



Eastern and western lineages of *Leucotaraxis argenticollis* (Diptera: Chamaemyiidae), a biological control agent for *Adelges tsugae* (Hemiptera: Adelgidae), are capable of hybridization in the laboratory

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Hybridization between eastern and western lineages of the biological control agent, *Leucotaraxis argenticollis* (Zetterstedt) (Diptera: Chamaemyiidae), was demonstrated in the laboratory. The western lineage is abundant on hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae) in western North America while a genetically distinct eastern lineage feeds mostly on pine adelgids (Hemiptera: Adelgidae: *Pineus* spp.). Western flies have been released in eastern North America but establishment has not been observed. When reared on *A. tsugae* in the laboratory, many eastern *L. argenticollis* larvae did not survive, and of those that did reach pupariation, 21% survived to the adult stage. To assess hybridization, eastern females were placed with western males for no-choice mating. They produced hybrid offspring with 8% survival to the adult stage when reared on *A. tsugae*. Further investigation of hybrid fitness, assortative mating, and the prey-seeking strategies of *Leucotaraxis* would aid in predicting the outcome of potential hybridization in the field.

Keywords: no-choice test, HWA, silver fly, forest pest.

Introduction

Classical biological control has a set of best practices (Van Driesche and Reardon 2004). Agents should be specialists, not cause ecological or economic harm, and show potential to establish and regulate pest populations, alone or in combination (Denoth et al. 2002). The potential of agents to hybridize with native species has been considered a concern (Hopper et al. 2006) or a facilitator of establishment (Yamaguchi et al. 2019) by helping to overcome mate-finding limitations at low population densities (Taylor and Hastings 2005).

An invasive lineage of hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Hemiptera: Adelgidae), from Japan, is a pest of hemlock (*Tsuga canadensis* and *T. caroliniana*) in eastern North America, where it causes widespread tree death and ecological change (Ellison et al. 2018). Biological control strategies include introducing predators from western North America (Mayfield et al. 2023), where there is an endemic lineage of HWA (Havill et al. 2016). Two species of western silver flies *Leucotaraxis argenticollis*

(Zetterstedt) (Diptera: Chamaemyiidae) and *Le. piniperda* (Malloch) are being released to complement 2 species of beetles, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) and *La. osakensis* Shiyake and Montgomery (Mayfield et al. 2023).

Both silver fly species have separate genetic lineages in western and eastern North America, with western lineages abundant on HWA, and eastern lineages abundant on pine adelgids (Hemiptera: Adelgidae: *Pineus* spp.) (Havill et al. 2023). It was suggested that after release, western flies might hybridize with eastern flies because of their close genetic relationship, so microsatellite loci were developed to detect introgression (Havill et al. 2023). Based on studies of phenology (Wantuch et al. 2019, Dietschler et al. 2021, Preston et al. 2023), it is possible that eastern and western lineages could overlap temporally as adults in biocontrol release areas, facilitating hybridization.

While performing post-release monitoring at a western silver fly release site in New York in June 2023, a heavy infestation of European pine adelgids, *Pineus pini* (Goeze) (Hemiptera: Adelgidae)

on Scots pine (*Pinus sylvestris* L.) was found being predated by eastern *Le. argenticollis*. This gave an opportunity to perform a development trial and laboratory crosses with their Western counterpart. Two no-choice scenarios were tested: (1) Will eastern flies oviposit and complete development on HWA, and (2) Will eastern females cross with western males to produce offspring that complete development on HWA?

Materials and Methods

Prior to our study, western *Leucotaraxis* was released at High Tor Wildlife Management Area, New York, USA (Lat: 42.63, Long: -77.36). Adult flies collected from quarantine (see Dietschler et al. 2021) were transported in 50-dram plastic vials to the selected field site and released under eastern hemlock trees infested with HWA. A mixed-species (*Le. argenticollis* and *Le. piniperda*) release of 603 flies occurred on 30 May 2019, and 772 *Le. piniperda* were released on 29 April 2020. Western flies have not been recovered at this site (NJ Dietschler, unpublished monitoring data).

Source of Eastern Flies

To obtain eastern *Leucotaraxis*, on 2 June 2023, two 2-gallon bags of terminal *Pinus sylvestris* branches infested with *P. pini* were collected from High Tor. Stems were cut and inserted in saturated floral foam in 2 acrylic insect cages (43 cm × 36 cm × 30 cm) placed at room temperature near a west-facing window. Cages were monitored daily for emergence and specimens were periodically preserved frozen in 95% ethanol throughout the study for morphological and genetic identification. Adult flies were sexed by observing the terminus of the abdomen and identified to species by observing post-pronotal setae for females and external genitalia for males (photos in Gaimari and Havill 2021, Dietschler et al. 2021 supplement).

