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Cold tolerance of Chinese emerald ash borer parasitoids: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae)

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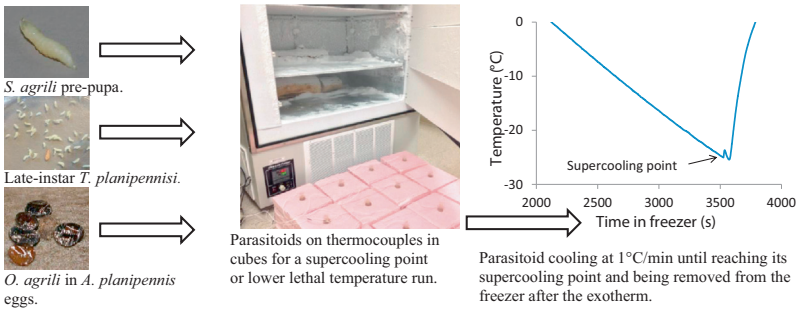
HIGHLIGHTS

- Cold acclimation decreased supercooling points of *Tetrastichus planipennisi and Spathius agrili*.
- 50% of cold acclimated *T. planipennisi* and *S. agrili* died at −19.9 and −27.3 °C.
- Mortality of *T. planipennisi* and *S. agrili* increased with cold exposure time.

GRAPHICAL ABSTRACT



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ABSTRACT

Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive insect that has caused significant ash (*Fraxinus* spp.) mortality in North America. Three Chinese parasitoids have been approved for release as part of a classical biological control program for *A. planipennis* in the United States: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae). This study was designed to measure the cold tolerance of the overwintering stage for each parasitoid species in the laboratory. We exposed cold-acclimated and non-cold-acclimated individuals to temperatures from 0 to −35 °C to determine temperatures that cause body fluids to freeze, mortality after brief exposure, and mortality after long-term exposure. Cold acclimation lowered the supercooling points of *S. agrili* (median −28.8 °C) and *T. planipennisi* (median −29.4 °C). Median supercooling point for *Oobius agrili* was −30.5 °C. Cold acclimation also increased survival of diapausing *S. agrili* (50% mortality at −27.3 versus −23.7 °C for non-diapausing *S. agrili*) during brief cold exposure. *T. planipennisi* and *S. agrili* mortality increased over long term cold exposure when held at constant temperature. Half of *T. planipennisi* are predicted to fail to eclose after exposure to 0, −5, −10, and −15 °C after >84, 82, 59, and 36 days, respectively, while 50% of *S. agrili* with diapause induced in one generation would be discolored from cold injury >84 days for all exposure temperatures. Our models characterizing parasitoid mortality due to cold exposure can be used to assess the climatic suitability of a location prior to release.

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1. Introduction

Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an Asian beetle that was first detected in North

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America (Michigan, USA and Ontario, Canada) in 2002 (McCullough and Katovich, 2004), but may have been present since the mid-1990s (Siegert et al., 2009). Currently, *A. planipennis* occurs in 21 US states and two Canadian provinces but may spread to 25 North American states and provinces over the next decade (Kovacs et al., 2010). The larvae feed on the cambium, phloem, and outer sapwood of ash (*Fraxinus* spp.). Under high larval densities, galleries from the tunneling larvae girdle the tree, thereby causing crown dieback and eventually tree death. Large trees typically die three to four years after an infestation starts (Poland and McCullough, 2006). *A. planipennis* has already killed at least 50 million ash trees in Michigan alone (Smith et al., 2009). The cost of removing infested trees on developed land to slow further infestation and prevent safety hazards caused by dead trees is estimated to be $10.7 billion from 2009 to 2019 in North America (Kovacs et al., 2010). However, *A. planipennis* is not a major pest in its native range of northeastern Asia. Damage from *A. planipennis* in North America has been attributed to the lack of natural enemies and host-plant resistance (Liu et al., 2007).

