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# Effects of larval stage and diapause status on cold hardiness of emerald ash borer (Coleoptera: Buprestidae): implications for winter survival and establishment

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Though a minor forest pest in its native range in Asia, the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire, causes widespread ash (*Fraxinus* spp) mortality throughout its invaded range in North America and Europe. The rapid range expansion demonstrated by EAB suggests this beetle possesses extraordinary tolerance for a broad range of climatic conditions. While previous studies have measured the cold tolerance of overwintering EAB larvae, none have considered the effect of developmental stage or diapause condition on cold hardiness. By testing the supercooling point (SCP) of lab-reared and field-collected larvae, we evaluated EAB cold hardiness at multiple developmental stages and diapause phases following exposure to different temperature regimes. Results of SCP testing indicated feeding larval stages and larvae in late diapause may be most susceptible to freeze-induced mortality, suggesting that semivoltinism and late-winter freeze events may limit winter survival within EAB populations. Results of field studies confirmed that cold hardiness in EAB is a plastic trait that increases with exposure to lower temperatures, but indicated plasticity may differ between populations. Together, these findings indicate that developmental stage, diapause phase, induction of cold hardiness, and geographic origin may all contribute to the ability of EAB larvae to acclimatize to and survive cold temperatures. Our findings contribute to our understanding of how environmental and physiological factors interact to impact winter survival of EAB and therefore, may improve estimates of the potential invasive range of EAB.

*Keywords: Agrilus planipennis*, cold tolerance, invasive, supercooling, voltinism

## Introduction

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is the most destructive invasive forest pest of North American ash (*Fraxinus* spp.) in the United States and has killed hundreds of millions of trees since it was first detected in Michigan in 2002 (Poland and McCullough 2006, Morin et al. 2017). As of 2025, EAB has been detected in 37 US states and 6 Canadian provinces (Emerald Ash Borer Information Network 2025) and will likely continue to spread throughout the range of ash in North America. The recent 2022 detection of an EAB population in Oregon has highlighted the serious concern about the expansion of EAB to and along the West Coast (Popkin 2022). The beetle has also spread rapidly in European landscapes. In 2010, EAB was detected in Moscow (Izhevskii and Mozolevskaya 2010) and has since spread northwest to Saint Petersburg and southwest into Ukraine as far as Kiev (Drogvalenko et al. 2019, Musolin et al. 2021, EPPO 2023). The ongoing dispersal and establishment of this Asiatic beetle across both North America and Europe suggest that EAB can survive a broad range of climatic conditions, including extreme winter cold events (Crosthwaite et al. 2011, Duell et al. 2022). To accurately predict the potential extent of the invasive range of EAB, it is necessary to understand the limits of this species' cold tolerance.

Winter mortality is one of the primary abiotic factors limiting the range of insect species (Bale 2002, Chown and Terblanche 2007, Bale and Hayward 2010), including invasive insects outside of their native range (e.g., Evans and Gregoire 2007, Paradis et al. 2008, Trotter and Shields 2009). As a means of surviving harsh environmental conditions, many insect species enter a seasonal period of diapause (Andrewartha 1952, Denlinger 2022) that generally consists of distinct phases

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including initiation, maintenance, termination, and postdiapause (Koštál 2006, Koštál et al. 2017). Upon the initiation of diapause, development halts and metabolism slows, and once diapause is terminated, insects resume development in response to favorable conditions (Andrewartha 1952, Denlinger 2002, 2022, Koštál 2006).

Insects that diapause during the winter may undergo physiological changes that increase their tolerance for cold temperatures (Andrewartha 1952, Somme 1982, Denlinger 2002, 2022, Chown and Terblanche 2007, Bale and Hayward 2010). One of the primary mechanisms by which insects increase cold hardiness is by lowering the temperature at which ice crystals form and the insect freezes, a process called supercooling. Insects can accomplish this by increasing the production of cryoprotectants (e.g., glycerol), reducing water content, and removing or inactivating nucleating agents (e.g., by clearing gut contents) that could initiate the formation of ice crystals (Somme 1982, Zachariassen 1985, Bale 1987, 2002, Block et al. 1990). Within an insect species, the supercooling point (SCP) is a plastic trait that can vary seasonally but can also change in response to exposure to low ambient temperature (Zachariassen 1985, Bale 1987, Lee et al. 1987). Though cold hardiness and diapause are not often studied concurrently, cold hardiness and supercooling ability may be dependent on the state and phase of diapause (Bale and Hayward 2010, Dang et al. 2025).

