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Modest Effects of Host on the Cold Hardiness of Emerald Ash Borer †

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Abstract: The emerald ash borer, *Agrilus planipennis* Fairmaire, is invading North America and Europe but has not yet reached its ultimate distribution. Geographic differences in host availability and winter temperatures might affect where this species will occur. In central North America, black ash (*Fraxinus nigra*) is more abundant than green ash (*F. pennsylvanica*) at northern latitudes, but much of our current understanding of *A. planipennis* cold tolerance is based on observations of overwintering larvae from green ash. The effects of black and green ash on the cold hardiness of *A. planipennis* larvae were measured over three winters. Supercooling point, the temperature at which insect bodily fluids spontaneously begin to freeze, was marginally greater for larvae from artificially-infested black ash than green ash in one trial, but not in three others. Host species also did not consistently affect mortality rates after larval exposure to subzero temperatures, but larvae from black ash were less cold hardy than larvae from green ash when there were differences. Comparisons of mortality rates among chilled (unfrozen) and frozen larvae indicated that overwintering *A. planipennis* larvae are primarily freeze avoidant, and this cold tolerance strategy is unaffected by host. All of our studies suggest that *A. planipennis* larvae from black ash are not more cold hardy that larvae from green ash. Where temperatures annually decline below ~−30 °C, overwintering morality may substantially affect the population dynamics and future impacts from this invasive alien species.

Keywords: *Fraxinus nigra*; *Fraxinus pennsylvanica*; biogeography; ecophysiology; overwintering mortality; freeze avoidance

1. Introduction

The invasions of North America and Eurasia by the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), portend significant ecological, economic, and social impacts, many of which have been realized and others theorized. Black ash, *Fraxinus nigra* Marsh., blue ash, *F. quadrangulata* Michx., green ash, *F. pennsylvanica* Marsh., and white ash, *F. americana* L. have been colonized naturally by this insect in eastern North America [1–3], and tens of millions of ash trees have died from infestation [4–6]. The loss of ash could detrimentally affect native arthropods [7] and ecosystem functions [8,9]. Where emerald ash borer has invaded, significant economic costs associated with mitigative treatments, tree removals, and tree replacements and lost home equity have been incurred [10,11]. Further, losses of ash trees may be associated with an increase in cardiopulmonary disease in urban areas [12] and threaten culturally significant uses of the resource by some Native Americans and First Nations [6,13]. The ultimate magnitude of these impacts depends, in part, on the final geographic distribution of the species.

Continued spread of emerald ash borer in North America and Europe indicates that the adventive populations of the species have not yet reached spatial equilibria with their environments [6,14,15]. For many insects, host availability and climate suitability are among the primary determinants of where a species may (not) persist [16]. A worst-case scenario for emerald ash borer assumes that all areas where *Fraxinus* spp. grow will also be climatically suitable for the insect. Inductive species distribution models, which compare the climates where emerald ash borer occurs (in Asia, North America, or both) to the rest of North America, suggest that 35% or more of the geographic range of ash may be climatically sub-optimal for emerald ash borer [17]. Variation in temperature (as opposed to precipitation) contributes to more than 50% of these models, and the mean cold temperature of the coldest quarter alone may contribute up to 35% [17]. Model forecasts of suitability differ considerably along the northern, western, and southern limits of the geographic range of ash [17]. Ecophysiological studies may be useful to resolve these differences, and studies of the cold tolerance of *A. planipennis* may be particularly informative at high elevations or northern latitudes.

Agrilus planipennis typically overwinter as late fourth instars (commonly called the "J-stage") in the outer sapwood but may overwinter as earlier instars in or near phloem when individuals undergo a two-year lifecycle [6]. A number of studies suggest that *A. planipennis* larvae might be freeze avoidant (=freeze intolerant) and capable of surviving some sub-zero temperatures. Insects that avoid freezing by supercooling produce polyols and thermal hysteresis proteins to stave off internal ice formation until low temperatures are reached [18,19]. The supercooling point is the temperature at which the spontaneous freezing of body fluids begins and has been used, with criticism [20,21], as a measure of the lowest temperature at which a freeze-avoidant insect species can survive [22,23]. Wu et al. [24] were the first to report supercooling points for the species between −23 and −26 °C in China. Venette and Abrahamson [25] similarly reported a mean supercooling point of −25 °C for winter-acclimated larvae from green ash. Crosthwaite et al. [26] reported seasonal changes in the supercooling point for larvae from green ash in southern Ontario and measured the lowest mean supercooling points from November through February of approximately −30 °C. They also provided the first direct evidence of freeze avoidance for the species [26]. Sobek-Swant et al. [27] found that once cold acclimated, *A. planipennis* supercooling points increased only marginally after one week when exposed to 10 °C or warmer, and only after two weeks at 0–4 °C. All North American studies of the cold tolerance of *A. planipennis* focused on larvae from green ash.