The US Department of Agriculture conducted surveys in northeastern China for potential natural enemies of *A. planipennis* to initiate classical biological control in the United States (Liu et al., 2003, 2007; Gould et al., 2005; Bauer et al., 2008, in press). Three parasitoids were approved for release in North America: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), and *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) (Yang et al., 2005, 2006; Zhang et al., 2005). *S. agrili*, a gregarious ectoparasitoid, and *T. planipennisi*, a gregarious endoparasitoid, both oviposit through the bark where the offspring feed on and kill the host *A. planipennis* larva (Wang et al., 2010; Yang et al., 2010; Ulyshen et al., 2010a). *T. planipennisi* larvae feed within the host and rupture from the host to pupate and eclose (Duan et al., 2011). In contrast, *S. agrili* larvae feed externally until the host is consumed; afterwards larvae enter a wandering stage and move away from the consumed host, then spin cocoons, pupate, and eclose (Yang et al., 2010). In both cases, the larvae are free of their host after feeding ceases. For both larval parasitoids, development from egg to adult takes 3–4 weeks when temperatures are between 22.5 and 26.5 °C, and adults exit the gallery by chewing through the bark (Lelito, *unpublished* data; Ulyshen et al., 2010b; Yang et al., 2010). Both *T. planipennisi* and *S. agrili* have been described as multivoltine (Yang et al., 2005; Liu et al., 2007a), but emergence of the overwintering generation of *S. agrili* may instead occur periodically throughout the spring and summer (Lelito, *unpublished data*).

Oobius agrili is a solitary egg parasitoid that is typically parthenogenetic. *O. agrili* eggs develop within the *A. planipennis* egg, and emerge as adults in approximately one month when temperatures are between 22.5 and 26.5 °C (Lelito, *unpublished data*; Bauer and Liu, 2006; Liu et al., 2007b).

Each species of emerald ash borer parasitoid overwinters in a different stage or location. *A. planipennis* overwinters inside the gallery or pupal cell, often as pre-pupae, but occasionally as first to third instars (Poland and McCullough, 2006; Crosthwaite et al., 2011). The two larval parasitoids typically are not in direct contact with the host while overwintering. *S. agrili* overwinter as diapausing pre-pupae within the host gallery (Yang et al., 2010). Diapause in *S. agrili* can be induced in one or two generations; individuals with diapause induced over two generations have greater survival rates during storage at 4 °C for 120 days, which is required to break diapause, than individuals that entered diapause in one generation (Lelito, *unpublished data*; Gould et al., 2011). *T. planipennisi* has been described overwintering as late-instar larvae or pupae in the host gallery (Bauer et al., 2008; Ulyshen et al., 2011). However, it has recently been found overwintering as young larvae inside the host (L. Bauer, *personal comm.*). *T. planipennisi* is not known to enter diapause in any stage (Ulyshen et al., 2011; Duan et al., 2011, 2013). *O. agrili* overwinter as diapausing pre-pupae within the host egg (Liu et al., 2007).

The parasitoids may reduce *A. planipennis* densities in North America because the parasitoids have high parasitism rates in China, especially on North American ash trees planted in Asia (Yang et al., 2005; Liu et al., 2007; Duan et al., 2012b). Average parasitism rates for *T. planipennisi* in the laboratory typically range from 60% to 80% with 4–172 offspring per host (Lelito, *pers. obs.*; Ulyshen et al., 2010b). Likewise, parasitism rates for *S. agrili* typically range from 30% to 90% with 1–18 offspring produced per host (Yang et al., 2005, 2010). *O. agrili* parasitize up to 82% of eggs in the lab; parasitized eggs turn black (Liu et al., 2007b). However, cold may limit the northern distribution of the parasitoids in North America. The Chinese parasitoids have overwintered in initial release sites in Michigan, Maryland, Ohio, Indiana, and Illinois (Bauer et al., 2012; Duan et al., 2013). However, states such as Michigan and Maryland also have relatively mild winters compared to other areas at similar latitudes (USDA, 2012). Additional time may be required to confidently assess establishment in states where the parasitoids have only been recently released (Ulyshen et al., 2011; Duan et al., 2012a).

For insects, three measures are commonly used to assess cold tolerance at the population level: supercooling points, lower lethal temperature, and lower lethal time (e.g., Eaton and Kells, 2011; Morey et al., 2012). The supercooling point is the temperature at which insect body fluids begin to change from a liquid to solid state and is the lowest temperature an insect reaches prior to an exotherm, an increase of temperature due to the heat released as water crystallizes, as reviewed in Denlinger and Lee (2010). Brief exposure to freezing temperatures is lethal for freeze-intolerant species, and most insects are freeze-intolerant, as reviewed in Sømme (1982). Two other mortality responses can also be classified relative to the supercooling point. An insect is chill-intolerant if it dies after brief exposure to cold but before the supercooling point is reached, and is freeze-tolerant if it survives exposure to temperatures below its supercooling point. Lower lethal temperature is a measure of mortality in a population after brief exposure to a given low temperature. However, both supercooling point and lower lethal temperature measurements are limited to characterizing the consequences of brief temperature exposures that may occur during an overnight low, but do not account for cold stress that accrues with prolonged cold exposure throughout winter (Renault et al., 2002). Lower lethal time is similar to lower lethal temperature, but measures mortality at multiple lengths of exposure to a constant low temperature to simulate long-term cold exposure.