EAB overwinters as both immature larvae (1st to 4th instars, hereafter referred to as L1 to L4) and mature J-shaped larvae (JL) in both its native range in northeast Asia (Wei et al. 2007, Wang et al. 2010) and its invasive range in North America (Cappaert et al. 2005, Duan et al. 2010, 2014). Throughout immature larval development (L1 to L4), EAB larvae molt 3 times while feeding on the phloem of host trees. Late L4 larvae chew and enter pupation chambers in the outer sapwood or bark where they stop feeding and develop into JL. In late fall, JL enter an obligatory diapause that terminates only after a winter chill period (Duan et al. 2010, 2014, 2021). With the onset of warming temperatures, larvae resume development and become prepupae (PP), a straightened and visibly shorter and rounder larval developmental stage, before pupating in spring and emerging as adults approximately 1 month later (Duan et al. 2010). EAB populations in North America are univoltine, semivoltine, or a mix of both, depending on climate, timing of oviposition, and host tree species and condition (Cappaert et al. 2005, Duan et al. 2010, 2014). Populations in colder climates are more likely to be semivoltine, as colder temperatures slow development so that larvae may not reach the JL stage before the onset of winter (Duan et al. 2021). EAB that overwinter as immature larvae require a second winter as diapausing JL, after which they continue development through the prepupa, pupa, and adult stages (Duan et al. 2010, 2014). Despite the successful establishment of EAB in North America, EAB larvae are susceptible to freeze-induced mortality, especially during extreme cold events such as polar vortices when rapid drops in temperature do not provide larvae with time to gradually acclimate to colder temperatures (Jones et al. 2017, Duan et al. 2020). However, phenotypic plasticity of cold hardiness (Venette and Abrahamson 2010, Sobek-Swant et al. 2012, Duell et al. 2022) and potential genetic selection for cold hardiness during invasion (Colautti and Lau 2015) have allowed survival, establishment, and northward range expansion of EAB despite subzero temperatures.

Previous studies have tested the cold hardiness of overwintering EAB larvae (Venette and Abrahamson 2010, Crosthwaite et al. 2011, Sobek-Swant et al. 2012, Duell et al. 2022). However, none of these studies assessed cold hardiness differences among immature larvae (L1 to L4), diapausing JL, postdiapause JL, and PP. Identifying these differences is likely critical to predicting cold hardiness and winter mortality of EAB since semivoltine populations overwinter as both immature and mature larvae (Cappaert et al. 2005, Duan et al. 2010, 2014), and developmental stage and diapause phase of mature larvae changes through winter. EAB form JL and initiate diapause in the late summer or fall, maintain diapause through late fall and early winter, then enter the postdiapause phase during which they may remain in a metabolically suppressed state but are temperature-sensitive and ready to resume development and become PP with the onset of warmer temperatures (Duan et al. 2021, Dang et al. 2025). In North America, extreme temperature events such as polar vortices occur most often in mid- to late winter (Cohen et al. 2018, Overland and Wang 2019), which overlaps with the temperature-sensitive postdiapause phase of JL EAB. As a result, overwintering larvae may experience greater winter mortality in the postdiapause JL phase. Phenology and climate suitability models have been published with the aim of aiding managers in determining when and where to monitor for EAB (Cuddington et al. 2018, Barker et al. 2023), but estimates of the potential geographic extent of invasive EAB establishment are limited by the lack of knowledge of how EAB cold hardiness varies throughout winter and among populations.

To evaluate the effects of developmental stage, diapause phase, and low temperature exposure on the cold hardiness of EAB larvae, we measured the SCP of both lab-reared larvae and field-collected larvae from 2 different geographic locations experiencing different climatic regimes. Using lab-reared larvae we tested 2 hypotheses: (H1) SCP of mature larvae (JL) is lower than SCP of immature larval stages (L1 to L4) before exposure to winter temperatures and (H2) SCP of JL changes throughout different stages of diapause with SCP lowest (cold hardiness highest) during deep diapause. By testing SCP of field-collected larvae from Western Massachusetts and Delaware, USA, we tested our third hypothesis: (H3) SCP temperatures of the 2 nonfeeding stages of EAB that overwinter (i.e., JL and PP) increase with exposure to increasing temperatures and this temperature-dependent response differs between populations.