For some insects, the host species on which immature stages develop can affect supercooling points and overwintering ability [28–33]. The objective of this study was to measure the effect of host species on the cold hardiness of *A. planipennis* larvae. Specifically, we hypothesized that the cold hardiness of larvae from black ash would differ from larvae from green ash. While both ash species are considered quality hosts for larval development, *A. planipennis* adults prefer green ash over black ash [2,3,34], and larvae extracted more essential and non-essential amino acids from green ash than from black ash [35]. Black ash tends to be more abundant than green ash in northern stands in the northeastern United States [36], so any impact of host on cold hardiness would have significant impacts on the potential geographic distribution of the species. In this study, supercooling points were measured for *A. planipennis* larvae from artificially- and naturally-infested hosts, and lower lethal temperatures were measured for larvae from naturally-infested hosts.

2. Materials and Methods

2.1. Supercooling Points of A. planipennis from Artificially-Infested Ash

Because of the low numbers of infested black ash within the distribution of *A. planipennis* in Minnesota, we artificially infested cut logs with laboratory reared larvae. *Agrilus planipennis* were reared by following methods modified from Keena et al. [37]. To obtain adults, multiple naturally-infested green ash were felled in Minneapolis and Saint Paul, MN, USA in late May and early June 2011, cut into 38–56 cm lengths, and placed in 66.8 (length) by 31.75 cm (diameter) cardboard rearing tubes with

vented plastic lids in a controlled temperature greenhouse. Rearing tubes were checked daily for newly emerged adults. Adults were transferred to 1.9 L plastic jars (United States Plastic Corporation, Lima, OH, USA) with a mesh lid (aluminum window screen with an opening size of 1.41 × 1.59 mm or stainless steel wire mesh with an opening size of 6.4 × 6.4 mm overlapped for openings approximately one quarter of the size). The bottom of the jar and the lid were lined with grocery store bleached coffee filter paper to provide a substrate for oviposition. Adults were also given a water pick and fresh green ash foliage from a mature tree. Jars were cleaned every other day, at which time the filter paper, water, and foliage were replaced. Eggs on the filter paper were counted and transferred to 37 mL sealed plastic cups (Solo Cup Company, Highland Park, IL, USA). Rearing jars and egg cups were stored in an illuminated growth chamber at 26 °C with a photoperiod of 16:8 (L:D) h. Cups were checked daily for newly emerged neonates, which were inserted into cut logs on the same day.

Green and black ash logs from Grand Rapids, MN, USA, far from any known infestations of *A. planipennis*, were cut in July 2011. Two logs, each from a different tree, from each host species were used in 2011. Logs ranged in size from 12–16 cm in diameter and 90–94 cm in length. A total of 26 neonates, all that were available, were infested from 11–18 July 2011, with 13 neonates in each species of ash (no more than 1 larva per 205 cm^2 of log surface area). Neonates were only placed in the top 60 cm of the log because each log was set in a plastic potting dish with 2.5–5 cm of water. Notches 2–3 cm wide and down to the phloem were made in the bark with a 1.25 cm chisel, and a neonate was placed at the bottom of each notch. Laboratory infested logs were kept in a greenhouse throughout August (mean temperature June–August 2011 = 27.6 ± 2.6 °C SD) before they were moved into an unheated garage in preparation for winter temperature acclimation. The bark was peeled from the logs, and larvae were extracted in November 2011. All recovered larvae had reached the "J"-stage, as characterized by Chamorro et al. [38]. Six of thirteen infested *A. planipennis* larvae had successfully developed in black ash, and five were extracted without injury. Seven of thirteen larvae developed in green ash, and four were successfully recovered.

The rearing process was repeated in 2012. Uninfested logs were cut in July 2012 from Grand Rapids, MN, USA and ranged in size from 25.5–33 cm in diameter and 118–125 cm in length. Two logs, each from a different tree, of each host species were artificially infested from 16 July–19 July 2012, with 28 neonates infested into green ash and 29 infested into black ash and held as before (mean temperature June–August 2012 = 28.4 ± 3.3 °C). Artificially infested logs were peeled, and larvae were extracted in January 2013. Eight of 28 larvae developed to the late fourth instar in green ash, and four were successfully recovered. Seven of 29 larvae developed to the same instar in black ash, and four were extracted.

Supercooling points of *A. planipennis* larvae were measured by following protocols modified from Carrillo et al. [39]. Each larva was weighed to the nearest tenth of a milligram before being placed in a trimmed 9 mm (diameter) × 27 mm (length) gelatin capsule (size 000) (Capsuline, Pompano Beach, FL, USA). Capsules prevented larvae from contacting the high vacuum grease (Dow Corning, Midland, MI, USA) that was used to attach the capsule to the thermocouple. Coiled copper-constantan thermocouples, as described in Hanson and Venette [40], were connected to multi-channel data loggers (USB-TC, Measurement Computing, Norton, MA, USA) so that temperatures for up to 16 insects could be recorded at the same time. Each insect and thermocouple was insulated within a polystyrene cube designed to provide a cooling rate of 1 °C min^{-1} below 0 °C in a −80 °C freezer [39]. Temperatures were recorded once per second using TracerDAQ software (Measurement Computing, Norton, MA, USA). Larvae were cooled until an exotherm was detected. The supercooling point was the lowest temperature reached before the onset of the exotherm.

