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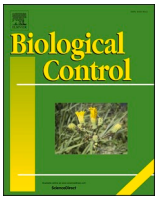

Overwintering diapause and survival of western *Leucotaraxis argenticollis,* a promising biological control agent for *Adelges tsugae,* in the eastern United States

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HIGHLIGHTS

- We found *Le. argenticollis* overwinters in the puparial stage.
- *Le. argenticollis* survived winter at all experimental sites; survival increased with latitude.
- Summer-fall abiotic factors influenced survival and dormancy.
- Adult *Le. argenticollis* emerged at the appropriate time for reproduction on *Adelges tsugae*.
- *Le. piniperda* appears to be using a different developmental stage or environmental cue for diapause.

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ABSTRACT

Successful use of biological control agents can be improved with a thorough understanding of the life history, survival, diapausing strategy, and synchrony with the host in the introduced range. Introduction of a novel organism can be influenced by abiotic factors that can impact the interactions with their target prey. *Leucotaraxis argenticollis* (Zetterstedt) and *Le. piniperda* (Malloch) (Diptera: Chamaemyiidae) are two species of predatory fly showing promise in the biological control program to manage the invasive hemlock woolly adelgid, *Adelges tsugae* (Annand)(Hemiptera: Adelgidae), in eastern North America. Field releases of these *Leucotaraxis* spp. began in 2015, but little is known about their potential for survival or how they overwinter. In an effort to explore *Le. argenticollis* establishment potential, laboratory reared puparia were observed at seven sites, across a latitudinal gradient, on the east coast and one site on the west coast (native range) of North America from July 20, 2020, to June 1, 2021. *Leucotaraxis piniperda* puparia were included at five sites and observed to be diapausing in a different stage or using a different cue. Adult *Le. argenticollis* emerged in spring 2021, after successfully

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overwintering as puparia, with a subset emerging in summer-fall 2020 at all observation sites. Results suggest that 1) the interaction of mean daily fall maximum temperatures and relative humidity has the most impact on survival, 2) mean daily minimum fall temperatures influence induction/maintenance of diapause, and 3) mean average daily fall temperatures and relative humidity impact survival to spring emergence. Substantial variation in survival and facultative diapause of *Le. argenicollis* was observed along a latitudinal gradient, potentially influencing predator establishment, supporting the need for continued research.

1. Introduction

Understanding predator–prey synchrony is one of the keys to successful biological control (Clausen 1936, Zilahi-Balogh et al., 2003a, 2003b). Organisms develop a unique phenology linking growth, development, and reproduction to annual cycles associated with biotic and abiotic factors. Conditions that influence phenology can be environmentally related such as temperature, light, moisture, and food resources (Tauber et al. 1986). Organisms can overcome harsh environmental conditions through quiescence (the short-term reduction in metabolism due to seasonal stress) or through diapause (Tauber et al. 1986). Diapause is a neurohormonal-mediated period with reduction in metabolism, activity, and morphogenesis leading to increased tolerance to unfavorable environmental conditions. Induction and maintenance of diapause occur at genetically pre-determined stages in response to seasonal environmental stimuli predicting adverse conditions (Tauber et al. 1986). Diapausing insects can tolerate extreme conditions, such as freezing winter temperatures, enabling them to withstand abiotic mortality factors (Lee and Denlinger 1985) until favorable conditions return and food resources are available. In this study, we explored the overwintering survival and diapause of a potentially important biological control agent of the invasive hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), throughout the pest's introduced range in eastern North America.

Adelges tsugae is a non-native pest of eastern and Carolina hemlock (*Tsuga canadensis* (L.) Carriere and *Tsuga caroliniana* Engelmann, respectively) in eastern North America (McClure 1987). Although *A. tsugae* was likely introduced sometime in the early 1900 s (Havill et al. 2006), it was first collected in the Richmond, Virginia area in 1951 (Stoetzel 2002). *Adelges tsugae* has spread throughout much of the native range of eastern hemlock and is now present in 21 US states and two Canadian provinces (USFS *A. tsugae* Distribution map, Morgantown Field Office, https://hiro.ento.vt.edu/hwa/wp-content/uploads/2023/02/HWAdistribution2022.pdf). A new infestation can begin with a single parthenogenic insect whose offspring spread rapidly throughout the stand (Tobin et al. 2013) and leads to tree decline and mortality (Orwig and Foster 1998, Trotter et al. 2013).

Current management techniques for *A. tsugae* focus on the use of chemical treatments (Vose et al. 2013) and release of biological control agents (Limbu et al. 2018) aiming for an integrated pest management approach (Mayfield et al. 2020). Multiple distinct genetic lineages of *A. tsugae,* each specializing on *Tsuga* spp., exist throughout Asia and western North America, and evidence suggests that the lineage introduced to the east coast originates from southern Japan (Havill et al. 2006, 2016). Significant research effort has focused on a suite of specialist predators native to western North America including a beetle species, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae), and two species of silver flies, *Leucotaraxis* spp. (Diptera: Chamaemyiidae, formerly in the genus *Leucopis*) (Limbu et al. 2018, Gaimari and Havill 2021). Silver flies have predaceous larvae that feed on sternorrhynchous Hemiptera (Gaimari 2010). *Leucotaraxis argenticollis* (Zetterstedt) and *Le. piniperda* (Malloch) (Diptera: Chamaemyiidae) are two specialist predators of adelgids that show promise as biological control agents for *A. tsugae* (Kohler et al. 2008). These two Holarctic species have genetically distinct lineages on the east and west coasts of North America (Havill et al. 2018) that have evolved specific host preferences (Havill et al. 2022 in Press). In the western range, *Le. argenticollis* and *Le. piniperda* represent two of the most abundant predators of *A. tsugae*, and two of three specialists (Kohler et al. 2008), with the eastern lineage specializing on pine bark adelgid (*Pineus strobi* Hartig, Hemiptera: Adelgidae)(Havill et al. 2018). While *La. nigrinus* only preys on the overwintering sistens generation (Zilahi-Balogh et al., 2003a, 2003b), *Leucotaraxis* spp. are most abundant during the egg laying stages of the sistens and progrediens (Grubin et al., 2011, Kohler et al. 2016). Recently, Crandall et al. (2022) demonstrated with predator exclusion experiments in Seattle, Washington that predators such as *Leucotaraxis* spp., active on both the winter sistens and spring/summer progrediens generation of *A. tsugae,* played a critical role in maintaining the very low densities of *A. tsugae* that exist throughout the northwestern US and Canada.