In this study, our primary objective to measure supercooling points, lower lethal temperature and lower lethal time for *O. agrili*, *T. planipennisi*, and *S. agrili*. Several insect species become more cold tolerant in response to environmental cues such as decreasing temperature or photoperiod (e.g., Kim and Song, 2000). We hypothesized that parasitoids reared under short photoperiods or cool temperatures would be more cold tolerant than parasitoids reared under long photoperiods and warm temperatures. We also hypothesized that *O. agrili* may be the most cold-tolerant species because it overwinters near the surface of the tree where it can experience lower temperatures than larval parasitoids overwintering in the host gallery.

2. Materials and methods

2.1. Rearing

O. agrili, *T. planipennisi*, and *S. agrili* were reared at the US Department of Agriculture–Animal and Plant Health Inspection

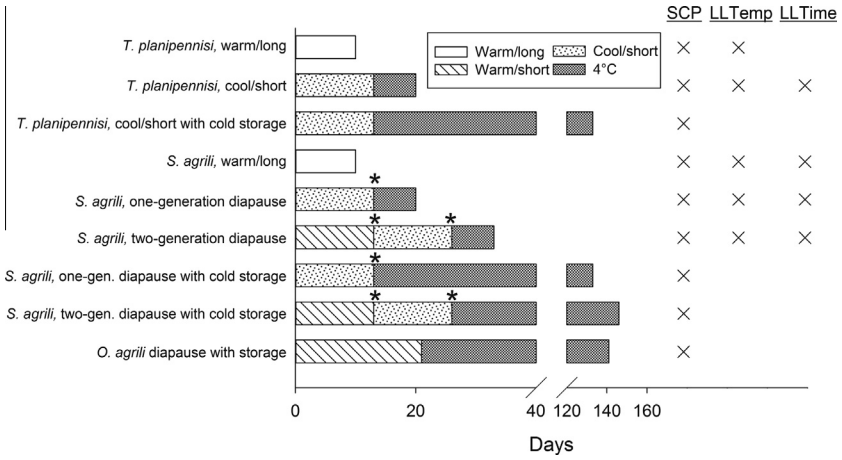

Fig. 1. Rearing conditions for *O. agrili*, *T. planipennisi*, and *S. agrili*. Species and rearing treatment combinations used during cold tolerance testing are shown on right: supercooling point (SCP), lower lethal temperature (LLTemp), and lower lethal time (LLTime). Each rearing condition occurs in a single generation excluding *S. agrili* diapause treatments where a new generation is marked with an asterisk.

Service, Emerald Ash Borer Biological Control Facility in Brighton, MI as part of a mass rearing program. Adults were allowed to parasitize the target life stage of *A. planipennis* under temperature and photoperiod conditions that would mimic summer-like conditions and potential cold-acclimating or diapause-inducing conditions in the fall and winter. Warm (26.5:22.5 °C for 16:8 h) or cool (20:15 °C for 8:16 h) rearing temperature regimes, and long (16:8 h L:D) or short (8:16 h L:D) photoperiod conditions were used in different combinations for each species (Fig. 1). In all cases, relative humidity was maintained at approximately 60–70%. Specific rearing conditions applied to each species are described below.

O. agrili were allowed to parasitize *A. planipennis* eggs under diapause-inducing conditions. *A. planipennis* eggs on coffee filter paper were presented to *O. agrili* under warm/short-photoperiod conditions to induce diapause (L. Bauer, *personal comm.*; Lelito *unpublished data*). Approximately 14 d after parasitism, *O. agrili* pupae within *A. planipennis* eggs were held at 4 °C for 4 months to simulate potential long term cold acclimation. Because the number of *O. agrili* was limited, only individuals from diapause conditions were tested.

Mated *T. planipennisi* females were allowed to parasitize *A. planipennis* larvae manually placed under the phloem of ash bolts (*Fraxinus* spp.) that were approximately 5 cm diameter × 10 cm in length (Liu and Bauer, 2007; Yang et al., 2008). Three rearing treatments were tested for late-instar *T. planipennisi*: warm/long-photoperiod conditions to produce non-cold acclimated individuals, cool/short-photoperiod conditions to produce cold acclimated individuals, and cool/short-photoperiod conditions followed by 4 °C for 4 months for long term cold acclimation (Fig. 1).