## Materials and Methods

### EAB Rearing

We used lab-reared EAB larvae to test the effects of EAB larval stage (H1) and diapause status (H2) on SCP. EAB larvae for laboratory experiments were reared at the United States Department of Agriculture (USDA), Agricultural Research Service (ARS) Beneficial Insects Introduction Research Unit (BIIRU) in Newark, DE from eggs laid by adults collected from infested green and white ash trees (*Fraxinus pennsylvanica* Marsh. and *Fraxinus americana* L., respectively) harvested in Maryland (38.5222° N, 77.1025° W) during the late fall and early winter of 2020. Emerging EAB adults were reared on bouquets of greenhouse-produced tropical evergreen ash (*Fraxinus uhdei Wenz.*) leaves for egg production according to methods described in previous publications (Duan et al. 2011,

2013a, 2013b, 2021). Briefly, approximately 20 500-ml cups containing 5 gravid females each (approximately 100 females total, mated from 14 to 17 days old) were provided coffee filter paper as substrate for oviposition. Freshly laid EAB eggs (laid within 48 to 72 h) were pooled and used to infest freshly cut evergreen ash twigs (1 to 2 cm in diameter, 14 to 16 cm long), which were then incubated in a growth chamber set at standard laboratory rearing conditions (27 °C to 30 °C, 65 ± 10% RH, and a photoperiod of 16:8 (L: D) h) for various lengths of times to produce larvae of different stages (Duan et al. 2021).

## Effects of EAB Larval Stage and Diapause Status on SCP

EAB larvae used in experiment 1 (examining the effect of larval stage of EAB on SCP before winter, H1) were reared under standard laboratory rearing conditions (described above) and dissected from rearing twigs as immature feeding larval stages (L2 to L4; $n = 19$) and nonfeeding J-shaped 4th instar larvae (JL; $n = 11$) for use in SCP tests. Due to their small size, L1 larvae were difficult to dissect out of bolts without incurring damage. Therefore, we tested only L2, L3, and L4 larval stages.

For experiment 2 (examining the effect of diapause status of EAB on SCP; H2), we tested the SCP of JL in different phases of diapause. To produce JL in different phases of diapause, lab-reared JL in evergreen ash twigs at 27 °C to 30 °C, with 60 ± 5% relative humidity (RH) and a photoperiod of 16:8 h (L: D), were divided into 3 groups and moved directly to 1 of 3 different temperatures where they were held for 3 months: (1) JL ($n = 23$) kept at a standard rearing temperature of 23.9 °C (75 °F) with 55% to 65% RH and a long-day photoperiod (16:8 h L: D) to produce larvae in the pre-overwinter diapausing state, (2) JL ($n = 15$) chilled at 1.7 °C (35 °F) with 30% to 85% RH, and a short-day photoperiod (8:16 h L: D) to induce and maintain an overwintering diapause state, and (3) JL ($n = 21$) chilled at 12.8 °C (55 °F) with 30% to 85% RH, and a short-day photoperiod (8:16 h L: D) to allow termination of diapause (Duan et al. 2021; Supplementary Table S1). We hereafter identify the 23.9 °C, 1.7 °C, and 12.8 °C treatment groups as early diapause, deep diapause, and postdiapause, respectively. For all treatment groups, we dissected larvae from peeled rearing twigs at USDA ARS BIIRU and shipped the larvae overnight to the University of Massachusetts Amherst (UMass) for SCP testing. Larvae exposed to the 2 colder chill treatments (deep and postdiapause) were shipped with cold packs to buffer larvae from warmer temperatures, whereas the early-diapause larvae (reared at 23.9 °C) were shipped at ambient temperature without cold packs. Upon arrival at UMass, SCP testing began immediately when possible. If SCP was not conducted on the day larvae were collected or received, they were held at temperatures consistent with their treatment temperature until SCP testing occurred. All SCP testing occurred within 2 days of arrival at UMass.