Adelges tsugae has a unique life history involving two parthenogenic generations in the introduced eastern range, with the sistens generation aestivating in the summer and developing throughout the winter and the progrediens generation developing in spring (McClure 1989). The winter growth pattern of *A. tsugae* means its predators must be cold-tolerant or able to diapause to feed on the eggs in late winter and spring. The specialist predatory beetle *La. nigrinus* undergoes an obligate summer diapause (aestivation) coinciding with *A. tsugae* aestivation and emerges in the fall to feed throughout the winter (Zilahi-Balogh et al., 2003a, 2003b). The supercooling point of *La. nigrinus* occurs at sub-zero temperatures for all life stages (Humble and Mavin, 2005), and varies based on geographic origin in the native western range (Mausel et al. 2011). Tolerance of sub-freezing temperatures is crucial to the survival of *A. tsugae* predators in the introduced range, because eastern hemlock occurs from Alabama to southern Canada and west to Wisconsin (Hardin et al. 2001), growing in USDA hardiness zones 4 through 7a (Gilman and Watson 1994, USDA plant hardiness zones: https://planthardiness.ars.usda.gov/).

Silver flies belong to a clade within Diptera known as Cyclorrhapha which also includes families such as Muscidae, Calliphoridae, and Sarcophagidae (Schoch et al. 2020). Cyclorrhaphous flies are unique among Diptera because they do not shed their larval cuticle before pupation, but instead form a puparium in which pupation occurs, referred to as pupariation (Fraenkel and Bhaskaran 1973, Martín-Vega et al. 2016). Following pupariation, cyclorrhaphous flies undergo three stages: cryptocephalic (immediately after formation of the puparium and prior to larval-pupal apolysis, Martín-Vega et al. 2016), phanerocephalic (head everts, and other events prior to pupal-adult apolysis), and pharate adult (metamorphosis complete but the fly has not yet eclosed), before eclosion of the imago (Pujol-Luz and Barros-Cordeiro 2012, Proença et al. 2014, Martín-Vega et al. 2017). At emergence, cyclorrhaphous flies simultaneously shed both their pupal cuticle and the third-instar larval cuticle that formed the puparium (Fraenkel and Bhaskaran 1973). Some flies in this group such as Sarcophagidae undergo a facultative pupal diapause (Fraenkel and Hsiao 1968, Denlinger 1972, Goto and Numata 2009), while other cyclorrhaphous flies such as Tephritidae have an obligatory diapause program (Papanastasiou and Papadopoulos 2014). *Sarcophaga argyostoma* (Robineau- Desvoidy) and *S. bullata* (Parker)(Diptera:Sarcophagidae) diapause as a phanerocephalic pupa (Fraenkel and Hsiao 1968). The majority of studies on diapause, phenology and development of these flies are in the forensic entomology literature (Fraenkel and Hsiao 1968, Denlinger 1972, Goto and Numata 2009, Pujol-Luz and Barros-Cordeiro 2012, Proença et al. 2014, Martín-Vega et al. 2017), while data for biocontrol and other

predator/prey systems are lacking. Identifying the developmental stage at which diapause occurs in *Leucotaraxis* spp. used for biocontrol would aid our understanding of phenological patterns in this system.

We reared *Le. argenticollis* and *Le. piniperda* in the laboratory to the puparial stage and exposed them to two field treatments representing exposure to 1) summer through spring conditions, or 2) fall through spring conditions, across a latitudinal gradient representing most of the currently infested range of eastern hemlock. The purpose of this study was to evaluate the mortality and emergence timing of puparia experiencing a range of environmental conditions and to compare the effect of summer and winter exposure. We also determined intrapuparial stage of uneclosed flies to infer the possible diapausing stage. This research was designed to provide more information about the suitability of *Leucotaraxis* spp. as biological control agents for *A. tsugae* in eastern North America.

2. Methods

2.1. Laboratory rearing

Emerging adult *Leucotaraxis* spp. collected from *A. tsugae*-infested western hemlock, *Tsuga heterophylla,* were identified to species and sexed using a dissecting microscope. Species were determined using male genitalia and post-pronotal setae (Dietschler et al. 2021). Due to the difficulty of identifying females to species using only post-pronotal setae, the propensity of adults to emerge in species clusters at equal sex ratios helped to determine female identity (Dietschler et al. 2021). All parental *Le. argenticollis* used in rearing were from the first of the two seasonal emergence periods identified by Dietschler et al. (2021). Parents grouped into varying sex ratios (Supp table 1) were placed into custom made acrylic insect cages (mating cages; 43 cm × 36 cm × 30 cm) with field-collected eastern hemlock infested with ovipositing *A. tsugae* sistens adults. Infested foliage twigs were inserted into saturated floral foam blocks wrapped with Parafilm, creating rearing foliage blocks. We placed 1–3 twigs per block allowing space for flies to move freely among the twigs. Parental flies were allowed to oviposit on infested foliage for four to six days before foliage was replaced inside the mating cages. Infested foliage used in rearing was collected from a location with no *A. tsugae* predator release history. Rearing cages were kept at room temperature (21–23 °C) under indirect natural lighting conditions from a west-facing window.

Upon removal of twigs with *Leucotaraxis* eggs from the mating cages, we added three to four twigs of supplemental HWA-infested foliage per block to supply hatching larvae with additional ovipositing *A. tsugae* as prey. Supplemental *A. tsugae* twigs were placed in contact with *Leucotaraxis* oviposition twigs to allow larvae to move among the twigs in search of food. Larval feeding blocks (rearing foliage blocks with supplemental foliage) were held in insect rearing cages (Bugdorm-1 cubic foot cage, MegaView Science Co. Ltd, Taiwan) placed inside growth chambers at 16 °C, 12:12 light. Growth chamber temperatures and light cycles were selected based on laboratory trials and pilot experiment data (Bittner and Dietschler, unpublished data). Rearing foliage blocks were rotated through mating cages following these methods for between 9 and 30 days (Supp table 1), as long as sufficient numbers of living adults remained. Larvae were given a minimum of 30 days to complete feeding with all oviposition taking place from March 9-April 15 for *Le. argenticollis*, and April 15-June 2 for *Le. argenticollis/Le. piniperda* mixed cages. Puparia were excised from rearing bouquets throughout June and July by clipping off small portions of twigs onto which puparia were naturally cemented (*Le. argenticollis* N = 975, *Le. argenticollis/Le. piniperda* N = 334). Puparia were then separated into 44 ml cups (hereafter referred to as puparia cups) and held in growth chambers at 16 °C and 12:12 L:D until shipment to observation sites.

Le. piniperda were laboratory reared to puparia following the same methods outlined above. Due to challenges identifying female *Leucotaraxis* spp. and periods of overlapping emergence between *Le. piniperda* and the second *Le. argenticollis* emergence, these parental cages were not pure. A substantial number of *Le. piniperda* adults emerged in the laboratory prior to shipment to field observation sites (*Le. piniderda* were shipped only on October 5, 2020), with the rest emerging before winter, suggesting *Le. piniperda* uses different life history strategies than *Le. argenticollis*. These emergence patterns limited the conclusions we could draw and thus *Le. piniperda* data were not included in the analysis.