Five rearing treatments were examined for *S. agrili* pre-pupae: warm/long-photoperiod summer conditions, one-generation diapause, two-generation diapause, and one- and two-generation diapause groups acclimated at for 4 °C for 4 months. Diapause was induced in *S. agrili* in one generation by allowing adults to mate under cool/short-photoperiod conditions for 3–4 d before presenting them with a piece of ash artificially infested with *A. planipennis* larvae (as described above for *T. planipennisi*). The resulting offspring entered diapause as pre-pupae. Diapause was induced over two generations by allowing adults to mate under warm/short-photoperiod conditions for 3–4 d and allowing them to oviposit (F1 generation) under those conditions. The F1 adults were then reared under cool/short-photoperiod conditions for 3–4 d before allowing them to oviposit. The resulting offspring (F2 generation) entered diapause as pre-pupae.

Approximately one week after being parasitized, *A. planipennis* with *T. planipennisi* or *S. agrili* larvae were removed from ash bolts. Late-instar parasitoids in or on hosts were shipped in Petri dishes without ice via overnight courier to St. Paul, MN for cold-tolerance testing, all in accordance with the terms and conditions of USDA APHIS Permit P526P-11-00136. When *T. planipennisi* and *S. agrili* larvae were received, they had developed to wandering-stage larvae and were free from their host. Individual immature parasitoids of each species were placed into 1.5 ml microcentrifuge tubes using a fine-tipped paintbrush. *O. agrili* pre-pupae were kept in the remnants of the host egg to avoid potential handling stress from dissection. Each parasitoid was inspected for damage or disease, and such individuals were discarded. The remaining individuals were assigned arbitrarily to cold exposure treatments. Individuals from warm/long-photoperiod conditions were placed in a reach-in growth chamber with warm temperature and long-photoperiod conditions until they could be tested. *S. agrili* and *O. agrili* in diapause, and *T. planipennisi* from cool/short-photoperiod conditions were placed in a reach-in refrigerator with no lighting at 10 °C for 2 d and then into a refrigerator at 4 °C for 7 or 120 days before cold tolerance testing.

After the specified acclimation periods, *O. agrili* pre-pupae, *S. agrili* pre-pupae, and late-instar *T. planipennisi* were used in all cold tolerance tests. All *O. agrili* pre-pupae were kept in the egg remnant because it was dry and did not interfere with supercooling point measurements (i.e., only one exotherm from the pre-pupae and not from the egg remnant). For the larval parasitoids, we used a free-living stage to reduce potential confounding factors that might affect parasitoid cold tolerance, particularly the potential for parasitoids still in association with the living host to experience inoculative freezing within the host should the host freeze. Occasionally, *S. agrili* larvae would construct cocoons, and cold tolerance testing was performed with the cocoon intact if one was constructed.

2.2. Cold tolerance testing

For supercooling point determination and lower lethal temperature studies, "cradle" thermocouples were constructed from 0.127 mm diameter copper and constantan wires that are able to detect exotherms >0.05 °C (Hanson and Venette, 2013). Thermocouples were connected to a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Temperatures (accuracy ± 0.17 °C) were recorded once per second at the first point of

contact between the copper and constantan wires (Carrillo et al., 2004).

Constraints on the availability of insects only allowed us to test supercooling points and lower lethal temperature of one species and rearing condition at a time. Supercooling points were measured for all species and rearing combinations previously described (Fig. 1). Due to limited availability of *T. planipennisi*, lower lethal temperature was only measured for individuals reared under warm/long-photoperiod and cool/short-photoperiod conditions, and lower lethal time was only measured for individuals from cool/short-photoperiod conditions. For *S. agrili*, lower lethal temperature and time were measured from warm/long-photoperiod, one-generation-, and two-generation-diapause conditions. Differences were compared among species and rearing treatments for each of the cold tolerance measures (Fig. 1). *Post hoc* comparisons among species or rearing conditions must be interpreted with caution because logistics prevented us from testing multiple species or rearing conditions at the same time. Thus, other factors which could not be controlled by our experimental design could influence apparent differences or lack thereof among treatments of interest. Lower lethal temperature and time were not measured for *O. agrili* because the number of parasitoids was limited.