Rearing Eastern Flies on HWA

On 12 June 2023, an acrylic mating cage in a growth chamber at 16 °C, 70% RH, and 12:12 L:D housed adult flies after they emerged from the pine foliage. Each cage included a saturated floral foam block and a paste of honey and wheat (Evergreen Growers Supply, Clackamas, OR) on yellow laboratory tape on the cage wall. A total of 22 females and 33 males were placed into the cage through 29 June.

HWA-infested branches for fly oviposition and feeding were collected from locations with no *Leucotaraxis* release history near Oswego, NY (43.4998, -76.4519) during the winter of 2022 to 2023 and stored at 3.5 °C in 5-gallon buckets at 15:9 L:D. These branches (about 1 m each) were collected when HWA nymphs were not yet mature and prior to winter mortality events and were maintained with biweekly trimming of the stems and water changes. Under these storage conditions, HWA mature and lay eggs, but the eggs do not hatch (Salom et al. 2002), thus producing large ovisacs. At the time of use, the branches had been in storage for about 6 mo and had overwintering HWA adults with eggs of the spring generation. Oviposition bouquets of 1 to 3 twigs (15 to 20 cm) inserted into cubes of parafilm-wrapped hydrated floral foam were placed into the mating cage for 1 to 7 days and then checked under a stereomicroscope for eggs.

Eastern fly eggs were either singly reared or mass reared. For single rearing, individual eggs were transferred to a new infested twig (2 to 4 cm) in a 44-ml deli cup. Eggs were checked every 2 to 3 days, and once hatched, a fresh twig was added every 2 to 3 days. Larvae moved among the twigs to find food and depleted twigs were

removed at the next feeding to avoid the buildup of plant material. For mass rearing, multiple bouquets with fly eggs were reared together in cages as described in Dietschler et al. (2023). Briefly, twigs used for oviposition were transferred to a new bouquet with the addition of fresh supplemental infested foliage in direct contact with the oviposition foliage and stored in ventilated jars at 20 °C and 12:12 L:D. Larvae were not disturbed until the cohort pupariated.

Crossing Experiment

A 30.5-cm³ mating cage (Bugdorm, MegaView Science, Taiwan) was established on 20 June 2023 with eastern females and western males. Eastern females were added as they emerged from the pine foliage collected on 2 June for a total of 15 females on 28 June. On 23 June 2023, 15 western males from the quarantine facility at Cornell University (USDA APHIS facility PPQ-NY-286783) were added to the cage. This facility provides containment of HWA-infested western hemlock foliage from which western flies emerge and are collected for study or release as described in Dietschler et al. (2021, 2023). The temperature of the cross-mating cage was initially 16 °C, but was raised to 20 °C on 3 July to increase the oviposition rate. Three oviposition bouquets from the same cold-storage branches described previously were exposed to the flies, on 5 to 11 July, 10 to 17 July, and 17 to 24 July, then reared with a combination of single-rearing and mass-rearing approaches as described previously.

Genetic Analyses

DNA was extracted nondestructively using the MagMAX DNA Ultra Kit (Applied Biosystems, Waltham, MA) on a KingFisher Flex instrument (Thermo Fisher, Waltham, MA). Vouchers of flies and *P. pini* were deposited in Yale University's Peabody Museum. Each individual was genotyped with 15 microsatellite loci as described in Havill et al. (2023). Eastern versus western lineage was evaluated with STRUCTURE 2.3.4 (Pritchard et al. 2000) which analyzes multilocus genotypes using an iterative Bayesian clustering algorithm to group similar patterns of variation. Program settings were: $K = 2$ clusters with correlated allele frequency, admixture ancestry, 50,000 burn-in, and 1,000,000 sample generations. The data set included reference sets of 76 *L. argenticollis* collected from *Pineus* spp. across eastern North America, and 83 were collected from HWA in western North America. These same genotypes were reported in Havill et al. (2023), clusters 1 and 4, respectively.

Results

Source of Eastern Flies

Eighty flies emerged from the pine foliage. Of these, 55 were used as parents for the eastern lineage development trial, 15 for crossing with western flies, and 10 were preserved as vouchers.

Rearing Eastern Flies on HWA

None of the 10 eggs reared individually developed to eclosion. One larva was lost, 5 died, and 4 pupariated; on 7 March 2024, puparia were dissected and found to be dead. For the mass-rearing, many dead larvae were observed on the twigs but were not counted or staged. The visible dead larvae were mostly second instar (based on size), but it is possible that smaller larvae also died and were missed in the inspection. The dead larvae appeared to have starved because they were often crowded together and HWA ovisacs were depleted. A total of 19 flies pupariated, and from these, 4 adults (21%) eclosed between November 2023 and January 2024. The remaining pupae were confirmed dead after dissection in July to August 2024.