Data were analyzed in R version 3.1.1 [41] or in SAS v. 9.4 [42]. Linear regression (in the R package "MASS") was used to assess the relationship between the supercooling point and larval mass. Larval supercooling points were compared between hosts in each year and between years for each host by using a Wilcoxon rank sum test (PROC NPAR1WAY in SAS), because not all data were normally distributed (Table 1). Statistical *p*-values were calculated exactly to account for small sample sizes.

Table 1. Median and mean (±SEM) supercooling points for larvae from green and black ash that were artificially infested (lab) or naturally infested (field) during the winters of 2011–2012, 2012–2013, or 2013–2014.

	n	Median (°C)	Mean ± SE (°C)	Shapiro-Wilk (W, *p*)
Lab 2011–2012				
Black ash	5	−25.5	−24.8 ± 0.97	0.91, 0.48
Green ash	4	−24.0	−22.1 ± 3.32	0.81, 0.13
Lab 2012–2013				
Black ash	4	−29.2 *	−30.4 ± 1.42	0.74, 0.03
Green ash	4	−34.2 *	−33.8 ± 1.59	0.93, 0.61
Field 2012–2013				
Black ash	41	−32.6	−31.2 ± 0.71	0.87, < 0.001
Green ash	30	−32.9	−29.8 ± 5.43	0.84, < 0.001
Field 2013–2014				
Black ash	25	−30.6	−31.3 ± 0.63	0.95, 0.3
Green ash	36	−32.1	−31.4 ± 0.61	0.95, 0.1

Shapiro-Wilk provides a test for normality in the distribution of supercooling points. * A Wilcoxon test indicates a moderately significant effect of host (Wilcoxon S = 24, $p = 0.06$).

2.2. Cold Tolerance of *A. planipennis* Larvae from Naturally-Infested Ash

Larvae of *A. planipennis* from naturally-infested green and black ash trees were collected in the winters of 2012–2013 and 2013–2014. In January 2013, six green ash and nine black ash trees were removed from the Fort Snelling Golf Course, in Minneapolis, MN (44.886286° latitude, −93.195189° longitude). *Agrilus planipennis* was confirmed at this location on 13 August 2012 by the Minnesota Department of Agriculture but could have been present for ≥3 years given the extent of infestation in some trees (RCV, personal observation). Logs (5–20 cm in diameter and approx. 90 cm in length) were stored outside our research facility in Saint Paul. Bark was peeled from logs and larvae were extracted between 10 January and 28 February 2013. In the winter of 2013–2014, at least 24 infested trees each of green and black ash were identified at Great River Bluffs State Park (approx. 43.944° latitude, −91.385° longitude) in Winona County, MN. *Agrilus planipennis* was first confirmed in the park on 14 September 2011. Trees were felled in November and December 2013 and January and early February 2014. Logs (5–15 cm in diameter and approx. 120 cm in length) were cut and returned to St. Paul, MN (Permit: Minnesota Department of Agriculture, State Formal Quarantine No. RF-1 036, RF-1076, RF-2074 Emerald Ash Borer, Section VI: Special Exemptions). Logs were again held outdoors and were peeled between 30 December 2013 and 27 March 2014 to extract larvae. All larvae were noted as early (first–third) instar or fourth instar (including the "J-stage"), whether or not a larva was recovered without injury, based on descriptions provided by Chamorro et al. [38]. The proportions of individuals that were fourth instars were compared between hosts in the same year and within the same host between years by using a *z*-test of proportions.

Extracted larvae from both winters were stored at 0 °C for 24–96 h before measuring larval mass and exposing larvae to cold. This holding time allowed minor injuries from extraction to become visible, but was not long enough for the overwintering larvae to lose cold acclimation [27].

Laboratory cooling experiments of field-collected larvae were performed from 11 January–1 March 2013 and 3 January–20 March 2014. In January 2013, supercooling points were measured for 13 larvae from green ash and 15 larvae from black ash, as previously described. Results informed cold exposure treatments for lower lethal temperature studies. The lower lethal temperature studies generally followed procedures described in Stephens et al. [43] and Hefty et al. [44]. The experimental design was a randomized-complete-block with time as the block. In each block, up to ten larvae from black and green ash (20 larvae total) were randomly assigned to one of five temperature treatments (i.e., two larvae from each host species per temperature). Treatment temperatures in the winter of 2012–2013 were −35 °C, −30 °C, −25 °C, −20 °C, and a room temperature control (approx. 25 °C). The temperatures were selected to detect pre-freeze mortality, if it occurred. The coldest exposure temperature was approximately 2.5 °C lower than the median supercooling point that we measured and

was expected to cause 60–80% mortality. This expectation came from the distribution of supercooling points and an assumption that the onset of an exotherm would cause death. Treatment temperatures in the winter of 2013–2014 were −40 °C, −35 °C, −30 °C, −25 °C, and a room temperature control (approx. 25 °C). These treatments provided a more thorough analysis of larval condition before or after freezing as they were balanced around the median supercooling point. A total of 111 larvae from green ash and 100 larvae from black ash were tested in the winter of 2012–2013; 47 larvae from green and black ash (94 larvae total) were tested in the winter of 2013–2014.