2.2.1. Supercooling point

Each insect was placed in a 5 × 14 mm clear gelatin capsule (Capsuline, Pompano Beach, FL). Capsules with insects to be cooled were held on the thermocouple with a small amount of high vacuum grease (Dow Corning, Midland, MI). The thermocouples with insects were placed in polystyrene cubes constructed to cool at a constant 1 °C per minute inside a −80 °C freezer (Carrillo et al., 2004). After detection of an exotherm, the individual cube was removed from the freezer once the temperature returned to the supercooling point as per Koch et al. (2004). The thermocouple was immediately removed from the cube and allowed to warm to room temperature (ca. 25 °C). Individuals were removed from the gelatin capsules, each placed in a microcentrifuge tube, and returned to warm/long-photoperiod rearing conditions to monitor development. One to sixteen cubes containing a single species × rearing combination were used per run. When more than one cube was used in a run, we considered the run to be a block, but when only one cube was used, all runs within a day were considered to be a block. (Blocking by date only applied to *S. agrili* and *T. planipennisi* held at 4 °C for 4 months.) The number of blocks and total number of individuals tested are reported (Supplemental Table), but in general supercooling points were measured for 10–57 individuals from each rearing treatment.

Data were analyzed with SAS 9.3 (SAS Institute, 2013). Supercooling point distributions were compared within species to test the effect of rearing condition and among species for the rearing condition that appeared to give the greatest cold tolerance. Initially, supercooling point data were analyzed for normality (PROC UNIVARIATE) and if data were not normally distributed, Box–Cox transformations (Box and Cox, 1964) were attempted. Supercooling points, in general, were not normally distributed and appropriate Box–Cox transformations could not be found (data not shown). Consequently, we first tested the effect of blocking within each species × rearing combination with a Kruskal–Wallis ANOVA with a critical $\alpha = 0.005$ after a Bonferroni correction for multiple comparisons to maintain an overall $\alpha = 0.05$. No block effects were detected (Supplemental Table), so data were pooled to test for effects of rearing condition or species on supercooling points. Supercooling point distributions among rearing treatments or between species were compared using pairwise nonparametric two-sample tests for equality of probability distributions [Kuiper (1960) test in PROC NPAR1WAY]. Probability values were adjusted for multiple comparisons to maintain an overall α of 0.05 using a Bonferroni correction (critical $\alpha = 0.006$ per comparison). Supercooling points of each rearing treatment within each species were compared, and the rearing treatment with the lowest median supercooling point for each species was selected for comparisons among species. We consider tests of the complete distribution of supercooling points to be more informative than tests of central tendency (i.e., based on the median or mean) because they are able to discern changes in the tails of the distributions (i.e., effects on the most or least cold hardy individuals).

2.2.2. Lower lethal temperature

Lower lethal temperature experiments followed a randomized block design. Within each block (i.e., run of the test), parasitoids from one species × rearing-condition were assigned arbitrarily to one of four target temperature treatments. Treatment temperatures were chosen to bracket the approximate mean supercooling point for each species × rearing group based on previously collected data. Each treatment was replicated four times for a total of 16 insects per run. Insects were placed individually into gelatin capsules, onto thermocouples, and cooled as described for supercooling point measures. Insects were not removed until the target temperature was reached, even if an exotherm was detected. The duration of exposure to the target temperature was about 5 s. After removal, samples were immediately warmed to room temperature. An additional four to ten insects in separate gelatin capsules were held at room temperature for the duration of the test as a control for each block and returned to warm/long-photoperiod conditions after completion of the run. Between five and ten blocks were run for each species × rearing group (Supplemental Table). After cold exposure, individuals were removed from the gelatin capsules, each placed in a microcentrifuge tube, and returned to warm/long-photoperiod rearing conditions for further observation.

Individuals were inspected at least twice to evaluate the effects of exposure to a temperature treatment. *T. planipennisi* and *S. agrili* were examined 3 d after cold exposure for black or brown discoloration and lack of movement. Both species were also examined for eclosion 30 d after return to warm/long-photoperiod after cold exposure. Diapausing *S. agrili* were also examined at 12 and 16 weeks when eclosion was expected (Lelito, *unpublished data*). Discoloration by 16 weeks was noted to determine if non-eclosed individuals were healthy but still in diapause (creamy white) or likely dead (black or brown). Discoloration and eclosion were assessed for *T. planipennisi* and *S. agrili* reared at warm/long-photoperiod conditions by cooling individuals to −15, −20, −25, or −30 °C before being returned to warm/long-photoperiod conditions (sample sizes in Supplemental Table). *T. planipennisi* from cool/short-photoperiod conditions and *S. agrili* from one- and two-generation diapause conditions were cooled to −20, −25, −30, or −35 °C before being returned to warm/long-photoperiod conditions (sample sizes in Supplemental Table). Some adult eclosion occurred for all *T. planipennisi* rearing treatments, so discoloration measures were not included in further analyses for this species. Cohen's kappa (Cohen, 1960) was used to measure agreement between the proportion of *S. agrili* from warm/long-photoperiod conditions that were discolored by 3 d after cold exposure and the proportion that failed to eclose. This test was meant to determine if discoloration and eclosion failure agreed. The proportion of individuals where both measures indicated possible cold injury (discoloration and failure to eclose) or survival (normal color and development) was calculated as a coarse measure of overall agreement.

