY was not used in 2022. The same field site that was used in Virginia in 2021 was also used in 2022. In the fall of 2021, 19 eastern hemlock branches containing medium–high *A. tsugae* densities (1–3 *A. tsugae*/cm) were selected. Eastern hemlock trees ranged in size from 0.3 to 11.7 cm DBH with a mean of 8.6 ± 2.2 cm. Nylon cages were secured over each branch just like in 2021. One pair of Onset

HOBO data loggers was set up at the site with 1 in a cage and the other outside of the cage on a nearby branch. Both data loggers were set to record temperature every 60 min.

Leucotaraxis argenticollis Adult Releases

In 2021 and 2022, *Le. argenticollis* adults, were obtained from *A. tsugae* infested hemlock branches that were located at several sites in the northern Puget Sound area in the Pacific Northwest. These were sexed and sorted at the Sarkaria Arthropod Research Laboratory quarantine facility at Cornell University, Ithaca NY. To transport to the field sites, 5 females and 5 males were placed into ~3.7 ml clear polystyrene plastic vials with caps, and provided with moistened filter paper to prevent dehydration. At each field site, *A. tsugae* phenology was checked to determine when *A. tsugae* adult sistens began oviposition. Once *A. tsugae* sistens had oviposited 1–5 eggs per ovisac, 10 adult flies (5 female: 5 male) were released into each cage at both field sites (Table 1). In 2022, 7 adult flies (4 female: 3 male) were released into each cage at the VA field site (Table 1).

Branch Collection and Sampling

Two weeks after flies were released into the cages, branch sampling occurred. Each sample consisted of three 5–15 cm branch clippings and all debris from each cage. Branch clippings were selected throughout the cage and consisted of 20–30 *A. tsugae* ovisacs. There were situations when no *A. tsugae* ovisacs were present on the branches within the cages. If no *A. tsugae* ovisacs were seen, branch clippings were randomly selected from the cage containing current year growth as well as 1–2 yr old growth. Before opening the cages, cages were checked for *Le. argenticollis* adults. If *Le. argenticollis* adults were present, an aspirator was used to collect as many adult specimens as possible.

At the NY site in 2021, sampling occurred every 2 wk starting on 13 April–20th July. Monthly sampling then occurred from 23 August–29 September. At the VA site in 2021, sampling occurred every 2 wk starting on 12 March–22 July. Monthly sampling then occurred from 19 August–24 September. After sampling in September, all cages were removed at both field sites, allowing remaining *Le. argenticollis* puparia to be completely exposed to the environmental conditions present at each site. In 2022, biweekly sampling started on 21 March and monthly sampling occurred from 4 August 2022–6 March 2023. Cages remained on until 6th March 2023. Branches were held in the freezer at −20 °C until they could be evaluated.

Branch Clipping Evaluation

Branch clippings were measured to the nearest 0.10 cm. *Adelges tsugae* phenology was recorded for each live adelgid present. *A. tsugae* were considered live if they produced red liquid hemolymph and *A. tsugae* were considered dead if no hemolymph was produced or if brown coagulated hemolymph was produced. All *A. tsugae* life stages were recorded. Sistens, progrediens, and sexupara crawlers that were present were classified into 4 categories (none, low: 1–10 per branch sample, medium: 11–30 per branch sample, and high: ≥31 per branch sample). Adult sistens and progrediens that were producing eggs were placed into 3 categories (low: containing 1–10 eggs, medium: containing 11–30 eggs, and high: containing ≥31 eggs). Categories included sistens crawlers, aestivating sistens, 2nd–4th instar sistens nymphs, the total number of sistens adults, sistens adults with low eggs/ovisac, sistens adults with medium eggs/ovisac, sistens adults with high eggs/ovisac, progrediens and sexupara crawlers, settled progrediens and sexupara, progrediens and sexupara 2nd–3rd instar nymphs, 4th instar sexupara, adult sexupara, 4th instar progrediens, the total number of progrediens adults, progrediens adults with low eggs/ovisac, progrediens adults with medium eggs/ovisac, and progrediens with high eggs/ovisac.

At the same time, all life stages of *Le. argenticollis* were collected and categorized as: egg, larva, puparia with no emergence hole, puparia with an emergence hole, and adult. All collected specimens were stored in 95% EtOH and at–20 °C.

2020–2021 Observation of *Le. argenticollis* Adult Emergence

For the first year of the study, 2021–2022, branches remained in the field from Oct–Feb. In February, branches were cut and brought back to the lab. Branches were placed in water saturated floral foam (Smithers-Oasis North America, Kent, OH) and then placed in modified Lari-Leuco containers (Mayfield et al. 2021, Fig. 1). The modified Lari-Leuco containers consisted of two 17 liter food grade Cambro containers (CAMWEAR CAMSQUARES Classic Series, product 18SFSCW, cambro.com), 1 with a solid bottom, and the other with a 1.27 cm hardwire cloth bottom. The second container with the 1.27cm hardwire cloth bottom nested inside the first container with the solid bottom. One 17 liter Cambro lid (CAMWEAR CAMSQUARES Classic Series, product SFC12SCPP, cambro.com) was used to cover the opening of the inside container. Two 6.3 cm diameter holes were made on the bottom sides of the outside container, and two 6.3 cm diameter holes were made on the top sides of the inside container. Polyester mesh (435 µm opening, NBC Meshtech Americas, Batavia, IL) was used to cover these holes and prevent flies from escaping. Two 12 cm diameter holes were made on the Cambro lid using a power drill with the appropriate drill bits. Two 12 cm diameter round funnels (HNBun, UPC 696629698126, amazon.com) were adhered, using hot glue, through the 2 holes in the Cambro lid. A 2.8 cm diameter hole was created in the lid of 118 ml round plastic screw top jars (Uline, Pleasant Prairie, WI), allowing the funnel tip to fit through. Hot glue was used to adhere the lids to the tips of the funnels.