2.2. Field observation

Field observation sites were selected to represent the variation in climatic conditions (USDA plant hardiness zones, based on average annual winter temperatures, https://planthardiness.ars.usda.gov/, Fig. 1) throughout the eastern hemlock native range, as well as current and future *A. tsugae* infested range. Sites from south to north were in Knoxville, Tennessee (TN, zone 7a); Weaverville, North Carolina (NC, zone 7a); Christiansburg, Virginia (VA, zone 6b); Lonaconing, Maryland (MD, zone 6b); Amherst, Massachusetts (MA, zone 5b); Locke in New York State's Finger Lakes Region (NY-FL, zone 5b); Au Sable Forks in New York State's Adirondacks Region (NY-ADK, zone 4a, outside the current infested range)(Fig. 1); and one site in Tacoma, Washington (WA, zone 8b, not pictured in the map) where this lineage of flies are native. Puparia cups were divided into two treatment groups and assigned to each site. Treatment group one, referred to as the summer (S) treatment group, were overnight shipped on July 20, 2020 (TN n = 60, NC n = 62, VA n = 47, MD n = 58, MA n = 61, NY-FL n = 58, NY-ADK n = 66, WA n = 61). Treatment group two, referred to as the winter (W) treatment group, were held at 16 °C, 12:12 (L:D) for an additional 11 weeks and shipped overnight on October 5, 2020 (TN n = 63, NC n = 62, VA n = 62, MD n = 64, MA n = 61, NY-FL n = 72, NY-ADK n = 59, WA n = 59). At each site, puparia cups were placed into plastic shoe boxes (Sterilite, 35.6 cm × 20.3 cm × 12.4 cm, Townsend, MA) inside outdoor wooden sheltered enclosures. Enclosures were elevated platforms with a roof and 12.5 mm hardware cloth to shelter from precipitation and animal disturbance and were placed out of direct sunlight. A temperature and relative humidity data logger was placed inside one shoebox per site, with logging intervals set to 30 min (Onset HOBO U23 Pro V2, Bourne, MA).

Frequency of checks for emergence were determined based on observations made in a 2019–2020 pilot experiment (Dietschler, unpublished data). Summer treatment puparia were checked once per week from July through September, when emergence was expected to be infrequent. Checks were increased to every other day during October and November when a subset of flies was expected to emerge. Frequency of checks was reduced to once per week from December through February, during the time flies are in diapause. Puparia checks were increased back to every other day from March through the experiment end date of June 1, 2021. Minor deviations to this general monitoring schedule were made occasionally due to schedule complications associated with the COVID-19 pandemic.

Eclosed adult flies were collected with an aspirator and transferred to 1.5 ml microcentrifuge tubes with 95% ethanol. All flies were kept separate by date, treatment, and cup. All adult flies were examined under dissecting microscope to confirm species identity using methods from Dietschler et al. (2021). At the end of the observation period, uneclosed puparia were overnight shipped back from field observation sites and immediately frozen (-20 °C), prior to being dissected under a 40X microscope to determine mortality and intrapuparial stage based on observable morphological traits (Martín-Vega et al. 2016, 2017; Pujol-Luz and Barros-Cordeiro 2012, Proença et al. 2014). We followed the suggested terminology and staging identifications of Martín-Vega et al. (2016) using characteristics observable under a dissecting microscope, which cannot pinpoint the exact completion of larval-pupal apolysis or pupal-adult apolysis.

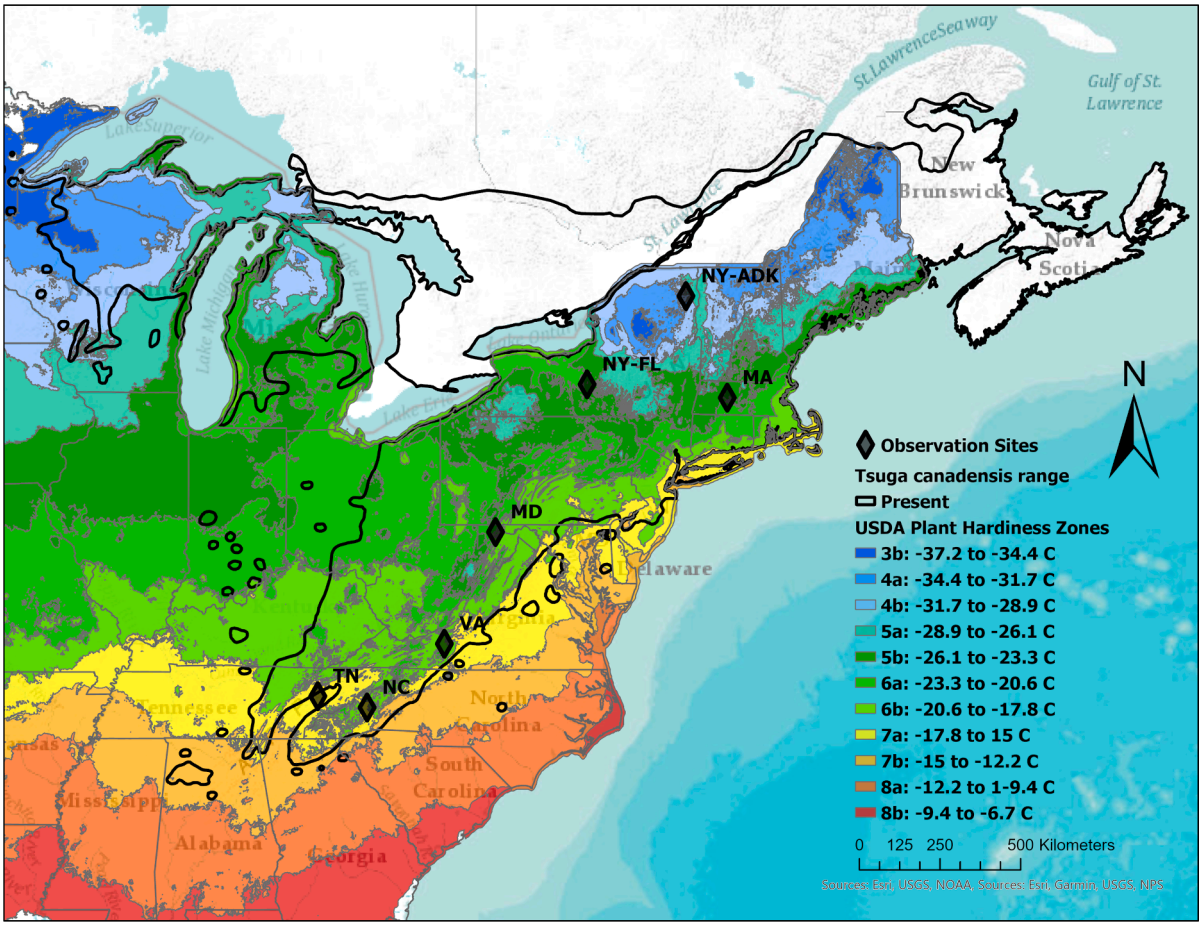

Fig. 1. The location of the seven east coast observations sites (black and grey diamonds) used as observation sites, USDA plant hardiness zone (zones 3b-8b, https://planthardiness.ars.usda.gov/), and eastern hemlock range (*Tsuga canadensis,* black line).