## Plasticity of SCP in Field Populations of EAB Larvae

To test hypothesis 3 (H3), we collected EAB larvae monthly from October 2021 to December 2022, excluding summer months (Supplementary Table S1 and Supplementary Fig. S1, see online supplementary data for a color version of this figure), from EAB-infested ash (*Fraxinus* spp.) logs. Larvae were collected from 2 locations separated by approximately 400 km and more than 2.5 degrees latitude in order to obtain larvae from different populations that experience different winter climate. The more southerly collection location in Newark Delaware, USA (39.6837° N, 75.7497° W) is in plant hardiness zone 7b and typically experiences warmer winters (average annual extreme winter minimums of −15 °C to −12.2 °C) than the collection location in Western MA (larvae collected from the UMass campus, Amherst, MA, 42.3965° N, 72.5314° W and nearby Quabbin Reservoir property, Belchertown, MA, 42.3048° N, 72.3786° W) which is in plant hardiness zone 6a (average annual extreme winter minimums of −23.3 °C to −20.6 °C; USDA Agricultural Research Service 2023). Infested logs were cut from multiple trees in Western Massachusetts and Newark, DE and stored near their collection location at outdoor ambient temperatures (Amherst, MA or Newark, DE) where they remained until peeled for larval extraction. Each month, logs were selected at random, peeled, and all EAB larval stages extracted until approximately 30 larvae were collected. Larvae from both populations (MA and DE) were collected within 1 wk of each other except for the latest spring collection (collection group 7, Supplementary Table S1 and Supplementary Fig. S1, see online supplementary data for a color version of this figure) which was timed to coincide with the development of pupae at each location to ensure that the entire spring larval period was captured. While L2—PP larvae were collected from MA trees, all larvae collected from DE were JL or PP larvae. Larvae were collected in 24-well cell culture plates and transported to the laboratory for SCP testing. Larvae from the Delaware population were shipped overnight to UMass with cold packs and moist tissue paper to prevent desiccation. The stage of each larvae collected was determined based on larval head capsule width and overall length (Chamorro et al. 2012), as well as shape and position in the host tree (Duan et al. 2010). All testing occurred within 2 days of removal of larvae from outdoor temperatures. If larvae were stored overnight in the lab prior to SCP testing, they were stored in a growth chamber at the average temperature forecasted for their collection location.

The outdoor temperatures to which the infested logs were exposed were recorded every 2 h using iButtons (Maxim Integrated Products, San Jose, CA) in Amherst, MA and hourly in Newark, DE using HOBO temperature loggers (Onset Computer, Bourne, MA). In cases where temperature loggers failed, temperature records were reconstructed using temperature records collected by National Oceanic and Atmospheric Administration (NOAA) weather stations (station GHCND: USC00190120 for MA temperatures and station GHCND: USC00076410 for DE temperatures, Menne et al. 2012). NOAA temperature records were adjusted to reflect local temperature logger records by first regressing logger records against NOAA records from the missing dates for a year with no missing records, then applying the resulting linear function to NOAA records from the dates with missing temperature logger readings.

## SCP Testing

We measured SCPs of individual EAB larvae by gradually lowering the temperature of larvae and documenting the temperature at which they froze. To measure SCP, we placed individual EAB larvae in 0.5-ml microcentrifuge tubes with a K-type thermocouple (Physitemp Inc., Clifton, NJ) inserted through a small hole drilled in the top of the tube. We secured thermocouples

in place with Fisherbrand label tape (Thermo Fisher Scientific, Waltham, MA) so that the thermocouple was in contact with the larva inside of the tube. We placed tubes containing larvae into a metal container filled with small steel beads, then partially immersed the container in a bath of antifreeze (50% ethylene glycol) inside a supercooling unit (NESLAB RTE-140, Thermo NESLAB Instruments, Inc., Newington, NH). The temperature of the bath was slowly reduced from a starting point of −5 °C down to −39 °C over the course of 185 min (an average decrease of approximately 1 °C every 4.2 min with the rate of change decreasing as temperature decreased; Elkinton et al. 2017, Chandler et al. 2020). Multiple individual larvae were run simultaneously and temperatures for the larvae were monitored using a multichannel thermocouple recorder (Physitemp Inc., Clifton NJ) at 10 s intervals. When liquids pass from the liquid to solid phase, heat is released. This release of latent heat due to crystallization produces an evident spike in the temperature record of each larva and documents the point at which it freezes. We used the temperature recorded immediately prior to the thermal spike as the SCP (Elkinton et al. 2017, Chandler et al. 2020) for each larva. If a spike in temperature was not apparent for a sample this typically indicated the thermocouple had pulled away from the larva inside the tube, and the larva was excluded from analyses.