The containers were placed below a window for natural lighting and remained at room temperature (20–22 °C). Containers were checked weekly for *Le. argenticollis* adult emergence. Emerging flies were collected and contained in 1.5 Eppendorf tubes with 95% EtOH.

Species Confirmation

To confirm the species of *Leucotaraxis* that was released into the cages, progeny (adults and puparia) of the released *Leucotaraxis* spp.

Table 1. Field site set up and *Le. argenticollis* adult release dates for each field site in 2021 and 2022

Year	Site	Field Site Set Up Date	Fly Release Date	Number of Male Flies Per Cage	Number of Female Flies Per Cage
2021	NY	13 Jan 2021	30 Mar 2021	5	5
2021	VA	23 Nov 2020	25 Feb 2021	5	5
2022	VA	10 Nov 2021	3 Mar 2022	3	4

Fig. 1. Modified Lari-Leuco containers containing *A. tsugae* infested hemlock branches that were used to observe *Le. argenticollis* adult emergence.

adults were collected and analyzed based on morphological characteristics that separate *Le. argenticollis* from *Le. piniperda* (Gaimari and Havill 2021) and by multiplex qPCR. Morphological characteristics that were used included the presence/absence of darkly sclerotized posterior spiracular tubercles on the puparia, and the presence/absence of 1 to several long setulae medially from the postpronotal seta on adults. In 2021, 50 adults collected from the NY site were analyzed and 10 adults and 40 puparia collected from the VA site were analyzed morphologically. In 2022, 26 adults and 24 puparia collected from the VA site were analyzed morphologically. Voucher specimens were deposited in the Virginia Tech Insect Collection, Blacksburg, VA (VTEC).

In 2021, 94 specimens, collected from cages at the VA site, were randomly selected for multiplex qPCR analyses. First, DNA extractions were conducted by digesting insect tissue in an extraction buffer (100 mM Tris–HCl with pH = 8.2, 1 mM EDTA, 25 MM NaCl) containing 2 mg/ml Proteinase K for 30 min at 37 °C and then 2 minutes at 95 °C. This created the template DNA that would be used in the multiplex qPCR analysis. Buffer volumes varied depending on the life stage; eggs were crushed in 10 µl of buffer and larvae and puparia were crushed in 100 µl of buffer. For 94 samples, in a 2 ml tube, 1 ml of 2 X Taqman PCR master mixture was added. This consisted of 618 µl of ddH_2O, 200 µl of 10 X *PerfectStart Taq* buffer, 160 µl of 2.5 mM dNTPs, 20 µl of *PerfectStart Taq* DNA polymerase (2.5 units/µl), and 2 µl of ROX (50 µM). In the same 2 ml tube, 120 µl of *Le. argenticollis* F Primer (10 µM), 120 µl of *Le. argenticollis* R Primer (10 µM), 120 µl of *Le. piniperda* F Primer (10 µM), 120 µl of *Le. piniperda* R Primer (10 µM), 60 µl of *Le. argenticollis* SUN-probe (10 µM), and 60 µl of *Le. piniperda* FAM-probe (10 µM) (IDT, Coralville, IA) were added (Kirtane et al. 2022). This was then vortexed and centrifuged for 5 s. Using an 8-channel pipettor, 18 µl of the mixture was added to each well on a 96 well-plate. In addition to the 94 samples, a positive control and a negative control (water + Taqman PCR master mix + primers + probes) were included for a total of 96 samples. For the 94 samples, 2 µl of the DNA template was added to each well, except for 2 wells which were for the positive control and negative control. After the positive control and negative control were added the plate was sealed using a clear adhesive seal, spun in a plate spinner, and placed in the qPCR machine (QuantStudio 3, Applied Biosystems). The thermocycler profiler consisted of 1 cycle of incubation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, and an annealing/extension step 60 °C for 45 s. Species was determined using the Design & Analysis version 2.2.0 software by Applied Biosystems.

Statistical Analyses

Year 2021.

To determine if the temperature inside and outside the mesh cages was different at the NY and VA field sites, a linear mixed-effects model, with a Gaussian distribution, was used for each site separately. Location inside vs outside of the cage, month, and the interaction between location and month were fixed effects and the tree was set as a random effect. If the interaction between month and location was not significant, then it was removed from the model. To determine if the relative humidity inside and outside the mesh cages was different at the NY field site, a generalized linear mixed-effects model using a beta distribution and logit link function was used to determine differences in relative humidity between cage locations over experimental months. Location inside vs outside of the cage, month, and their interaction were included as fixed effects and individual trees were included as random effects. Relative humidity was not recorded at the VA site since the data loggers used at the site could not measure relative humidity. The Tukey's HSD *P*-value ($P < 0.05$) adjustment was used for pairwise comparisons for both models. R packages that were used for these analyses were "glmmTMB", "car", and "emmeans" (RStudio Team 2022).

Year 2022.

To determine if the temperature inside and outside the mesh cages was different at the VA field site a multiple linear regression model was used. Location of the data loggers, month, year, and the interaction between location and month were fixed effects. If the interaction term was not significant, then it was removed from the model. The Tukey's HSD *P*-value ($P < 0.05$) adjustment was used for pairwise comparisons.

Comparing Monthly Temperatures Among Field Sites and Years

To determine if monthly temperatures within the mesh cages were different among field sites and between 2021 and 2022, a multiple linear regression model was used. Temperature was the response variable. The field site, including year, month, and the interaction between field site, including year, and month were fixed effects. If the interaction was not significant, then it was removed from the model. The Tukey's HSD *P*-value ($P < 0.05$) adjustment was used for pairwise comparisons. R packages that were used for this analysis were "glmmTMB", "car", and "emmeans" (RStudio Team 2022). All statistical analyses were performed with R version 2022.7.1.554 (RStudio Team 2022).