2.3. Data analysis

Analysis was performed using R version 4.0.2 (R Core Team 2022). Binomial logistic regression, using the lme4 package (Bates et al. 2015), analyzed survival (adult emergence/alive = 1, no adult emergence/dead = 0), overwintering diapause (Spring emergence = 1, Fall emergence = 0), and survival to spring emergence (alive and Spring emergence = 1, dead or fall emergence = 0). For regression analysis, flies were considered "alive" when an eclosed adult was collected. Fall emergence was defined as emergence between July 20, 2020 and December 31, 2020, and spring emergence was defined as emergence between January 1, 2021 and June 1, 2021. These timeframes captured the two observed non-overlapping emergence periods (Supp table 2). All three models included the interaction between observation site and treatment with cup as random variables. All models were back transformed to probability and odds ratio for interpretation using the emmeans package (Length 2022). Binomial logistic regression and multi-model inference using the MuMIn package (Barton 2022) were run to test which abiotic variables best explained the variation in each model. Each abiotic model included site and cup as random variables. Predictor variables tested in the model selection were the average minimum, maximum and mean daily temperatures for the summer-fall, winter-spring, and entire experimental period (summer-fall = July 20, 2020 – December 31, 2020 and winter-spring = January 1, 2021 – June 1, 2021); season low temperature (lowest temperature recorded); the average of the mean daily relative humidity for the summer-fall, winter-spring, and entire experimental period; and latitude (Supp table 3). Models exploring abiotic variables were selected using the lowest Akaike information criterion (AIC) value, with a ΔAIC cutoff of < 2.0. When there were two abiotic variable models with a ΔAIC of < 2.0, an interaction and additive model were included into the model selection. Collinearity was tested for using variance inflation factor (VIF), and a VIF < 2.5 was used as the cut off (Fox and Monette 1992). Chi-square analysis was conducted on puparial dissection data to explore the differences in puparial survival (alive versus dead) and intrapuparial stage (phanerocephalic, pharate adult, imago) at which mortality occurred across sites.

3. Results

3.1. Survival, diapause, and spring emergence

In the binomial logistic regression model for survival (emergence = 1, no-emergence = 0), there was a significant site by treatment interaction, when compared to the null model (X^2 = 93.528, df = 15, p = <0.001). At sites in MA, MD, VA, NC, and TN, the probability of survival to adult was significantly higher in the winter (W) treatment group (puparia deployed to field conditions in the fall, October 5, 2020) than in the summer (S) group (deployed to field temperatures in mid-summer, July 20, 2020) (Table 1, Fig. 2a). Probability of survival did not differ by treatment group at sites in NY-ADK, NY-FL, and WA (Table 1, Fig. 2a) (see Supp table 4 for non-transformed model output).

In the model evaluating the probability of spring 2021 versus fall 2020 emergence of living adult flies (spring emergence = 1, fall emergence = 0), the site by treatment interaction was significant, when compared to the null model (X^2 = 30.543, df = 2,17, p = 0.01). At sites in NY-ADK, MD, and TN, *Le. argenticollis* adults were more likely to emerge in the spring if the puparia were shipped to the field in the

Table 1
Probability and odds ratios from binomial logistic regressions (1 = adult emergence, 0 = no emergence) of survival to emerged adult of *Leucotaraxis argenticolllis*. The probability explores, out of all the puparia what is the probability of surviving to emerge as an adult fly. Odds ratio is a calculation of the chances of survival in the summer compared to the winter treatment (summer treatment = S, winter treatment = W).

Site	Treatment	Probability	Odds Ratio	*p value* (α = 0.05)
Survival				
NY-ADK	S	0.833 ± 0.0459	0.6731 ± 0.3505	0.4471
	W	0.881 ± 0.0421		
NY-FL	S	0.914 ± 0.0369	0.9636 ± 0.61	0.9533
	W	0.917 ± 0.0326		
MA	S	0.623 ± 0.0621	0.0842 ± 0.0855	0.0001
	W	0.951 ± 0.0277		
MD	S	0.776 ± 0.0548	0.0842 ± 0.1649	0.0292
	W	0.922 ± 0.0335		
VA	S	0.617 ± 0.0709	0.1111 ± 0.0664	0.0002
	W	0.935 ± 0.0312		
NC	S	0.790 ± 0.0517	0.1917 ± 0.1282	0.0135
	W	0.952 ± 0.0273		
TN	S	0.367 ± 0.0622	0.0842 ± 0.0390	0.0002
	W	0.873 ± 0.0419		
WA	S	0.738 ± 0.0563	0.5740 ± 0.26	0.2203
	W	0.831 ± 0.0488		

preceding summer (S treatment group) versus those shipped the preceding fall (W treatment group) (Table 2, Fig. 2b). Although the probability of spring emergence did not differ by treatment group at the other sites, data at three sites (NY-FL, MA, and VA) were weakly suggestive of higher probability of spring emergence in the summer group, as indicated by an odds ratio > 1.0 (Table 2, Fig. 2b) (see Supp Table 5 for non-transformed model output).

For the model evaluating the probability of adult *Le. argenticollis* surviving the winter to emerge in spring (alive and spring emergence = 1, dead or fall emergence = 0), the interaction between site and treatment was significant, when compared to the null model ($X^2 = 41.646$, df = 2,17, p = <0.001). The data were weakly suggestive of a higher probability of survival to spring emergence in the summer group at sites NY-FL, NY-ADK, and MD (as indicated by an odds ratio > 1.0) and in the winter group at sites MA, VA, NC, TN, and WA (as indicated by an odds ratio < 1.0) (Table 3, Fig. 2c) (see Supp table 6 for non-transformed model output).

3.2. Abiotic effects on survival, diapause, and spring emergence

In the model evaluating the effect of abiotic factors on probability of survival to adult, only data from the summer treatment were included, because this treatment group had the most variation (Fig. 2a,Table 1) and was most biologically relevant since wild populations will experience summer conditions. Probability of survival was best explained by the interaction of fall mean daily relative humidity and fall mean daily max temperature (Supp table 7). The two abiotic factors in the model were tested for collinearity, and the VIF was found to be < 2.5.The slope of the relationship between temperature and humidity was statistically significant at 22 °C (p = <0.0001), 20 °C (p = <0.0001), 18 °C (p = <0.001), 16 °C (p = <0.0001), and 14 °C (p = 0.0021) (Supp table 8). At 12 °C the slope of the relationship between increasing humidity and temperature was non-significant (p = 0.1844, Fig. 3a). Temperature ranges were selected based on temperature ranges observed across observation sites (Supp table 3).