### Data Analysis

All statistical analyses were conducted in R Core Team (2024). We examined differences in the SCP of nonfeeding (JL) vs. feeding (L2 to L4) larval stages (H1) using a two-sample *t*-test. We tested the differences between diapause chill treatment groups (H2) using a Kruskal–Wallis test followed by a Wilcoxon rank sum test with Bonferroni corrections for multiple comparisons. Nonparametric tests were used to analyze H2 since data were not normally distributed.

To examine the relationship between SCP and the pretesting temperature experienced by larvae from 2 different locations (H3), we fit 2 separate multiple linear regression models, 1 for JL and 1 for PP larvae, with SCP temperature as the dependent variable and lowest minimum temperature preceding collection, field location, and their interaction as fixed effects. The inclusion of field location as a fixed effect served to test whether the influence of ambient temperature on SCP differs with collection location. To achieve normality of the response (SCP temperature) and predictor (minimum temperature) variables for the JL model, we added a constant (one plus the absolute value of the minimum of each variable) to temperatures to achieve values greater than or equal to 1, then took the square root. The conditioning of larvae resulting from exposure to low temperatures has been shown to influence insect cold hardiness (Bale 1987, Lee et al. 1987, Sobek-Swant et al. 2012, Elkinton et al. 2017, Duell et al. 2022) and minimum winter temperature has been used previously to predict variation in SCP across insect populations (Ditrich et al. 2018). However, the period over which this acclimatization occurs is variable and difficult to test under field conditions. Therefore, to determine the optimal period (i.e., number of days) for calculation of minimum temperature prior to collection as a predictor of SCP, we fit a model for each temperature window ranging from 1 to 21 days prior to collection for both JL and PP larval stages separately. We selected the final model for each larval stage (JL and PP) based on model AIC (Supplementary Tables S2A and S2B). The JL and PP model with the temperature window yielding the lowest AIC for each of the 2 developmental stages (5 days for JL and 14 days for PP) was used to evaluate how SCP varies as a function collection location and its interaction with lowest minimum temperature preceding collection (Supplementary Table S3). We evaluated model assumptions and fit using Q–Q and residual plots. Although immature larvae and pupae were also collected and tested, we analyzed the effect of temperature exposure on SCP of JL and PP only, as these were the only stages that were consistently present across multiple collection events at both collection locations.

## Results

### Experiment 1: Larval Stage

Larval stage significantly influenced SCP of lab-reared EAB larvae. The mean SCP of L2 to L4 (−12.46 ± 1.03 °C, mean ± SE) was significantly higher than JL (−22.44 ± 0.73 °C) reared at the same temperature ($t = 7.91$; df = 27.95; $P < 0.001$, Fig. 1).

### Experiment 2: Diapause Status

The effect of diapause temperature treatments on overwintering JL was not significant ($X^2 = 5.06$, df = 2, $P = 0.080$; early diapause = −20.67 ± 1.35 °C; deep diapause = −19.31 ± 1.61 °C; postdiapause = −15.97 ± 1.99 °C; Fig. 2). Pairwise comparisons indicate SCP is not significantly different between early- and deep-diapause JL ($P = 1.00$), between deep- and postdiapause JL ($P = 0.540$), nor between early- and postdiapause JL ($P = 0.066$). However, trends in the data and *P*-values approaching significance suggest that increases in mean SCP from early diapause to postdiapause may have biological significance and warrant further examination.