Results

Year 2021 and 2022 Season Cage Effects

In 2021, at the NY site, overall, the temperature outside of the cage, was significantly cooler by 0.24 °C compared to inside of the cage, (χ^2 = 33.06, df = 1, $P < 0.0001$). There were statistically significant differences in temperature by month (χ^2 = 190336.71, df = 8, $P < 0.0001$, Supplementary Table 1A). Overall relative humidity was significantly higher by 21.0% outside of the cage compared to inside of the cage (χ^2 = 21.77, df = 1, $P < 0.0001$), but this varied by month, which was statistically significant (χ^2 = 5210.11, df = 8, $P < 0.0001$, Supplementary Table 2A). The interaction between location and month was also statistically significant (χ^2 = 2590.64, df = 8, $P < 0.0001$).

In 2021, at the VA site, overall, the temperature outside of the cage were significantly cooler by 0.15 °C compared to inside the cage (χ^2 = 4.38, df = 1, $P = 0.04$). There were statistically significant differences in temperature by month (χ^2 = 55900.97, df = 10, $P < 0.0001$, Supplementary Table 3A).

In 2022, at the VA site, overall, the temperature outside of the cage was significantly cooler by 0.21 °C compared to inside of the cage (χ^2 = 5.18, df = 1, $P = 0.02$). Monthly temperatures were statistically significant from each other (χ^2 =18811.14, df = 11, $P < 0.0001$) (Supplementary Table 4).

Comparing Monthly Temperatures Among Field Sites and Years

Overall, temperatures were statistically different between field site and year (χ^2 =351.9, df = 2, $P < 0.0001$). The VA field site was 4.7 °C warmer, in 2021, and 2.9 °C warmer, in 2022, compared to the NY field site. The VA field site was 0.8 °C warmer in 2022 compared to the VA field site in 2021. Monthly temperatures were statistically significant from each other (χ^2 =59988.6, df = 7, $P < 0.0001$), and the interaction between field site and month was statistically significant (χ^2 =1131.4, df = 14, $P < 0.0001$, Fig. 2).

Year 2021: NY *A. tsugae* and *Leucotaraxis argenticollis* Phenology

Adelges tsugae sistens 2nd–4th instar nymphs were present in April and were no longer present in May (Fig. 3A). *Adelges tsugae* sistens adults with eggs occurred from mid-April to mid-May. Progrediens and sexupara 2nd–3rd instar nymphs were observed from mid-May to mid-July. A very low number of progrediens adults with eggs were present from mid-June to mid-July. Sexupara 4th instar nymphs were

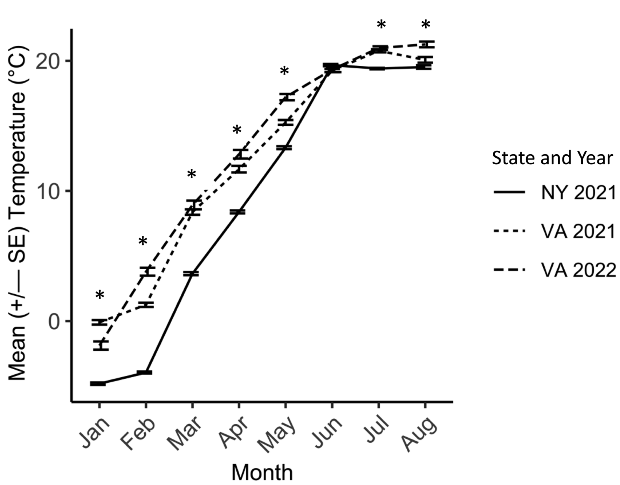

Fig. 2. Mean (± SE) monthly temperatures for the NY and VA field sites in 2021 and for the VA field site in 2022. The symbol * indicates statistical significance ($P < 0.05$) among the field sites and across months using the Tukey post hoc adjustment.