Variation in the probability of winter diapause among sites was

Table 2
Probability and odds ratios from binomial logistic regressions (1 = Spring adult emergence, 0 = Fall adult emergence) of overwintering diapause of *Leucotaraxis argenticolllis*. The probability explores, out of all flies that survived to adult, what is the probability of spring emergence. Odds ratio is a calculation of the chances of spring emergence in the summer compared to the winter treatment (summer treatment = S, winter treatment = W).

Site	Treatment	Probability	Odds Ratio	*p value* (α = 0.05)
Overwinter Diapause				
NY-ADK	S	0.873 ± 0.0449	3.63 ± 1.81	0.0097
	W	0.654 ± 0.0587		
NY-FL	S	0.774 ± 0.0575	1.828 ± 0.736	0.1489
	W	0.652 ± 0.0587		
MA	S	0.711 ± 0.0736	1.86 ± 0.828	0.1636
	W	0.569 ± 0.065		
MD	S	0.800 ± 0.0596	3.152 ± 1.436	0.0118
	W	0.559 ± 0.0646		
VA	S	0.690 ± 0.0859	1.170 ± 0.570	0.7478
	W	0.655 ± 0.0624		
NC	S	0.612 ± 0.0696	0.939 ± 0.374	0.874
	W	0.627 ± 0.0630		
TN	S	0.727 ± 0.095	2.974 ± 1.635	0.0474
	W	0.473 ± 0.0673		
WA	S	0.711 ± 0.0676	0.889 ± 0.41	0.7985
	W	0.735 ± 0.0631		

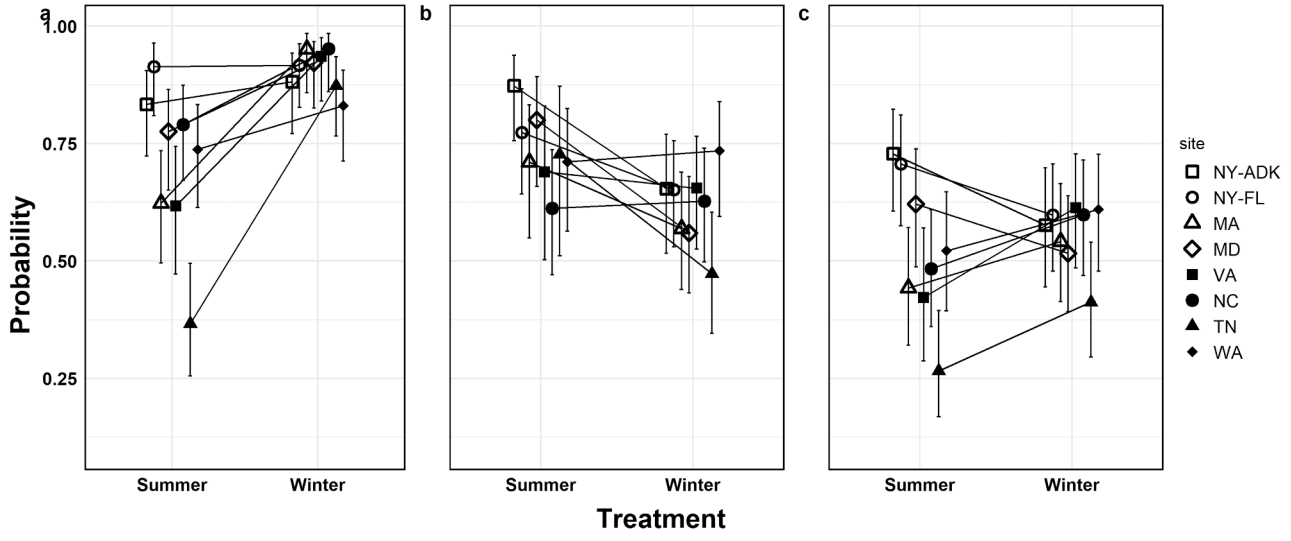

Fig. 2. (a) Probability of survival for *Leucotaraxis argenticolllis* by treatment at each observation site, (b) probability of spring emergence at each site between treatments (c) probability of survival and spring emergence. Summer treatment. puparia shipped to the observation sites on July 20, 2020, and Winter treatment: puparia shipped to the observation sites on October 5, 2020.

Table 3
Probability and odds ratios from binomial logistic regressions (Spring adult emergence, 0 = Fall adult emergence or no emergence) of survival to spring emergence of *Leucotaraxis argenticolllis*. The probability explores, out of all the puparia what is the probability of survival and spring emergence. Odds ratio is a calculation of the chances of survival and spring emergence in the summer compared to the winter treatment (summer treatment = S, winter treatment = W).

Site	Treatment	Probability	Odds Ratio	*p value* (α = 0.05)
Spring Emergence				
NY-ADK	S	0.728 ± 0.0559	1.971 ± 0.771	0.0829
	W	0.576 ± 0.0661		
NY-FL	S	0.707 ± 0.061	1.624 ± 0.623	0.2064
	W	0.598 ± 0.0592		
MA	S	0.443 ± 0.0652	0.672 ± 0.251	0.2873
	W	0.542 ± 0.0654		
MD	S	0.621 ± 0.0653	1.534 ± 0.58	0.2576
	W	0.516 ± 0.0642		
VA	S	0.423 ± 0.0742	0.461 ± 0.187	0.0561
	W	0.614 ± 0.0633		
NC	S	0.484 ± 0.065	0.629 ± 0.234	0.2133
	W	0.598 ± 0.0639		
TN	S	0.266 ± 0.0582	0.517 ± 0.205	0.0963
	W	0.412 ± 0.0635		
WA	S	0.522 ± 0.0663	0.699 ± 0.266	0.3458
	W	0.61 ± 0.0649		

similar between the two treatment groups; therefore, data from both treatments were included in the model using abiotic cues to predict overwintering diapause (i.e., spring emergence). The fall mean daily minimum temperature model best described the variation in overwintering diapause (Supp table 7). Although there was a decreasing trend in the probability of diapausing through winter as fall minimum temperatures increased, the slope was non-significant (p = 0.1082, Fig. 3b) (Supp table 9).