Larvae were prepared and cooled as previously described for the measurement of supercooling points. In this case, larvae were removed from the freezer and held at room temperature within ~5 s of when they reached the treatment temperature, whether an exotherm was detected during the course of cooling or not. If an exotherm was detected, the supercooling point was noted. Control larvae (held at room temperature) were placed in gelatin capsules and affixed to thermocouples with high vacuum grease, just as the chilled larvae were.

To determine if larvae had survived, cold-exposed and control larvae were removed from capsules and placed in covered 24-well cell culture plates (Corning, Tewksbury, MA, USA) at room temperature in the dark. Culture plates were kept in a plastic storage container with a loose fitting lid for gas exchange and wet paper towels to keep the humidity high. First observations were taken 72 h after cold exposure and then every other day until pupation. Color and movement were noted. Larvae that were active during the observation period or had pupated (only applicable to fourth instars) were classified as having survived. Larvae that had not molted or pupated at 13 days after cold exposure, did not move, or were discolored, were categorized as dead. For the study in the winter of 2012–2013, we focused our analysis on larval condition at one week post cold exposure and at the time of pupation, while in the winter of 2013–2014, we focused on observations at one week because mold had overtaken larvae thereafter. Larvae <1 mg were not included in lower lethal temperature studies because initial work suggested high mortality from extraction or handling, even among larvae that showed no initial signs of injury.

The relationship between supercooling point and mass, a proxy for development, was analyzed by linear regression in R. Because no relationship between these two measures was found ($F = 0.654$, $df = 1, 129$, $p = 0.420$), supercooling points were not analyzed by size class.

Supercooling point frequency distributions by host were tested for normality with the Shapiro-Wilk test in R (function *shapiro.test*). Because supercooling points of larvae from naturally infested ash in the winter of 2012–2013 were not normally distributed (Table 1), supercooling point data were analyzed by using non-parametric survival analysis in the survival package in R [45]. This statistical approach also allowed us to take advantage of information provided by individuals that were chilled but did not give an exotherm before being removed from the freezer. In our case, temperature (measured as the difference from room temperature) replaced time in the survival analysis, and the binary independent variable was whether each larva had an exotherm or not. When exotherms were observed, the supercooling points provided the exact temperatures at which freezing began, and these data are considered non-censored. A larva that did not give an exotherm before reaching the target temperature was considered a right-censored observation (i.e., we presumed that the supercooling point occurred at an unspecified temperature colder than the removal temperature). To estimate the probability that an individual would begin to freeze, given that it had been cooled to a particular temperature, non-parametric Kaplan-Meier curves were estimated from non-censored and right-censored observations using the *survfit* function in the R survival package [45]. We used the *survdiff* function to test for differences ($\alpha = 0.05$) in Kaplan-Meier curves between each host in the same year or between years for the same host.

Host influence on larval mortality after cold exposure was analyzed in two ways. The potential effect of host species on the extent of larval mortality at each target exposure temperature was tested by a z-test of proportions. Mixed linear models with a logit-link function were used to relate the extent

of mortality to exposure temperature, host, and the interaction of host and exposure temperature (Proc GLIMMIX in SAS). Block and thermocouple within block were included as random effects.

The determination of a species' cold-tolerance strategy, i.e., chill intolerant, freeze avoidant, or freeze tolerant, depends on a comparison of mortality rates among chilled and frozen individuals at a particular temperature [46]. In our studies, "frozen" equated to the onset of freezing, as evident from the detection of an exotherm; the extent of freezing was not measured. We followed the statistical procedures described in Cira et al. [47]. For each host species within a winter, the difference in mortality rates between chilled and frozen individuals was tested by using Fisher's exact test (Chi square) in SAS (Proc TABULATE). The exact test accounted for small sample sizes ($n < 5$) in some cases and converged to the large sample approximation as sample size increased. If the mortality rates of chilled individuals were equal to the mortality rates of frozen individuals and the mortality rates of chilled individuals increased as exposure temperature decreased, chill intolerance would be indicated. If the mortality rates of chilled individuals were less than the mortality rates of frozen individuals and the mortality rates of chilled individuals remained constant as exposure temperature decreased, freeze avoidance would be indicated. If the mortality rates of frozen individuals were less than 1, freeze tolerance (or perhaps partial freeze tolerance) would be suggested. We also tested whether the mortality rate of chilled (unfrozen) individuals differed from the mortality of controls kept at room temperature, again using Fisher's exact test. Because no differences were detected, likely as a joint consequence of relatively high mortality among control larvae and small sample sizes, we pooled mortality results across exposure temperatures (excluding controls) for chilled and frozen individuals, respectively, and tested for differences in mortality rates by using Fisher's exact test.