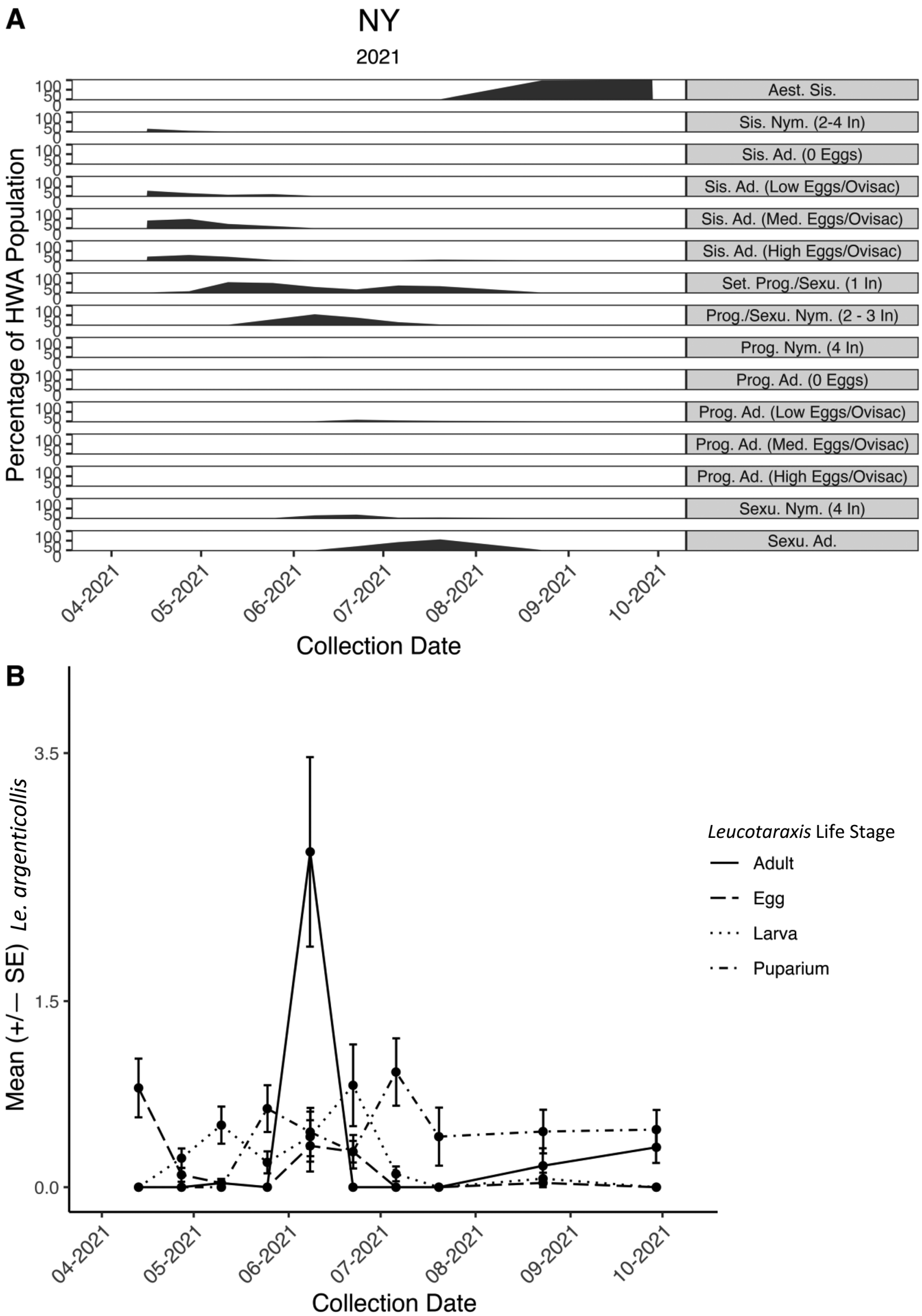

Fig. 3. *Adelges tsugae* and *Le. argenticollis* phenology in 2021 at the NY field site. A) Presents the percentage of the *A. tsugae* population per collection date for each *A. tsugae* life stage. Aest. Sis. stands for aestivating sistens, Sis. Nym. stands for sistens nymphs, In stands for instar, Set. stands for settled, Prog. stands for progrediens, and Sexu. stands for sexupara. Sistens adults (Sis. Ad) were categorized as having 0 eggs, low eggs per ovisac (1 – 10 eggs/ovisac), Medium eggs per ovisac (11- 30 eggs/ovisac), and high eggs per ovisac (31+ eggs/ovisac). Progrediens adults (Prog. Ad.) were categorized in the same way as the sistens adults. B) Mean *Le. argenticollis* (+/— SE) collected for each collection date and for each life stage.

observed in June and July, and sexupara adults were observed from mid-June to mid-August. Aestivating sistens nymphs were observed from mid-July until the end of September.

All *Leucotaraxis* adults and puparia from the NY site in 2021 that were analyzed were confirmed to be *Le. argenticollis*. *Leucotaraxis argenticollis* adults were initially released into the cages on 30 March 2021 (Table 1). *Leucotaraxis argenticollis* eggs were first observed on 13 April 2021 (Fig. 3B). Eggs were also observed on 8 June, 22 June, and 23 August. The first observance of *Le. argenticollis* eggs coincided with the presence of *A. tsugae* sistens adults with eggs (Fig. 3). The second occurrence of *Le. argenticollis* eggs coincided with the presence of *A. tsugae* progrediens adults with eggs. *Leucotaraxis argenticollis* larvae first occurred on 27 April 2021 and were present from 27 April–20 July and were observed again on 23 August 2021 (Fig. 3B). *Leucotaraxis argenticollis* puparia were first observed on 25 May and were observed from 25 May–29 September. Adults were first observed on 10 May 2021. Peaks with the highest mean of adults observed occurred on 8 June and 29 September.

Branches were brought into the lab in February 2022 to observe adult emergence after puparia were exposed to winter conditions. The first adult emerged on 1 March 2022 and a total of 17 adults emerged.

Year 2021: VA *Adelges tsugae* and *Leucotaraxis argenticollis* Phenology

Adelges tsugae sistens 2nd–4th instar nymphs occurred in April (Fig. 4A). *Adelges tsugae* sistens adults with eggs occurred in April until the end of May. Settled progrediens and sexupara 1st instar nymphs were observed from mid-April to mid-June. Progrediens and sexupara 2nd–3rd instar nymphs were observed in May and June and progrediens 4th instar nymphs were observed in very low numbers in June. Very low number of progrediens adults with eggs were observed from June to mid-July. No sexupara 4th instar nymphs and adults were observed in 2021.