Analysis of abiotic effects on the probability of survival to spring emergence was performed using only the summer treatment group because, similar to the analysis of survival, this is where the most variation occurred (Fig. 3c, Table 3) and is the most biologically relevant. Mean daily fall relative humidity was most predictive, but temperature and humidity are known to be linked in insect physiology. Additive and interaction models using the lowest AIC temperature variable, mean of the fall daily mean temperatures, were run with mean fall daily relative humidity. The additive model including relative humidity and temperature had a similar AIC to relative humidity alone, so the additive model was used (Supp table 7). The two abiotic factors in the model were tested for collinearity, and the VIF was found to be < 2.5. There was a decrease in the probability of survival and spring emergence across all temperatures (8 °C – 16 °C), with a statistically significant slope (p = 0.0173), as relative humidity increased (Supp table 10). Temperature ranges were selected based on temperature ranges observed across observation sites (Supp table 3).

3.3. Intrapuparial stage of mortality

Uneclosed puparia that were dissected to assess mortality and identify stage of mortality were analyzed using Chi-square to better understand whether the intrapuparial effects were different across sites. The proportion of uneclosed living puparia was statistically different across sites in the study (Table 4). Out of all the uneclosed puparia, living puparia were found at observation site NY-ADK, MA, NC, and WA, while all the uneclosed puparia at NY-FL, MD, VA, and TN were found to be dead. All living puparia were in the phanerocephalic stage. The proportion of puparia that experienced intrapuparial mortality at each stage was statistically different across sites in the study (Table 5). A larger proportion of puparia experienced mortality at the phanerocephalic stage in MA, MD, VA, and TN, a trend suggesting warmer sites with lower survival also generally had more dead in that stage. The proportion of intrapuparial mortality was found to be more even between the phanerocephalic and pharate adult stage at NY-FL, MD, NC, and WA, with NY-ADK experience the highest mortality at the pharate adult stage. Mortality at the imago stage consisted of fully formed adult flies that did not fully emerge and died exiting the puparium.

Table 4
Chi-square results of the proportion of living to dead uneclosed puparia of *Leucotaraxis argenticolllis* by site.

Observation Site	Dead Puparia	Living Puparia	Test statistics and *P*-value (α = 0.01)
NY-ADK	15	3	$X^2 = 21.683$, df = 17,
NY-FL	11	0	$P = 0.002$
MA	25	1	
MD	18	0	
VA	23	0	
NC	16	1	
TN	45	0	
WA	18	5	

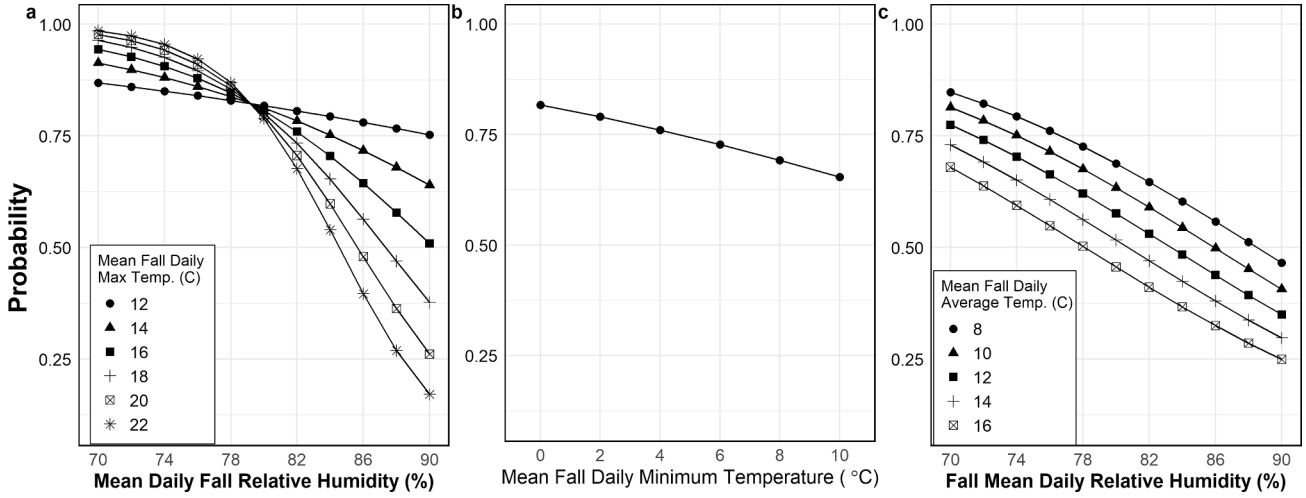

Fig. 3. (**a**) Effect of the interaction of mean fall daily max temperature (°C) and mean fall daily relative humidity on survival of *Leucotaraxis argenticolllis* to adult. (**b**) Effect of mean fall daily minimum temperature (°C) on number of adults that diapause to emergence in the spring (fall versus spring adult emergence). (**c**) The additive effect of mean fall daily average temperature (°C) and mean fall daily relative humidity on the spring emergence of adults (fall emergence and dead versus spring emergence).

Table 5
Chi square results of the proportion of intrapuparial mortality stage of *Leucotaraxis argenticolllis* at each site.

Observation Site	Imago	Pharate Adult	Phanerocephalic Pupae	Test statistics and *P*-value (α = 0.01)
NY-ADK	0	12	3	$X^2 = 38.194$, df = 14, $<P = 0.001$
NY-FL	1	6	4	
MA	0	6	19	
MD	2	7	9	
VA	0	6	17	
NC	0	8	8	
TN	2	6	37	
WA	2	7	9	

4. Discussion

Field collections of *Le. argenticollis* from the western native range have a bi-modal adult emergence that coincides with the two generations of *A. tsugae* (Dietschler et al. 2021). The second adult emergence likely represents the offspring of the first emergence, supported by observations that the secondary emergence only occurs after the primary emergence has completed in the field (Dietschler et al. 2021). The current study used only the first emergence as parents to produce puparia, thus exploring the possible variation in diapause among individuals of the second generation. Prey availability (oviposition of both *A. tsugae* generations) occurs between February and July in the native and introduced range, with substantial variation by latitude (McClure 1987, Gray and Salom 1996, Zilahi-Balogh et al., 2003a, 2003b, Mausel et al. 2008, Joseph et al. 2011). Adult *Leucotaraxis* spp. emergence and seasonal abundances of larvae have been shown to synchronize with the *A. tsugae* life cycle in the native range (Grubin et al. 2011, Rose et al. 2019, Dietschler et al. 2021). Our results show that spring emergence of diapaused adult *Le. argenticollis* will be synchronized with overwintering sistens oviposition, but summer-fall emergence appeared to be delayed. The rearing and holding temperature of 16 °C may have slowed the overall development of the second generation reared in the laboratory which could explain summer emergence of some individuals occurring later than would be expected for prey availability. However, this study shows that puparia exposed to a wide range of natural conditions can complete diapause and survive to emerge as adults during the *A. tsugae* oviposition stage in the following year.