Logistic regression (PROC LOGISTIC; SAS Institute, 2012) was used to express cold injury as a function of brief exposure to cold temperature. Modified Abbott corrected injury measures (Appendix) were calculated for each cold exposure treatment to account for any discoloration or eclosion failure in the unexposed control group (Rosenheim and Hoy, 1989). Treatment sample size was

multiplied by the proportion of individuals surviving in the control group to give an adjusted sample size. The adjusted sample size was then multiplied by the adjusted injury percentage to calculate the number of injured individuals in the treatment group attributable to cold exposure. The adjusted number of deaths and adjusted sample size were used in logistic regression models. Initially, a logistic regression model was developed with block as the only main effect to determine if differences in cold injury occurred between runs. No block effects were detected (Supplemental Table), so final logistic regression models followed the form:

$$\text{Discoloration or eclosion failure} = \frac{1}{1 + e^{-(b_0 + b_1 t)}} \quad (1)$$

where b_0 is the intercept, which reflects the projected degree of injury at 0 °C, b_1 reflects the rate of change in injury with the change in the coldest temperature, t, to which an insect was exposed. A separate regression model was estimated for each species and rearing combination. Differences between two models were tested in PROC LOGISTIC by including a binomial categorical variable, c, with values of 0 for a reference group and 1 for a test group (Suits, 1957). The resulting model followed the form:

$$\text{Discoloration or eclosion failure} = \frac{1}{1 + e^{-(b_0 + b_1 t + b_2 c + b_3 tc)}} \quad (2)$$

where b_0, b_1, and t are as defined previously, but now for the reference group; b_2 measures the difference in injury at 0 °C (i.e., intercept) between the test and reference group; and b_3 measures the difference in rates of change in injury with change in exposure temperature between the test and reference group. To measure differences in cold tolerance between species from similar rearing conditions, *T. planipennisi* was assigned to 0 and *S. agrili* was assigned to 1. A Bonferroni correction was used to adjust probability values for the number of comparisons within species and rearing treatments to maintain an overall $\alpha = 0.05$. Exposure temperatures resulting in 50% and 90% mortality were calculated from logistic regression models for each species and rearing combination. The delta method was used to estimate 95% confidence intervals for each estimate, as reviewed in Faraggi et al. (2003).

2.2.3. Lower lethal time

Species and rearing treatments for lower lethal time measures included *T. planipennisi* from cool/short-photoperiod conditions and *S. agrili* from warm/long-photoperiod, one-generation-, and two-generation-diapause conditions (Fig. 1). Lower lethal time studies followed a factorial design with four exposure temperatures (0, −5, −10, or −15 °C) and five exposure periods (3, 14, 28, 56, or 84 d). A control group was also placed in warm/long-photoperiod rearing conditions. After receiving a shipment of parasitoids, insects were placed in microcentrifuge tubes, and individuals were randomly assigned to a temperature and time exposure. Additional blocks of the test were performed as more parasitoids became available. Before being placed in reach-in freezers (Freezer Concepts, Southbury, CT), *S. agrili* from warm/long-photoperiod conditions were held at 10 °C for 24 h to reduce the potential for cold shock, while *T. planipennisi* and diapausing *S. agrili* were moved from 4 °C coolers to their respective freezers. Parasitoids were held at constant temperature (±1 °C) and removed at the end of each assigned exposure period (sample sizes in Supplemental Table). After the specified length of exposure, treatment individuals were held at 10 °C for 24 h, followed by warm/long-photoperiod rearing conditions. Discoloration was assessed for control and exposure groups 3 d after being placed in warm/long-photoperiod conditions, and eclosion was monitored as described for lower lethal temperature studies. Due to the limited availability of *S. agrili* from one and two-generation diapause over time, individuals from the first shipment of each rearing condition were only assigned to 84-d exposures and room-temperature controls. This long exposure period had to be started first to have sufficient time for the treatment and time to break diapause (at least 192 days). After we noticed that none of the diapausing *S. agrili* were eclosing from the control or the three day exposure treatments, discoloration was noted at 16 weeks after removal from cold exposure for the remaining 14, 28, 56, and 84 d time treatments.