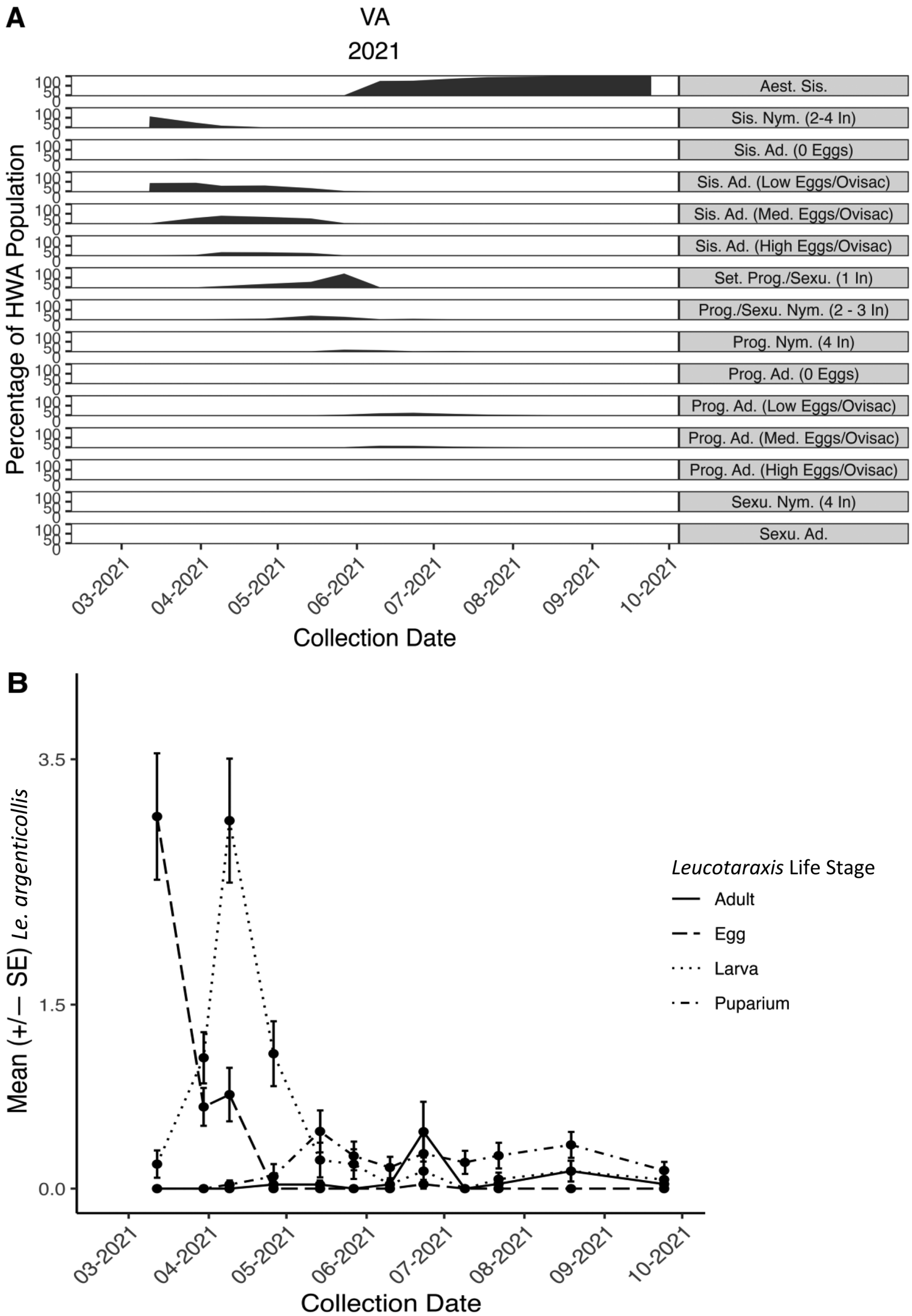

Fig. 4. *Adelges tsugae* phenology and *Le. argenticollis* phenology in 2021 at the VA field site. A) Presents the percentage of the *A. tsugae* population per collection date for each *A. tsugae* life stage. Aest. Sis. stands for aestivating sistens, Sis. Nym. stands for sistens nymphs, In stands for instar, Set. stands for settled, Prog. stands for progrediens, and Sexu. stands for sexupara. Sistens adults (Sis. Ad) were categorized as having 0 eggs, low eggs per ovisac (1 – 10 eggs/ovisac), Medium eggs per ovisac (11- 30 eggs/ovisac), and high eggs per ovisac (31+ eggs/ovisac). Progrediens adults (Prog. Ad.) were categorized in the same way as the sistens adults. B) Mean *Le. argenticollis* (+/— SE) collected for each collection date and for each life stage.

All *Leucotaraxis* adults and puparia from the VA site in 2021 that were analyzed morphologically were confirmed to be *Le. argenticollis*. From the molecular analysis 70 out of the 94 samples were identified as *Le. argenticollis*, 24 out of the 94 samples were undetermined. This could be due to degradation of the DNA or the specimen was of eastern lineage so the sample did not amplify with the primers that were used. However, it is most likely that the undetermined samples were undetermined due to DNA degradation, since cages were applied to branches and prevented the eastern lineage of *Le. argenticollis* from getting to the branches. Therefore, we can assume that the flies released into the cages, at the beginning of the study, were the western lineage of *Le. argenticollis*. *Leucotaraxis argenticollis* adults were initially released on 25 February 2021 (Table 1). *Leucotaraxis argenticollis* eggs were first observed on 12 March 2021 and were present from 12 March–9 April (Fig. 4B). A smaller peak of eggs was observed on 23 June. Larvae were first observed on 12 March 2021 and were present from 12 March–24 September. Puparia were first observed on 9 April 2021 and were present from 9 April–24 September. Adults were first observed on 26 April 2021, and were present from 26 April–30 May, 10 June–23 June, and from 22 July–24 September. The main peaks of adults occurred on 23 June and 19 August.

Branches were brought into the lab in January 2022 to observe the first emergence of adults after puparia were exposed to winter conditions. The first adult emergence occurred on 19 February 2022, with a total of 8 adults emerging.