3. Results

3.1. Supercooling Points of A. planipennis Larvae from Artificially-Infested Ash

In 2011, the supercooling points of larvae from artificially-infested green and black ash were not significantly different (Table 1; Wilcoxon S = 21, $p = 0.45$). Supercooling points of larvae from black ash ranged from −26.7 to −22.0 °C and from green ash ranged from −27.1 to −12.4 °C. The supercooling point of −12.4 °C was atypically high, but removing this potential outlier (adjusted mean ± SE, −25.4 ± 1.0 °C) still did not reveal an effect of host (Wilcoxon S = 12, $p = 0.39$). In 2013, host had a marginally significant effect on the supercooling points of artificially-infested larvae (Wilcoxon S = 21, $p = 0.06$). Supercooling points of larvae from black ash, ranging from −34.6 to −28.5 °C, were greater than larvae from green ash, ranging from −36.8 to −29.3 °C (Table 1). Supercooling points occurred at warmer temperatures in 2011 (November) than in 2013 (January) for larvae from black ash (difference in medians = 3.7 °C, Wilcoxon S = 10, $p < 0.01$) and green ash (difference in medians = 9.7 °C, Wilcoxon S = 26, $p = 0.01$).

3.2. Cold Tolerance of A. planipennis Larvae from Naturally-Infested Ash

Stage distributions. In the winter of 2012–2013, 165 *A. planipennis* larvae were recovered from green ash, and a significantly greater percentage (99.4% ± 0.6%; [$\hat{p}$ ± SE] × 100) were fourth instars than among the 209 larvae recovered from black ash (92.3% ± 1.8%; Z = 3.64, $p < 0.001$). In the winter of 2013–2014, 203 and 170 *A. planipennis* larvae were recovered from green ash and black ash, respectively, and the percentages that were fourth instars (46.8% ± 3.5% and 53.5% ± 3.8%) did not differ (Z = 1.30, $p = 0.099$). A significantly greater percentage of larvae were fourth instars in the winter of 2012–2013 (Minneapolis) than 2013–2014 (Great River Bluffs State Park) for green ash (Z = 14.80, $p < 0.001$) and black ash (Z = 9.14, $p < 0.001$).

Supercooling points. Supercooling points were not significantly different between host species in the winter of 2012–2013 ($\chi^2 = 1.9$, df = 1, $p = 0.17$; Figure 1) or 2013–2014 ($\chi^2 = 0$, df = 1, $p = 0.90$; Figure 1). In the winter of 2012–2013, the lowest supercooling points of larvae from black ash and green ash were −37.5 °C and −36.2 °C, respectively, and in the winter of 2013–2014, they were −37.3 °C and

−38.0 °C, respectively (Figure 1). Supercooling points of larvae from black ash were not significantly different between winters (χ^2 = 1.2, df = 1, p = 0.28; Figure 1), but were different for larvae from green ash (χ^2 = 6.4, df = 1, p = 0.01; Figure 1).

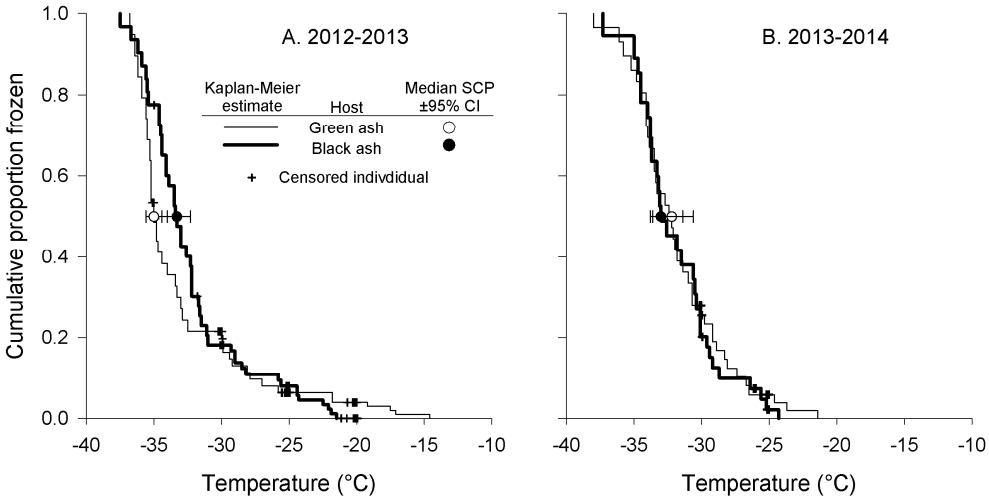

Figure 1. Kaplan-Meier estimates of the likelihood that an *A. planipennis* larva will begin to freeze (i.e., give a supercooling point) after exposure to a particular temperature, by tree species, in (**A**) the winter of 2012-2013 and (**B**) the winter of 2013-2014. Censored individuals did not start to freeze by their lowest exposure temperature.