### Experiment 3: Plasticity of SCP

The SCP of JL was a function of the lowest minimum ambient temperature during the 5-day window prior to collection ($F = 11.45$; df = 1, 304; $P < 0.001$), the geographic source of the larvae (field location, $F = 12.28$; df = 1, 304; $P < 0.001$), and their interaction ($F = 11.03$; df = 1, 304; $P = 0.001$; Fig. 3A and Supplementary Table S3). The significant interaction between larval source and minimum ambient temperature indicates that the slope of the linear regression of minimum temperature on

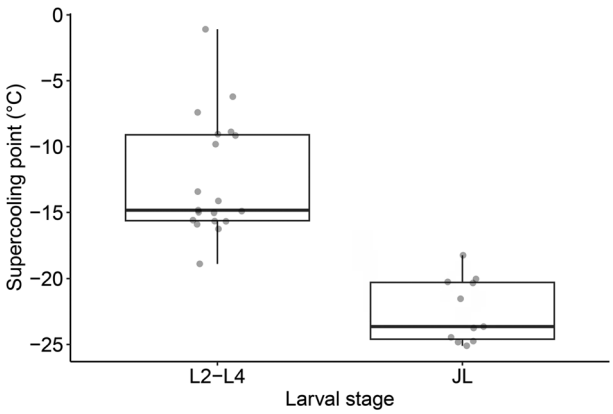


**Fig. 1.** Supercooling points of immature feeding (2nd to 4th instar; L2 to L4) and mature nonfeeding (J-shaped; JL) emerald ash borer (*Agrilus planipennis*) larvae reared under standard laboratory rearing conditions.

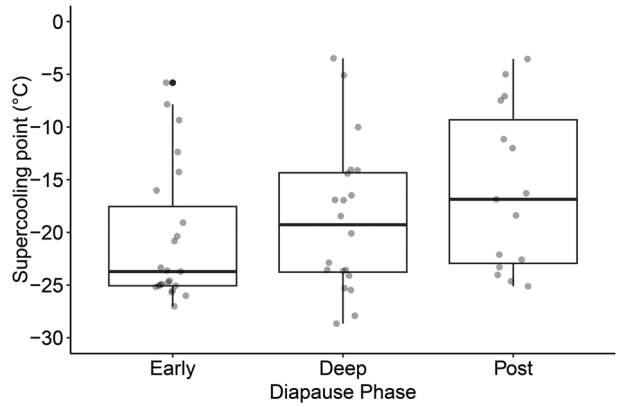


**Fig. 2.** Supercooling points of emerald ash borer (*Agrilus planipennis*) larvae reared under standard laboratory conditions then exposed to 1 of 3 temperature treatments for 3 months during the J-shaped larva stage to induce different phases of diapause: the standard laboratory rearing temperature of 23.9 °C to induce early diapause, chilled at 1.7 °C to induce and maintain deep diapause, and chilled at 12.8 °C to allow termination of diapause and begin the postdiapause phase.

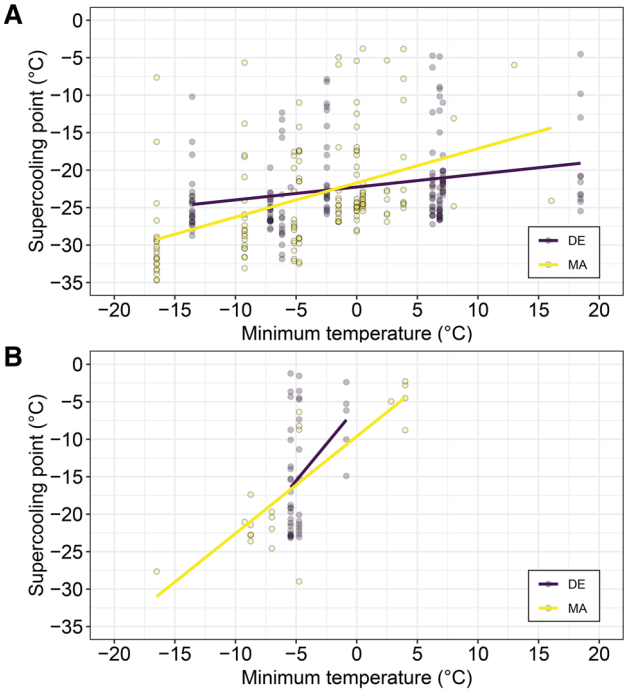


**Fig. 3.** Supercooling points of field-collected emerald ash borer (*Agrilus planipennis*) A) J-shaped larvae (JL) and B) prepupae as a function of the minimum temperature experienced during the temperature-sensitive window preceding testing. In panel A, nontransformed data from JL supercooling point testing are shown although data were square-root transformed prior to analyses.