Year 2022: VA *A. tsugae* and *Leucotaraxis argenticollis* Phenology

Adelges tsugae sistens adults with eggs occurred in March until the end of April 2022 (Fig. 5A). Settled progrediens and sexupara 1st instar nymphs occurred at the end of March–June 2022. Progrediens and sexupara 2nd–3rd instar nymphs were observed from mid-May to mid-June 2022. Progrediens 4th instar nymphs were observed in June 2022. Adult progrediens with eggs occurred at the end of May to July 2022 in very low numbers. Sexupara 4th instar nymphs were observed from the end of May to mid-June 2022, and sexupara adults were observed from the end of May to July 2022. Aestivating sistens occurred from June–December 2022. Sistens 2nd–3rd instar nymphs were observed from October 2022–February 2023. Sistens adults without eggs were observed from December 2022–March 2023 and sistens adults with eggs were observed at the end of January 2023–March 2023.

All *Leucotaraxis* adults and puparia from the VA site that were analyzed were confirmed to be *Le. argenticollis*. *Adelges tsugae* sistens started to oviposit approximately 1 wk later than in 2021. Therefore, *Le. argenticollis* adults were released 1 wk later on 3 March 2022 (Table 1). Due to fly availability, fewer flies were released into each cage with a sex ratio of 4F:3M instead of 5F:5M (Table 1). Even though fewer flies were released, we were still able to observe successful reproduction and could observe *Le. argenticollis* phenology. *Leucotaraxis argenticollis* eggs first occurred on 21 March 2022, and were also observed on 14 June 2022 (Fig. 5B). *Leucotaraxis argenticollis* larvae first occurred on 4 April 2022 and were present from 4 April–14 June, 11 July–21 July, and 1 September 2022–6 March 2023. Puparia first occurred on 2 May 2022 and were consistently found throughout the study. Adults first occurred on 21 March 2022, which were likely remaining parents that were released into the cages. Adults that were the offspring of the parents were observed on 30 May 2022 and the main peaks of adults occurred on 30 May 2022, 11 July 2022, 2 December 2022, and 6 March 2023.

Discussion

These results confirm that the western lineage of *Le. argenticollis* is capable of surviving year-round in VA and at least for the majority of the year in NY. Past research has indicated that immature life stages of *Le. argenticollis* were capable of tolerating environmental conditions during the spring into early summer (Motley et al. 2017), and that *Le. argenticollis* puparia were capable of overwintering at sites in the eastern United States, in plant hardiness zones 4a–7a (Dietschler et al. 2023). In our study, we were able to determine that *Le. argenticollis* was capable of completing its life cycle at 1 field site in NY and 1 site in VA. This indicates that establishment of the western lineage of *Le. argenticollis* is possible and supports the continued release and study of this biological control agent for *A. tsugae* management.

Potential Cage Effects

In 2021, using traditional sampling techniques, the western lineage of *Le. argenticollis* has not been found to be established at release sites in the eastern United States. Therefore, to observe the phenology of *Le. argenticollis* at field sites in NY and VA, large nylon mesh cages were applied over *A. tsugae* infested hemlock branches for our study. Our study compared mean temperature and mean relative humidity across several months, and found significant differences outside vs inside of the cages, but these differences were small. Temperature and relative humidity have been shown to influence the survival and diapause of *Le. argenticollis* (Dietschler et al. 2023). However, we believe that the cage effect on the phenology of *Le. argenticollis* within the cages was minimal.

Temperatures Among NY and VA Field Sites in 2021 and VA Field Site in 2022

From January–May, temperatures at the VA field site in 2021 and 2022 were warmer compared to temperatures at the NY field site (Fig. 2). *Adelges tsugae* phenology is known to vary depending on elevation, latitude, and temperature (Havill et al. 2014), and *Leucotaraxis* spp. adult emergence is tied closely to temperature (Dietschler et al. 2021). For our study, *A. tsugae* sistens oviposited eggs earlier at the VA field site compared to the NY field site. In addition, we released *Le. argenticollis* adults into mesh cages at the time when *A. tsugae* sistens started ovipositing. For the VA field site, in 2021, this occurred almost 1 mo earlier than at the NY field site (Table 1). In January, the temperature was cooler at the VA field site in 2022 compared to 2021 at the same site (Fig. 2). As a result, *A. tsugae* sistens started to oviposit eggs 1 wk later in 2022, and therefore *Le. argenticollis* adults were released into cages at a later date compared to 2021. After May, temperatures in June at the VA field site in 2021 and in 2022 were similar to temperatures in June at the NY field site (Fig. 2). This is also around the time when the second emergence of *Le. argenticollis* adults occurred at both field sites. Therefore, it would seem that the phenology of *Le. argenticollis,* in 2021, may be similar during the later summer months due to similarities in temperature at these field sites.

Year 2021: NY and VA *A. tsugae* and *Le. argenticollis* Phenology

At the NY and VA field sites, *Le. argenticollis* eggs were present when both *A. tsugae* sistens and progrediens were producing eggs. However, at the VA field site, *Le. argenticollis* eggs were found in March, approximately 1 mo earlier than the NY field site (Fig. 4B). The reason for this would be due to when *Le. argenticollis* adults were released into cages at each site, which was based on when *A.*