The proportion of *S. agrili* from warm/long-photoperiod conditions that were discolored or failed to eclose were compared with Cohen's kappa to assess the extent of agreement between the two measures over the course of the long-term cold exposure. Logistic regression models were fitted to data to describe injury over time at a constant temperature after adjusting for control injury (Appendix). Logistic regression models for lower lethal time followed the form:

$$\text{Discoloration or eclosion failure} = \frac{1}{1 + e^{-(b_0 + b_1 t + b_2 d + b_3 td)}} \quad (3)$$

where b_0, b_1, and t are as defined previously (see Eq. (1)), b_2 now measures the change in injury within increasing days of exposure, d, and b_3 measures the interaction of temperature and time ($t * d$) on changes in injury with respect to changing temperature and time exposures. The temperature by time interaction term was only included in the model for a species and rearing condition if there was a significant effect of both temperature and time, and the interaction term was also significant. A block effect was also included to determine if cold injury was different for each shipment of parasitoids received, but was excluded from the final model because no significant effect of block was detected (Supplemental Table). The effects of rearing conditions on injury were compared as for lower lethal temperature by including the categorical variable c to test for differences between reference and test groups. Exposure times resulting in 50% and 90% mortality were calculated from logistic regression models for each temperature, species, and rearing combination. The delta method was used to estimate 95% confidence intervals for each estimate.

Because few *T. planipennisi* from warm/long rearing conditions eclosed after lower lethal temperature exposures, the data from lower lethal temperature and 3 d observations from the lower lethal time studies were combined. Eclosion rates for room temperature controls from lower lethal temperature studies and lower lethal time studies were not different (data not shown). The combined data were used to generate a logistic function to describe the probability of eclosion failure as a function of brief exposure to temperatures from 0 °C to the lowest lower lethal temperature exposure.

3. Results

3.1. Supercooling point

The median supercooling point for late instar *T. planipennisi* was between −27.3 and −29.4 °C and was not the same for all rearing conditions (Table 1). *T. planipennisi* reared in cool/short-photoperiod conditions with an additional 4 mo. at 4 °C had median supercooling points that were 1.3 °C lower than those individuals that had only been exposed to cool/short-photoperiod conditions ($df = 1$; test statistic $Ka = 2.233$; $p = 0.002$; Table 1; Fig. 2A). Supercooling points for *T. planipennisi* were not different between warm/long-photoperiod and cool/short-photoperiod conditions ($df = 1$; $Ka = 1.610$, $p = 0.105$). Supercooling points were also not significantly different between runs for each rearing combination (Supplemental Table). No *T. planipennisi* adults eclosed after freezing.

Table 1
Comparison of *Tetrastichus planipennisi*, *Spathius agrili*, and *Oobius agrili* supercooling points from different rearing conditions.

Species	Rearing	Median	90th percentile	Within Sp.[a]	Among Spp.[b]
T. planipennisi	Warm/long	−27.34	−28.71	A	–
T. planipennisi	Cool/short	−28.14	−28.83	A	–
T. planipennisi	Cool/short, 4 °C acc.	−29.42	−31.11	B	ab
S. agrili	Warm/long	−25.19	−26.75	E	–
S. agrili	1 gen. diapause	−28.77	−31.02	F	a
S. agrili	1 gen. diapause, 4 °C acc.	−29.17	−31.22	F	–
S. agrili	2 gen. diapause	−29.06	−31.12	F	–
S. agrili	2 gen. diapause, 4 °C acc.	−28.09	−30.01	F	–
O. agrili	Diapause, 4 °C acc.	−30.49	−31.40	–	b

Rearing conditions are summarized in Fig. 1.
[a] Comparison of the statistical distributions of supercooling points within a species from different rearing conditions. Rearing conditions marked with the same letter are not statistically different. Comparisons of distributions were based on pairwise Kuiper's tests with a Bonferroni adjustment to maintain an overall $\alpha = 0.05$.
[b] Comparison of the statistical distributions of supercooling points among species from rearing conditions that gave the most cold-tolerant individuals. Rearing conditions marked with the same letter are not statistically different. Comparisons of distributions were based on pairwise Kuiper's tests with a Bonferroni adjustment to maintain an overall $\alpha = 0.05$.