Mortality from cold exposure. In the winter of 2012–2013, no effect of host on mortality rates of *A. planipennis* larvae could be detected one week after cold exposure (Figure 2A). Mortality rates of control larvae were 5.0% ± 4.9% (SE) from black ash and 20.8% ± 8.3% from green ash at this time. At 0 °C (i.e., the intercept of the statistical model), no effect of host on mortality rate was detectable (F = 1.3, df = 1, 170, p = 0.25). The mortality rate increased significantly as the coldest exposure temperature decreased (F = 9.3, df = 1, 170, p = 0.003). When exposure temperatures reached −35 °C, mortality was 59.1% ± 10.5% in larvae from black ash and 45.4% ± 10.6% in larvae from green ash. Host species did not affect the change in larval mortality rate as the coldest exposure temperatures declined (F = 1.5, df = 1, 170, p = 0.22).

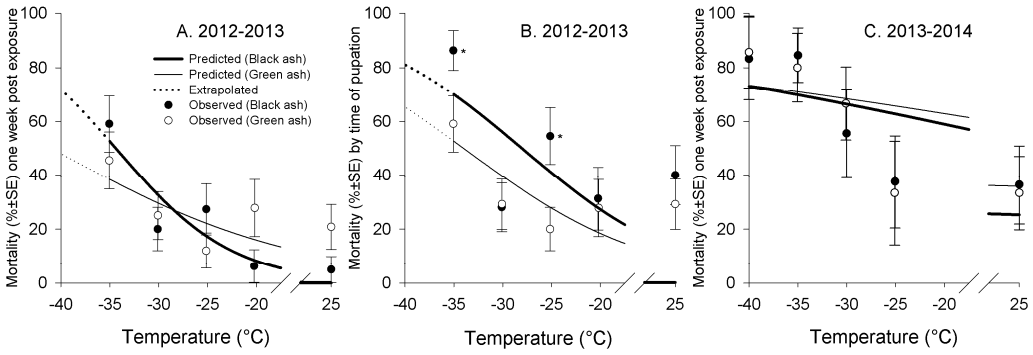

Figure 2. Mortality of *A. planipennis* larvae from black and green ash in the winters of 2012–2013 and 2013–2014 in response to acute exposure to different low temperatures. Mortality was assessed at: (**A**) one week after exposure; (**B**) again 13 days after exposure at pupation; and (**C**) one week after exposure. Asterisks indicate statistically significant differences in morality between larvae from black and green ash at that temperature. Host did not significantly affect the predicted mortality rates at 0 °C or the change in mortality rates as temperatures changed.

Mortality rates by the time of pupation (within two weeks of cold exposure) were typically ~40% greater than at one week after cold exposure (increase ranged from zero- to five-fold; Figure 2A,B). At this time, mortality rates were greater for larvae from black ash than green ash at −35 °C ($Z = 2.13$, $p = 0.017$) and –25 °C ($Z = 2.50$, $p = 0.0062$; Figure 2B). No statistically significant differences were detected when the coldest exposure temperature was –30 °C, –20 °C, or room temperature ($Z \leq 0.09$, $p \geq 0.18$). Although larvae from black ash had greater mortality rates than larvae from green ash in four of the five exposure treatments, host species did not affect the projected level of mortality at 0 °C ($F = 0.1$, df = 1, 170, $p = 0.92$) or the change in mortality rate as the coldest exposure temperatures declined ($F = 0.08$, df = 1, 170, $p = 0.77$). The mortality rate increased as the coldest exposure temperatures decreased for larvae from both host species ($F = 7.2$, df = 1, 170, $p = 0.008$; Figure 2B).

In the winter of 2013–2014, no differences were detected in mortality rates between *A. planipennis* larvae from black and green ash at one week after cold exposure ($Z \leq 0.52$, $p \geq 0.31$; Figure 2C). Mortality rates among larvae that were kept at room temperature were ~37%. Although mortality rates increased as the coldest exposure temperatures declined ($F = 5.1$, df = 1, 87, $p = 0.03$), host species had no discernable effect on the projected level of mortality at 0 °C ($F = 0.3$, df = 1, 87, $p = 0.6$) or on the change in mortality rate as exposure temperatures declined ($F = 0.2$, df = 1, 87, $p = 0.7$). Of larvae that were briefly exposed to –40 °C, ~85% were dead 7 days later. In the winter of 2012–2013, the mortality rates of control *A. planipennis* larvae from black and green ash were ~10% at one week after testing (Table 2). Mortality levels among chilled (unfrozen) individuals did not significantly differ from controls at any subzero temperature exposure. No larvae from black or green ash began to freeze (i.e., gave an exotherm) at temperatures ≥−20 °C (Table 2). When chilled (unfrozen) and frozen larvae were present after being exposed to the same temperature, the mortality rate of chilled larvae was less than one third of the mortality rate of frozen larvae; the difference was statistically significant for individuals from black ash exposed to −25 °C and from green ash exposed to −30 °C or −35 °C (Table 2). When observations among exposure temperatures were combined, the proportion of larvae that were dead one week after being chilled was significantly less than after being frozen. The mortality rate among all chilled individuals combined within a host species was not different from controls for larvae from black ($\chi^2 = 0.29$, df = 1, $p = 0.59$) or green ash ($\chi^2 = 1.14$, df = 1, $p = 0.28$). The proportions of larvae that were dead one week after being frozen were significantly less than 1.0 for larvae from black ash (exact upper 95% confidence interval = 0.87) and green ash (exact upper 95% confidence interval = 0.89).