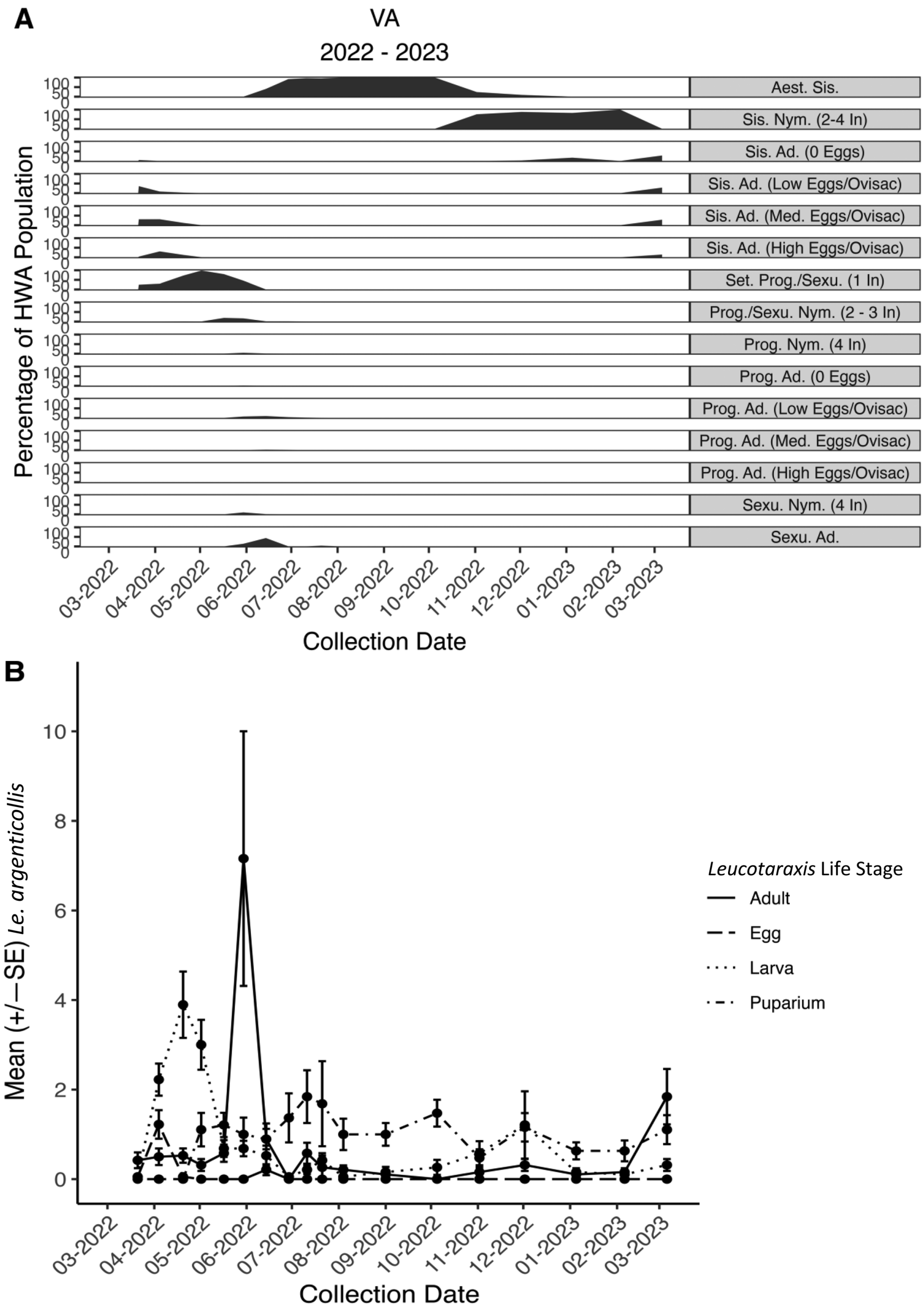

Fig. 5. *Adelges tsugae* phenology and *Le. argenticollis* phenology in 2022 - 2023 at the VA field site. A) Presents the percentage of the *A. tsugae* population per collection date for each *A. tsugae* life stage. Aest. Sis. stands for aestivating sistens, Sis. Nym. stands for sistens nymphs, In stands for instar, Set. stands for settled, Prog. stands for progrediens, and Sexu. stands for sexupara. Sistens adults (Sis. Ad) were categorized as having 0 eggs, low eggs per ovisac (1 – 10 eggs/ovisac), Medium eggs per ovisac (11- 30 eggs/ovisac), and high eggs per ovisac (31+ eggs/ovisac). Progrediens adults (Prog. Ad.) were categorized in the same way as the sistens adults. B) Mean *Le. argenticollis* (+/− SE) collected for each collection date and for each life stage.

tsugae sistens initiated oviposition. In VA, *A. tsugae* sistens started oviposition near the end of February, and in NY, *A. tsugae* sistens began ovipositing near the end of March. This supports the methodology to release *Le. argenticollis* adults at the time when *A. tsugae* sistens begin oviposition in order to match western lineage *Le. argenticollis* phenology with *A. tsugae* phenology in the eastern United States. There has been some concern about release at this time due to fluctuating temperatures, especially in the Northeast. Low temperatures of −8.13 °C were recorded in NY 3 days post release (2 April 2022) in addition to snow, providing the first anecdotal evidence that *Le. argenticollis* adults can survive drastic swings in temperature. At the NY field site, a low number of *Le. argenticollis* eggs were found in August (Fig. 3B), but *Le. argenticollis* eggs were not found in August at the VA field site. In the Pacific Northwest, *Leucotaraxis* spp. eggs were present from March–early August (Grubin et al. 2011), indicating that the phenology of the western lineage of *Le. argenticollis* in NY is similar to what it is in its native range, when released during *A. tsugae* sistens oviposition.

At the NY and VA field sites, *Le. argenticollis* larvae were found when *A. tsugae* sistens and progrediens were producing eggs. Larvae were also observed when only aestivating sistens nymphs and sexupara adults were present. In the Pacific Northwest, *Leucotaraxis* spp. larvae were also found during the time when *A. tsugae* adults of both generations were producing eggs (Kohler et al. 2008, 2016, Grubin et al. 2011, Rose et al. 2020), and when aestivating sistens were present (Grubin et al. 2011). It is possible that *Le. argenticollis* larvae enter diapause until *A. tsugae* sistens resume development and produce eggs, or may be feeding on aestivating sistens through the summer into early fall, or ran out of time to develop into puparia before their food supply was gone. Based on our study, we cannot confirm nor deny which situation is most probable. Future work is needed to determine if larvae do feed on *A. tsugae* aestivating nymphs or if they enter diapause when food availability is low.