JL SCP is lower in DE compared to MA (Fig. 3A and Supplementary Table S3).

The SCP of PP was a function of the lowest minimum ambient during the 15-day window preceding collection ($F = 7.78$; df = 1, 59; $P = 0.007$). However, the main effect of field location was not significant ($F = 0.96$; df = 1, 59; $P = 0.331$), and there was no significant interaction between minimum ambient temperature and field location ($F = 0.79$; df = 1, 59; $P = 0.379$; Fig. 3B and Supplementary Table S3).

## Discussion

SCP tests of lab-reared and field-collected EAB larvae indicate that developmental stage and recent exposure to cold temperatures influence cold hardiness of EAB. Results also suggest lower cold hardiness in the postdiapause phase. J-shaped larvae (JL, a mature, nonfeeding larval stage) are significantly more cold-hardy than immature feeding larval stages (L2 to L4; Fig. 1) and although the effect of diapause phase was not significant, trends in the data suggest early-diapause JL may be slightly more cold-hardy than those in postdiapause (Fig. 2). SCP positively correlates with the minimum temperature experienced in the prior 5 and 15 days, for JL and PP respectively, however source population influences the effect of cold exposure on SCP during the JL stage (Fig. 3). Together, our findings indicate that differences in cold hardiness throughout larval development and between different populations have potential to mediate the impact of winter temperatures on survival and establishment of EAB.

Reduced cold hardiness of immature larval stages (L2 to L4) compared to JL may lead to lower winter survival in semivoltine populations. Semivoltine EAB populations are more likely to occur in colder climates where development is too slow for larvae to mature to the JL stage in a single year (Duan et al. 2021). Semivoltine EAB overwinter first as immature larvae (L1 to L4) in year 1, then require a second winter as JL to complete development (Cappaert et al. 2005, Duan et al. 2010, 2014). Given that the SCP of immature larvae is nearly 10 °C higher than that of mature larvae, even before the onset of winter temperatures, we expect that winter mortality may be higher for immature larvae. Hemolymph composition (e.g., water, polyol, and sugar content) affects cold hardiness in insects (Block et al. 1990, Bale 2002) and previous studies have demonstrated that the accumulation of cryoprotectants in hemolymph increases the supercooling ability of EAB larvae (Crosthwaite et al. 2011, Sobek-Swant et al. 2012). While the differences we observed in cold hardiness between lab-reared immature and JL larvae are likely a result of hemolymph composition differences at different stages of development, gut contents may also play a role. Whereas JL do not feed, the gut contents of immature larvae may act as a nucleating agents for ice crystal formation (Somme 1982, Zachariassen 1985, Block et al. 1990, Bale 2002). Gut content evacuation in preparation for winter is a means by which some insects increase supercooling ability (Somme 1982), but immature EAB larvae do not evacuate their gut contents in preparation for winter (Duan, personal observ.) and immature larvae collected during winter readily resume feeding when artificially inserted into ash bolts (Ulyshen et al. 2010). Since the lab-reared larvae we tested in for stage-mediated differences in SCP (experiment 1; Fig. 1) were not acclimated to winter temperatures, further study of the relative ability of immature compared to mature larvae to supercool after gradual acclimation to winter temperatures may add to our knowledge of the differences in cold hardiness among larvae in semivoltine EAB populations. However, we expect that the reduced cold hardiness of immature larval stages persists throughout the winter, therefore the combination of a reduced winter survival rate for immature larvae in semivoltine populations and longer generation time may act in combination to reduce population growth rates in cooler climates.