The results between the east and west were consistent, supporting that the East Coast is suitable for *Le. argenticollis*. There was no difference in the probability of survival between treatments at the WA site, which is consistent with the northern most East Coast sites (NY-FL and NY-ADK), which saw the highest survival (Table 1, Fig. 2a). No difference in the probability of diapause was observed between WA treatments, similar to diapause results in NC, VA, MA, and NY-FL (Table 2, Fig. 2b). The probability of survival to spring emergence in WA summer treatment fell in the middle of the East Coast sites, with four sites having lower probability (TN, NC, VA, MA) and three sites having a higher probability (MD, NY-FL, NY-ADK) (Table 3, Fig. 2c). The conclusions we can draw on the variation of survival and diapause in the native western range are limited due to the inclusion of only one western site in the study, but it provides ample support that the east coast is suitable for *Le. argenticollis*.

To align with prey availability, puparied flies that do not emerge in early summer need to undergo winter diapause and emerge in the spring. Our data suggest that summer conditions may have a detrimental effect on survival (Table 1, Fig. 2a), but conversely summer cues may be necessary for the induction and/or maintenance of winter diapause. This is supported by observations of more *Le. argenticollis* in the summer treatment, where puparia were exposed to summer conditions, emerging as adults the following spring. This trend was observed at all sites except in NC, VA, and WA (Table 2, Fig. 2b). Taken together, these "summer effects" result in no significant treatment difference of flies that survived to emerge in spring within sites (Table 3, Fig. 2c). Considering only the summer treatment, an increasing probability of overwinter survival and spring emergence is observed moving from southern to more northern sites (Table 2, Fig. 2c). More research is needed to identify the cues that determine whether development will lead to a second spring/summer emergence (on the progrediens generation) or diapause through the winter to emerge the following spring (on the following year's sistens generation).

Based on dissections of uneclosed puparia at the end of this study, most of the uneclosed puparia were dead (Table 4), which indicates that the experiment was of sufficient duration to capture all potential emergence. It seems likely that diapause occurs during the phanerocephalic stage of intrapuparial development, because all of the living puparia and most of the dead puparia contained the phanerocephalic stage (Table 4 and 5), which is consistent with other groups classified as cyclorrhaphous (Fraenkel and Hsiao 1968), but the counts varied by site with a trend suggesting warmer sites with lower survival also generally had more dead in that stage. All sites experienced some intrapuparial mortality of insects in the pharate adult stage, which had broken dormancy and failed to emerge.

While this study attempted to explore the overwintering survival and diapause of both *Leucotaraxis* species that specialize on *A. tsugae* in western North America, it is clear that *Le. piniperda* uses a different strategy (i.e., developmental stage and/or environmental cue) for diapause. The observations in this study of lab-reared *Le. piniperda* puparia completing emergence prior to winter are consistent with other laboratory trials exploring development rates (TDB pers. obs.). *Leucotaraxis* spp. larvae are present overwinter in the native western range (Grubin et al. 2011, NJD pers. obs.) suggesting *Le. piniperda* could be overwintering in the larval stage. We were able to demonstrate that both species can successfully be laboratory reared to adult. However, more research is needed to explore how *Le. piniperda* overwinters, the implications of survival in the east, and how they will interact with other specialist predators.

4.1. Survival and phenological mismatch

Survival to adult in the winter treatment, that avoided summer conditions, was similar across sites (Fig. 2a), suggesting that winter conditions had minimal influence on survival, contrary to other predators in this system (i.e., *La. nigrinus*, see Mausel et al. 2010). Seasonal cold hardening of diapausing insects through physiological mechanisms such as altered cryoprotectant concentrations (glycerol, glucose, alanine) and upregulation of proteins (heat shock proteins) among others lead to increased cold tolerance (Teets and Denlinger 2013). Overwintering diapause of *Sarcophaga crassipalpis* Macquart (a cyclorrhaphous species, Diptera:Sarcophagidae) occurs during the phanerocephalic stage (Denlinger 1972), the same stage of winter diapause in *Le. argenticollis* suggested by our data. Studies show *S. crassipalpis* phanerocephalic pupae increase glycerol production (Lee et al. 1987), elevate concentrations of heat shock proteins (Flannagan et al. 1998, Hayward et al. 2005) and have the lowest super cooling point of any other developmental phase (Lee and Denlinger 1985). Our results suggest that winter temperatures throughout the climatic range of eastern hemlock will have little effect on the survival of *Le. argenticollis*, whereas temperatures have been shown to impact establishment of other predators in this system (Mausel et al. 2010). In contrast, we found higher temperatures and higher relative humidity in the summer-fall negatively impact overall survival and the number of adults that survived diapause to emerge in the spring. Summers in the native Pacific Northwestern range of *Le. argenticollis* are characteristically dry with mild temperatures, indicating potential adaptation to these conditions. Climate variation in temperature and humidity has been found to impact the survival of both hosts and their parasitoids in addition to parasitoid emergence in the emerald ash borer system (Wetherington et al. 2017).

The seemingly robust tolerance of *Le. argenticollis* to winter conditions on the east coast provides optimism for success of this species to establish throughout the introduced eastern range, but further exploration of the effects of high temperature and humidity are needed.

The introduced range of *A. tsugae* is spread across a large latitudinal gradient from Georgia to Nova Scotia with significant environmental variation, meaning biological control agents need to adapt to and synchronize with prey in diverse locations. Introducing species from one part of the world to another can lead to phenological and/or climate mismatch. These mismatches can be particularly impactful on biological control programs leading to incomplete control throughout the introduced range (Harms et al. 2021). The life cycles of *A. tsugae* specialist predators are closely tied to those of the pest (Kohler et al. 2016, Rose et al. 2019, Neidermeier et al. 2020, Dietschler et al. 2021) and disruptions may affect their food supply. We found that higher minimum temperatures in the fall through early winter (July 20 - December 31) led to fewer *Le. argenticollis* overwintering to emerge in the spring (Fig. 3b). Winter temperatures have been found to be a key predictor in biological control temporal synchronization and effective control, with temperature (i.e., warmer winter and/or summer temperatures) leading to early emergence and mismatch with prey (Wetherington et al. 2017, Ferracini et al. 2022). High summer temperatures have been linked to significant aestival mortality of *A. tsugae* in the introduced range (Sussky and Elkinton 2015, Mech et al. 2018). This combined with our results showing increasing mortality as mean fall daily max temperatures increase (mean of daily max from July 20 – December 31, 2020, Fig. 3a) suggest this system is susceptible to summer abiotic factors. Insect development is closely tied to temperature and the increasing effects of climate change are likely to lead to more variation in phenological synchrony (Wetherington et al. 2017, Damien and Tougeron 2019, Ferracini et al. 2022, Grevstad et al., 2022). Understanding how variation in abiotic factors influence predator synchronization throughout the introduced range is important to establishing effective biological control.