In the winter of 2013–2014, host species, chilling, and freezing had qualitatively similar effects on mortality rates (one week post exposure) of *A. planipennis* larvae as in the winter of 2012–2013. The mortality rates of chilled (unfrozen) individuals did not significantly differ from the mortality rates of control larvae (~44% for both host species) at any exposure temperature (Table 2). No larvae began to freeze at temperatures ≥−30 °C. The mortality rates of chilled (unfrozen) larvae were numerically less for frozen larvae from black and green ash at each exposure temperature, but the differences were not statistically different until observations from all exposure treatments were combined (Table 2). Then, the mortality rates of frozen individuals were twice as great as the mortality of chilled individuals for both host species. The combined mortality rate of chilled individuals was not different from the control for larvae from black ($\chi^2 = 0.18$, df = 1, $p = 0.67$) or green ash ($\chi^2 = 0.25$, df = 1, $p = 0.62$). The combined mortality rate of frozen individuals was less than 1.0 for larvae from black ash (exact upper 95% confidence interval = 0.99) and green ash (exact upper 95% confidence interval = 0.97).

Table 2. Mortality of chilled and frozen *A. planipennis* larvae from green and black ash in the winters of 2012–2013 and 2013–2014 at one week after acute exposure to a subzero temperature.

	Chilled [a]		Frozen		Chilled		Frozen	
Temp. (°C)	***n*** [b]	**Mortality %**	***n***	**Mortality %**	***n***	**Mortality %**	***n***	**Mortality %**
2012–2013		*Black ash*				*Green ash*		
−35	2	0.0	20	65.0	8	0.0	14	71.4 *
−30	22	13.6	3	66.7	19	5.3	5	100 *
−25	17	5.9	5	100 *	22	9.1	3	33.3
−20	16	6.3	0	-	18	27.8	0	-
25	20	5.0	0	-	24	20.1	0	-
Combined [c]	57	8.8	28	71.4 *,†	67	11.9	22	72.3 *,†
2013–2014								
−40	0	-	6	83.3	0	-	7	85.7
−35	1	0.0	12	91.7	1	0.0	9	88.9
−30	9	55.6	0	-	7	57.1	5	80.0
−25	8	37.5	0	-	6	33.3	0	-
25	11	36.4	0	-	12	33.3	0	-
Combined [c]	18	44.4	18	88.9 *,†	14	42.9	21	85.7 *,†

[a] an exotherm was not observed for chilled larvae but was for frozen larvae; the extent of freezing was not measured. [b] n = number of individuals that were chilled or had started to freeze by the exposure temperature. [c] combined values did not include 25 °C (room temperature controls). * mortality rates from chilling and freezing were significantly different ($p < 0.05$) as determined by Fisher's exact test (Chi square) with one degree of freedom. † combined mortality of frozen individuals was significantly less than one, as determined by the lack of overlap with exact 95% confidence intervals. For each host species in each winter, the mortality of chilled (unfrozen) larvae at each exposure temperature was not statistically different ($p > 0.05$) from larvae held at room temperature, as determined by Fisher's exact test (Chi square) with one degree of freedom.

4. Discussion

Host species had idiosyncratic effects on the cold tolerance of *A. planipennis* larvae. In only one of four trials did host have an effect on the supercooling point, and in this case, the effect was statistically marginal (Table 1). Similarly, in 16 comparisons of larval mortality after exposure to subzero temperatures, statistically-significant effects of host were only detected in two instances, both in the winter of 2012–2013 for larvae whose coldest exposure temperatures were −35 or −25 °C (Figure 2). In all cases where a possible effect was detected, larvae from black ash were less cold hardy than larvae from green ash. While these sporadic differences are intriguing to us, most of the data from this study indicate that the cold tolerance of *A. planipennis* larvae from black ash and green ash does not differ. In some cases, small sample sizes reduced the power of the statistical tests to detect a difference. Thus, we believe that the correct interpretation of these findings, in aggregate, is that overwintering *A. planipennis* larvae from black ash were not more cold tolerant than larvae from green ash.

This interpretation of our findings has important ramifications. Much of the previous knowledge of *A. planipennis* cold tolerance was based on larvae that had developed in green ash, and this information had been used to forecast where *A. planipennis* might (not) be able to overwinter, irrespective of the host species that were present [48]. Our findings suggest that mortality levels of larvae in black ash should be expected to be the same as larvae in green ash when exposed to the same temperature, but occasionally might be greater. Accurate forecasts depend on reliable information about the relationship between temperature and mortality and on measures of the temperature(s) that overwintering larvae experience. Although Vermunt et al. [49] developed models to forecast temperatures beneath the bark of green ash and white ash (*F. americana*), similar relationships have yet to be developed for black ash in mesic or hydric sites (but see Christianson [50]).