Puparia were first observed at the VA field site at the beginning of April, and at the NY field site puparia were first observed at the end of May. Based on when *Le. argenticollis* flies were initially released at these field sites, that would mean that puparia were present 8 days earlier at the VA site compared to the NY site. Once puparia were first observed, they remained present throughout the rest of the collecting dates, similar to what was observed at the NY field site, indicating that puparia remain present for the majority of the year. This is indicative of the year-long facultative puparial diapause observed (Dietschler et al. 2023) and provides evidence that the necessary environmental conditions are present to induce diapause in *Le. argenticollis* reproducing in the field. Grubin et al. (2011) found no *Leucotaraxis* spp. puparia in September–January, which could be an artifact of not sampling far enough back (sampled 6–15cm long terminal shoots) and missing settled puparia that fed on the pervious years *A. tsugae* sistens generation. This was not the case at the NY and VA field sites, since branches were brought back to the lab after the September collection and *Le. argenticollis* adults emerged the following March and February, respectively. Since puparia resulted in the observation of emerging *Le. argenticollis* adults, we can confirm that the western lineage of *Le. argenticollis* can reproduce and overwinter as puparia in the eastern United States.

Year 2022: VA *A. tsugae* and *Le. argenticollis* Phenology

Compared to what was seen in 2021 at the VA field site, *Le. argenticollis* eggs were observed 1 wk later in March, at the time when *A. tsugae* sistens adults were still ovipositing (Fig. 5). This was due to the fact that, in 2022, *Le. argenticollis* adults were released 1 wk later compared to when they were released in 2021. As in 2021, eggs were also found in June when *A. tsugae* progrediens adults were ovipositing (Fig. 5). In 2022, larvae were first observed when *A. tsugae* sistens adults were ovipositing. Larvae were then present throughout most of the year, except at the end of June and early August, this further supports that larvae are not only present while *A. tsugae* adults are ovipositing, but when *A. tsugae* nymphs are also present. Puparia were first observed approximately 3 wks later in 2022 compared to 2021, and continued to be observed throughout the rest of the year and into 2023 (Fig. 5B). This supports our findings in 2021 at the NY and VA field sites that puparia are present for the majority of the year. In 2022, *Le. argenticollis* adults were found on the first collection date, since eggs and larvae, not puparia, were present at this time, we conclude that these adults were surviving adults from the initial release. Since puparia were first seen at the beginning of May, we conclude that the next generation of *Le. argenticollis* adults would have emerged by mid-May, which would be 1 mo later than in 2021 at the VA field site. Adults continued to be observed into June with a small emergence occurring in July (Fig. 5B). These instances coincided with the presence of both generations of *A. tsugae* adults with eggs, just like in 2021 at the NY and VA field sites. However, the adult emergence, starting in July, lingered through September at a time when only aestivating sistens were present (Fig. 5). With the lingering emergence of adults occurring through September, we conclude that these adults could be either a late emergence of the second generation or were an early emergence of adults that were to emerge when *A. tsugae* sistens adults with eggs were present the following year. Dietschler et al. (2023), also observed a bimodal adult emergence, with puparial eclosion in summer-fall and winter-spring at sites throughout the eastern United States and 1 site in the native western range. Our results support the occurrence of an extended summer-fall emergence occurring in the field at the VA site. In the Pacific Northwest, aestivating sistens are present between August–November (Grubin et al. 2011, Kohler et al. 2016), so it is unclear as to why the late summer- fall emergence is occurring in the field. This could possibly be due to a phenological mis-match.

With a longer observation period during the 2nd year of the study, live adults were also found in November 2022–March 2023. This coincided with the presence of *A. tsugae* sistens adults before and after oviposition occurred and indicates that adult flies emerge throughout the fall and winter months (Fig. 5). This confirms that the phenology of the western lineage of *Le. argenticollis,* when initially released at the time of *A. tsugae* sistens adult oviposition, synchronizes well with *A. tsugae* phenology in VA.

Significance for *A. tsugae* Management

This study provides insight on the phenology of the western lineage of *Le. argenticollis* and how it relates with the phenology of *A. tsugae* in the eastern United States. It confirms that *Le. argenticollis* is capable of feeding on *A. tsugae* in the eastern United States and targets both generations of *A. tsugae*. The western lineage of *Le. argenticollis* is also capable of reproducing and surviving the environmental conditions present in the eastern United States, signifying that it is possible for *Le. argenticollis* to establish there. It also supports release timing suggestions made by Dietschler et al. (2021) for *Le. argenticollis* and when it would be the best time to release this biological control agent so that its phenology synchronizes with its prey. In the eastern United States, *La. nigrinus* has been successful in establishing populations in multiple locations, continues to spread, and effectively reduces the sistens population. However, the pressure this predator provides is not enough, and an additional predator that also targets the progrediens generation is needed. Based on this study, *Le. argenticollis* may be able to take that role and provide additional pressure on *A. tsugae*, creating a natural enemy complex similar to what has been observed in the Pacific Northwest which effectively mitigates *A. tsugae* population growth (Crandall et al. 2022).

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Author Contributions

Carrie Preston (Conceptualization [Lead], Data curation [Lead], Formal analysis [Lead], Investigation [Lead], Methodology [Lead], Project administration [Lead], Software [Lead], Writing – original draft [Lead]), Nicholas Dietschler (Investigation [Equal], Methodology [Equal], Writing – review & editing [Supporting]), Mark Whitmore (Methodology [Supporting], Resources [Equal], Writing – review & editing [Supporting]), and Scott Salom (Funding acquisition [Lead], Methodology [Supporting], Resources [Equal], Supervision [Lead], Writing – review & editing [Equal])

Supplementary Material

Supplementary material is available at *Environmental Entomology* online.

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