4.2. Bet hedging

Bet-hedging, or risk-spreading, is the idea that a population reduces potential exposure to adverse environmental conditions through phenotypic variation in physiological or behavioral traits (Hopper 1999, Simons 2011). The observation of facultative diapause in *Le. argenticollis* could be explained by the evolution of a bet-hedging strategy. In the native western range of *Le. argenticollis*, *A. tsugae* populations are controlled by predators and fluctuate widely (Crandall et al. 2022) leading to inconsistent food supply. Both the sistens and progrediens generations of *A. tsugae* experience predation by specialist and generalist predators (Kohler et al. 2008), with three specialist predators (*La. nigrinus* and two *Leucotaraxis* spp.) being present in abundance throughout the life cycle (Grubin et al. 2011, Kohler et al. 2016, Rose et al. 2019, Dietschler et al. 2021). Heavy predation and low survival of the spring-summer progrediens generation of *A. tsugae* (Crandall et al. 2022) could lead to resource competition for future *Le. argenticollis* offspring to complete development, making a facultative diapause advantageous as it provides an opportunity for prey populations to rebound. In addition, Weed et al. (2016) observed diapause of the spring-summer progrediens generation in the native western range, and while thought to be a rare occurrence, this removed an entire generation of prey resources from the landscape. Diapause of *A. tsugae* sistens has been shown to be facultative and induced by warm temperatures (Salom et al. 2001), providing another explanation why facultative diapause of *Le. argenticollis* could benefit synchronization with prey. A diversified diapause strategy can be beneficial in systems with unpredictable biotic and/or abiotic environmental factors (Le Lann et al. 2021). In the eastern US, *La. nigrinus* has become established in many areas (Mausel et al. 2010, Foley et al. 2019, Jubb et al. 2021) and has been shown to reduce population of the overwintering sistens generation of *A. tsugae* (Mausel et al. 2017, Jubb et al. 2020). Despite substantial predation by *La. nigrinus,* prey populations have been observed to rebound in a single generation with no significant impact on the spring-summer progrediens generation (Crandall et al. 2020), which is explained by density-dependent survival and fecundity in *A. tsugae* (Sussky and Elkinton 2014, Elkinton et al., 2011). The significant reduction in the overwintering generation could lead to prey resource competition for *Le. argenticollis* co-occurring with *La. nigrinus*. However, facultative diapause of *Le. argenticollis* would allow for increased predator population building on the spring-summer generation of *A. tsugae*, and/or the opportunity for a population rebound, replenishing prey resources for the following year. A bet-hedging strategy could be beneficial in eastern North America where both abiotic and biotic factors may reduce overwintering populations of *A. tsugae*. *Adelges tsugae* often experiences substantial winter mortality in the north (Trotter and Shields 2009, McAvoy et al. 2017, Tobin et al. 2017) and can take multiple years to fully rebound.

A *meta*-analysis of insect diapause in the northern hemisphere indicates little support for environmental cues facilitating bet-hedging but does indicate that onset of winter correlates well with induction of diapause (Joschinski and Bonte 2021), aligning with our observation that lower fall temperatures promote winter diapause induction and/or maintenance. However, strong maternal effects on diapause were observed in hymenopteran parasitoids (*Aphidius avenae* Haliday and *A. rhopalosiphi* De Stefani-Perez, Hymenoptera: Braconidae) of aphids, in which diapausing offspring increase as a function of maternal competition, believed to be a means to avoid superparasitism (Tougeron et al. 2018). A next step could be to investigate whether maternal *Le. argenticollis* have a similar level of control over the facultative diapause of their offspring based on cues about overall food availability/density or competition for oviposition sites (i.e., phenotypic plasticity of polyphenism; Joschinski and Bonte 2021). Alternatively, *Le. argenticollis* may be using diversified bet-hedging, producing some of each phenotype every year regardless of prey cues. Our study provides the first extensive look at the emergence trends of *Le. argenticollis*, and bet-hedging as a means of improving fitness could be a fruitful avenue of research.

4.3. Implications for Adelges tsugae management using biological control

Specialist predators and prey in the *A. tsugae* system are adapted for cool weather activity, with prey and predators all spending the summer in dormancy. Although research has led to concern around winter's effects on establishment probability of *A. tsugae* predators, summer drought and/or extreme high temperature events might pose a greater threat to some predators in this system, potentially causing predator populations to crash (Harvey et al. 2020). Negative impacts of summer temperatures and other abiotic summer factors on the survival and behavior of *A. tsugae* have been explored in the introduced range (see; Sussky and Elkinton 2015, Mech et al. 2018, Mayfield and Jetton 2020), with our study representing a first look at how summer affects their predators. *Leucotaraxis* spp. puparia are located on branches (Kohler et al. 2016, Celis et al. 2022), relatively exposed to high temperatures, while their competitors *La. nigrinus* are pupating and aestivating as adults in the soil (Zilahi-Balogh et al., 2003a, 2003b). Winter temperatures are thought to be one of the main factors influencing establishment of *La. nigrinus* in the eastern US, with lower rates of establishment in hardiness zones 5b and colder (Mausel et al. 2010). Increased survival of *Le. argenticollis* to adulthood in the winter treatment (in which summer temperatures were avoided), and at colder sites in the summer treatment (in which flies were exposed to summer temperatures), suggests it may be valuable to evaluate *Le. argenticollis* in areas where *La. nigrinus* has struggled to become established. These results provide additional hope for successful management of *A. tsugae* using biological control, with indications that *Le. argenticollis* can survive on the east coast to compliment *La. nigrinus* feeding and offer control in areas where *La. nigrinus* struggles to establish. Establishing multiple biocontrol agents with complimentary feeding strategies can often be necessary and

beneficial to effective management (Crandall et al. 2020, 2022, Stiling and Cornelissen 2005), and our results support continued research into *Leucotaraxis* spp. as potentially effective agents for biological control of *A. tsugae*.

CRediT authorship contribution statement

Nicholas J. Dietschler: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Tonya D. Bittner:** Methodology, Writing – original draft. **Nina G. Devine:** Methodology, Investigation, Writing – review & editing. **Albert Mayfield III:** Investigation, Writing – review & editing. **Carrie Preston:** Investigation, Writing – review & editing. **Ryan S. Crandall:** Investigation, Writing – review & editing. **James Parkman:** Investigation, Writing – review & editing. **Zachary Simek:** Investigation, Writing – review & editing. **Biff Thompson:** Investigation, Writing – review & editing. **Matthew E. Lonsdale:** Investigation, Writing – review & editing. **Budd Veverka:** Investigation, Writing – review & editing. **Joseph S. Elkinton:** Supervision, Writing – review & editing. **Scott M. Salom:** Methodology, Supervision, Writing – review & editing. **Mark C. Whitmore:** Methodology, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2023.105233.

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