Our results are based on acute exposures of larvae to subzero temperatures and need to be interpreted with caution. The mortality measured in this study does not account for the potential effects of prolonged exposure to subzero temperatures. In general, mortality increases within limits as exposure time to a constant, low temperature increases [51]. In this respect, our estimates of mortality are conservative because larvae in the field would be expected to experience some of these

temperatures for minutes, hours, or days when they occur, not seconds. Conversely, our estimates of mortality may be liberal because they are based on a cooling rate of 1 °C/min. Although this rate has been widely accepted as a standard laboratory protocol [52,53], it has been challenged for its ecological relevance [54]. With slower cooling rates that are common in temperate climates, supercooling points can be different, as can temperature-mortality relationships, e.g., [55]. The utility of these laboratory-based measures of cold tolerance, irrespective of the cooling rate that was used, can only be evaluated through comparisons with independent observations of mortality from the field. Such comparisons were beyond the scope of this study.

Our study indicates that overwintering *A. planipennis* larvae, whether developing in black or green ash, are primarily freeze avoidant. If larvae stay in an unfrozen state, mortality rates appear to remain unaffected, even as the coldest exposure temperature decreases. Any effects of cold on the mortality of these individuals could not be distinguished from the effects of larvae being extricated from their hosts and handled during the study, despite efforts to exclude injured individuals from the experiments. However, once larvae begin to freeze, mortality rates increase markedly. Freeze avoidant insects often have a low supercooling point, whilst freeze tolerant insects have a greater supercooling point [18]. The results of the present study are consistent with this pattern. The results are also in agreement with the conclusions of Crosthwaite et al. [26]; yet, in the winter of 2012–2013, we were surprised to find four larvae that had frozen, or had at least started to freeze (as evident by the detection of an exotherm), and continued to live for up to 13 days after cold exposure. Because we did not measure the extent of ice formation, it would be presumptuous to conclude that these individuals were freeze tolerant. Such individuals might be classified as partially-freeze tolerant [46,56], but the ecological ramifications of such a cold tolerance strategy are debatable [56,57].

Our studies help to provide a more robust characterization of the cold tolerance of *A. planipennis* by measuring the extent of mortality and the proportion of insects that may have frozen at different exposure temperatures [46]. Because the vast majority of overwintering *A. planipennis* larvae die upon freezing, the supercooling point provides a convenient indicator of the expected level of mortality at different temperatures. In portions of our study, the supercooling points of *A. planipennis* larvae were lower than previously reported. The supercooling points of artificially infested larvae measured in November 2011 (~−25 °C) were similar to previously reported values. However, the mean supercooling points of artificially-infested larvae in January 2013 and field-collected larvae in January-March 2013 and January–March 2014 were nearly 2–6 °C lower than previously reported mean supercooling points and were significantly colder than those from November 2011.

The differences in supercooling points from previous studies and ours could also be related to the extent of cold acclimation that occurs during the fall and early winter. The warmer supercooling points we recorded in November 2011 could have been because testing was performed before the insects had fully acclimatized to winter or the insects were responding to a warmer than average fall, which preceded a warmer than average winter [58]. However, two years of study by Crosthwaite et al. [26] showed that the mean supercooling point declined from October through mid-November, but did not change statistically thereafter for the remainder of the winter. The annual variation in supercooling points and temperature-mortality relationships measured in this study hint that the degree of cold tolerance of *A. planipennis* is a plastic response to as yet undefined cues experienced during autumn or winter. If *A. planipennis* larvae are able to adjust their cold hardiness in response to these cues, our data seem to show a lower limit to *A. planipennis*' capacity to cold harden: the fall and winter of 2013–2014 were colder than average, but supercooling points and the mortality from freezing were not statistically different from the results collected during the more typical Minnesota winter of 2012–2013. Potential genetic differences in populations of *A. planipennis* could also explain different reports of cold tolerance, but genetic testing suggests that the U.S. populations most likely stem from a single introduction [59]. Regional differences in the quality of host species could also be a factor, but the attribute of the host that could drive these differences remains to be identified.

Cold often determines the northern limits of an invading insect's range, e.g., [60,61]. *Agrilus planipennis* has yet to achieve its ultimate distribution in North America. The potential geographic range expansion by *A. planipennis* into northern Minnesota where black ash is abundant in lowland black ash-American elm-red maple forests [62] is a particular concern. Minnesota has more than 900 million ash trees, about 75% of which are black ash [63]. Black ash and green ash comprise almost half of Minnesota's timber resource by volume in lowland forests and about one quarter of Minnesota's total forest resource [63,64]. Black ash is one of the few native tree species that grows in bogs and poorly drained soils [65,66]. Green ash is a popular boulevard tree in communities throughout the state and is a common native species to the central and southern part of Minnesota [64].

Seven years after first being detected in North America, *A. planipennis* was found in Minnesota for the first time in Saint Paul [67]. Eight years after the insect arrived in the state, most of Minnesota's ash remains uninfested. The effect of temperatures <-30 °C on the distribution and impact of *A. planipennis* in this state and elsewhere in North America remain to be determined, but overwintering mortality in these areas could have substantial impacts on the rates of population growth and spread. Areas with regular exposure to temperatures <-35 °C may provide thermal refugia that are vital to the local persistence of native ash stands.

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