Near-significant differences in SCP between JL in the early- and postdiapause phases ($P = 0.066$; Fig. 2) in combination with

results from recent work showing a significant drop in cryoprotectant concentration in JL during late diapause (Dang et al. 2025) suggests cold hardiness decreases in late diapause, though further study of EAB cold hardiness during diapause is needed to confirm the biological significance of the trends we observed. The postdiapause JL stage occurs in late winter and early spring in response to warming temperatures after a winter chill period (Duan et al. 2013b, 2021). In an Ontario (42.98° N, 82.38° W) population, SCPs of overwintering mature larvae were −30 °C on average, but varied seasonally; SCPs were 10 °C higher in spring compared to winter (Crosthwaite et al. 2011; Supplementary Table S4). The authors of this study did not distinguish between JL and PP or between diapause phases, but our results in combination with evidence of biochemical differences throughout diapause (Dang et al. 2025), suggest that the seasonal differences observed in SCP of EAB in Ontario may correspond with the progression from deep diapause to postdiapause in the late winter or spring. While daily temperatures trend upward in late winter, extreme temperature events such as polar vortices can occur with increasing frequency during this period of the year (Cohen et al. 2018, Overland and Wang 2019), potentially exposing the most cold-susceptible diapause phase of EAB to extreme and sudden low temperatures without time for larvae to acclimatize to colder temperatures (Duan et al. 2020). Consequently, JL EAB may be most susceptible to winter mortality during the postdiapause phase.

Cold hardiness of JL and PP from wild EAB populations increased in response to cold temperature exposure. However, in JL this response varied between sampled populations (i.e., collection locations). Variation in cold acclimatization among populations may indicate that, although cold hardiness in EAB larvae is a plastic trait that changes as a function of cold exposure, the plastic response is variable among geographic populations for some developmental stages. Our analyses indicated significant variation due to population, even when accounting for variation due to minimum temperature exposure (Fig. 3). Other studies have observed the persistence of a small population of EAB in Winnipeg, Manitoba, Canada (49.88° N, 97.12° W) through several severe polar vortex events (Duell et al. 2022) and overwintering larvae of nonfeeding overwintering stages (JL and PP) within this population appeared to be extremely cold tolerant with SCPs as low as −52 °C (compared to the −32 °C in Ontario reported by Duell et al. 2022). Previous studies demonstrate large variation in cold hardiness and winter survival at both landscape (Duell et al. 2022) and local scales (Duan et al. 2020). Although Duell et al. (2022) concluded that plasticity drives variation in EAB cold hardiness between populations, they were unable to rule out among-population variation in plasticity. Reciprocal transplant studies of EAB winter cold tolerance and genomic screening for traits that increase cold tolerance plasticity are needed to investigate potential evolution of increased plasticity in invading EAB populations. Our results indicate that while cold hardiness is indeed a plastic trait, the genetic or epigenetic basis for the mechanisms driving plasticity itself (or the range of temperatures to which a larva is able to acclimatize) may vary with geographic population.

Larval development, abiotic conditions, and diapause phase may interact to influence cold hardiness of EAB. Given the capacity for winter survival to determine successful establishment of this destructive invasive species in its nonnative range, future models of potential EAB distribution and range expansion should consider variation in voltinism and phenotypic plasticity among invasive populations. Understanding how factors that influence cold hardiness interact with local climate to affect EAB winter mortality may improve accuracy of current and potential EAB range maps enabling more effective invasive species monitoring and risk assessment, especially in geographic regions novel to the species (Izhevskii and Mozolevskaya 2010, Drogvalenko et al. 2019, Popkin 2022). Future studies to determine the genetic mechanisms underlying thermal tolerance of different EAB populations would allow for optimization of management options such as biological control introduction (Duan et al. 2023) by allowing managers to prioritize treatment of EAB populations with greater capacity to survive local winter climate.

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## Author contributions

Jennifer L. Chandler (Conceptualization [equal], Data curation [equal], Formal analysis [lead], Investigation [equal], Methodology [equal], Visualization [lead], Writing—original draft [lead], Writing—review & editing [equal]), Jian Duan (Conceptualization [equal], Funding acquisition [equal], Project administration [equal], Resources [equal], Supervision [equal], Writing—original draft [equal], Writing—review & editing [equal]), Talbot Trotter (Formal analysis [supporting], Writing—review & editing [equal]), and Joseph S. Elkinton (Funding acquisition [equal], Project administration [equal], Resources [equal], Supervision [equal], Writing—review & editing [equal])

## Supplementary material

Supplementary material is available at *Journal of Economic Entomology* online.

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## Conflicts of interest

None declared.

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