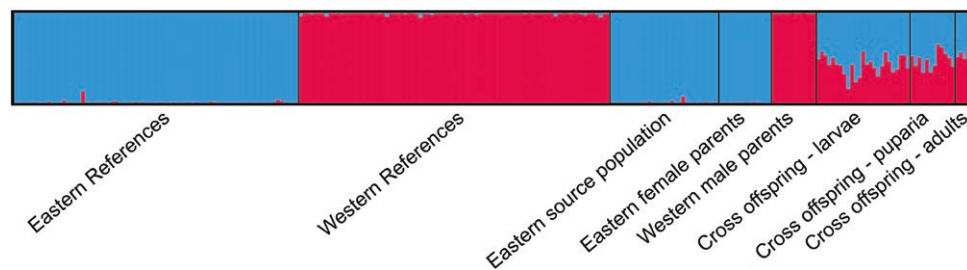


Figure 1. STRUCTURE plot for *Leucotaraxis argenticollis* individuals genotyped with 15 microsatellite loci. Each thin vertical bar represents 1 individual and the height of each color within the bar represents the probability of assignment to each of 2 clusters. Vertical black lines separate categories of individuals. Eastern and western reference genotypes are from Havill et al. (2023), clusters 1 and 4, respectively. Sample sizes: 76 eastern reference specimens, 83 western reference specimens, 29 eastern source specimens (mixed sexes from the caged pine), 14 eastern female parents, 12 western male parents, 25 larvae, 12 puparia, and 4 adult offspring from the crossing experiment. (Eastern offspring from the feeding trial were not genotyped.)

Crossing Experiment

From the cross between eastern females and western males, there was an excess of eggs that could not be accurately counted because they are often deposited hidden deep within HWA ovisacs. Of these, 68 eggs hatched and were observed and followed individually. Nine late-instar larvae and 10 puparia were preserved to ensure fresh specimens for genotyping. Four larvae did not develop after hatching, and 19 more died before pupariating. The dead larvae were mostly second instar and appeared to develop an infection, possibly fungal, causing discoloration and rigidity of the cuticle. Twenty-six pupariated, 4 of which emerged as adults (3F:1M). Survival is estimated at 8% (larva to adult) based on the 49 individuals that were allowed to progress. All dead individuals (parents and offspring) were preserved for genotyping, but not all were successfully genotyped due to DNA degradation.

Genetic Analysis

STRUCTURE analysis confirmed that the flies collected from *P. pini* used to initiate the development and crossing studies were eastern lineage *Le. argenticollis* and that the parents from the biocontrol rearing facility were western *Le. argenticollis* (Fig. 1). As seen by vertical bars with colors corresponding to probability of assignment to western (red) or eastern (blue) lineage, all of the parents ($n = 26$) were assigned to their corresponding reference cluster (lineage) with $P > 0.9$. The offspring of the cross ($n = 41$) were confirmed to be hybrids, with probability of assignment of $0.16 < P < 0.65$ (mean 0.45) to the western cluster and $0.35 < P < 0.84$ (mean 0.55) to the eastern cluster (Fig. 1).

Discussion

Given no choice, eastern *Le. argenticollis* oviposited on eastern hemlock infested with HWA. Larval development to the adult stage occurred at low rates with 0 of 10 reared individually, and 4 of 19 (for pupa to adult: 21% survival) in mass rearing. This low developmental success is consistent with the low incidence of eastern *Le. argenticollis* on HWA in eastern North America (Havill et al. 2023); however, our study cannot evaluate the impact of prey species on development. Rearing of silver flies in the laboratory is difficult due to timing the collection of high-quality prey to support development, so is not an accurate estimate of success in the field. The HWA-infested foliage had been stored for six months before being fed to flies, with fungal growth on adelgid honeydew around many ovisacs, so the survival rate may have been affected by low food quality or quantity.

When given no choice of mates, eastern *Le. argenticollis* females mated with western males and produced hybrid offspring. Some of

these developed to the adult stage on HWA (4 out of 49 larvae, 8%). Genetic analysis confirmed the eastern and western lineage membership of parents and the hybrid identity of offspring. The developmental success of hybrids compared to parental lineages on the same prey was not directly tested. The adult hybrid offspring (3F:1M) appeared healthy and normal, but due to a lack of available prey for further rearing, it was not possible to test later-generation crosses.

The impact of hybridization on HWA biological control is not known, but confirmation of a successful east–west cross in the lab merits further investigation. We recommend robust post-release monitoring of *Leucotaraxis* populations in eastern North America for evidence of hybridization with the western lineages. Further investigation of eastern lineage phenology, hybrid fitness, and assortative mating would aid in predicting the outcome of hybridization, if it were to occur in the field.

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Author contributions

Tonya Bittner (Conceptualization [Lead], Investigation [Lead], Methodology [Equal], Writing—original draft [Lead]), Nathan Havill (Formal analysis [Lead], Funding acquisition [Equal], Visualization [Lead], Writing—review & editing [Lead]), Nicholas Dietschler (Investigation [Equal], Methodology [Equal], Writing—review & editing [Equal]), Zephyr Zembrzski (Data curation [Lead], Investigation [Equal], Writing—review & editing [Supporting]), and Mark Whitmore (Funding acquisition [Equal], Project administration [Lead], Writing—review & editing [Equal])

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