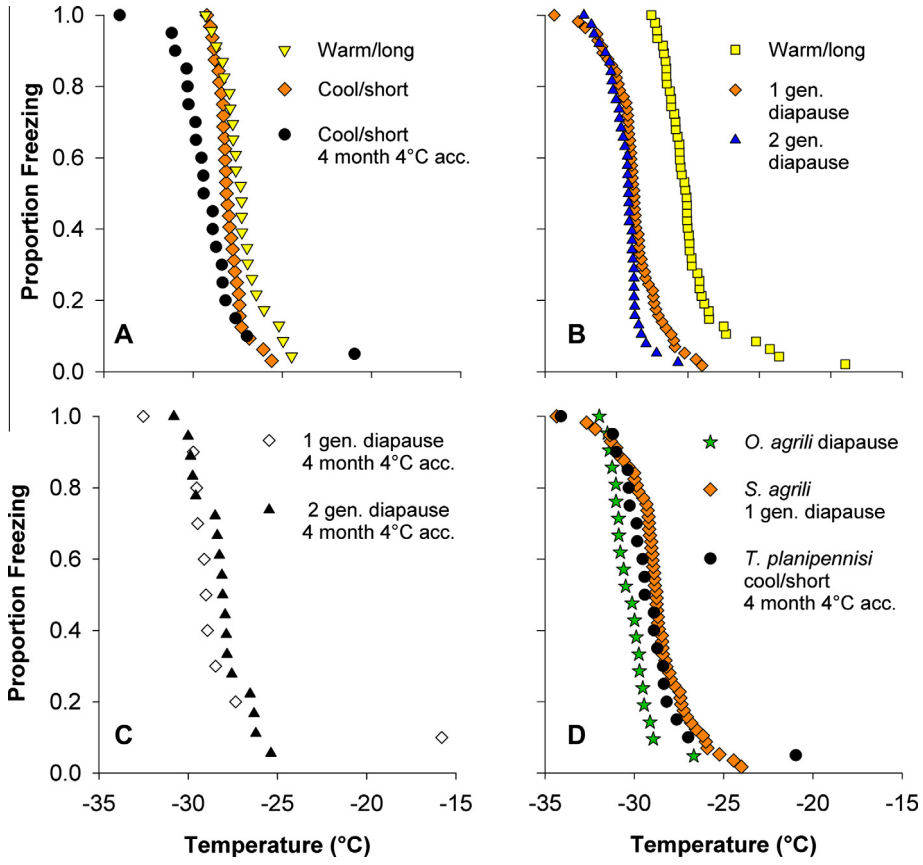

Fig. 2. Observed supercooling points for: (A) *Tetrastichus planipennisi* larvae from three rearing conditions; (B) *Spathius agrili* from three rearing conditions without an additional four months at 4 °C (C) *S. agrili* from two diapause-inducing conditions with an additional four months at 4 °C. (D) *Oobius agrili* and species from rearing combinations with the lowest supercooling points.

The median supercooling point for *S. agrili* ranged from −25.2 to −29.2 °C and was not the same for all rearing conditions (Table 1; Fig. 2B). *S. agrili* from warm/long-photoperiod conditions had supercooling points that were 3–4 °C warmer than *S. agrili* from one- or two-generation-diapause conditions without acclimation ($df = 1$; smallest $Ka = 4.077$; largest $p \leqslant 0.001$; Table 1). Supercooling points did not change when *S. agrili* from diapause conditions were acclimated at 4 °C for 4 months ($df = 1$; largest $Ka = 2.064$; smallest $p = 0.006$; Table 1; Fig. 2C). Supercooling points were also not significantly different among runs for each rearing combination (Supplemental Table). After freezing, all pre-pupae from warm/long conditions were discolored, and 91.2% ($n = 57$) from one-generation-diapause and 97.4% ($n = 38$) from two-generation-diapause were discolored.

Diapausing *O. agrili* had a median supercooling point of −30.5 (Table 1; Fig 2D). One of 21 *O. agrili* eclosed 30 d after freezing. When compared with the other parasitoid species from rearing conditions that yielded the lowest supercooling points, supercooling points of *O. agrili* were 1.3 °C lower than *S. agrili* from one-generation-diapause conditions with an additional 4 mo. at 4 °C ($df = 1$; $Ka = 2.601$, $p < 0.001$) (Table 1, Fig. 2D). Supercooling points for *T. planipennisi* reared under cool/short-photoperiod conditions

Table 2
Results of lower lethal temperature studies for *Tetrastichus planipennisi* and *Spathius agrili* from different rearing conditions: number of individuals that froze and died after freezing and estimated logistic-regression parameters to characterize discoloration or failure of adults to eclose as a function of brief exposure to low temperatures.

Species & rearing	*N* frozen	Freeze-mortality	Intercept (b_0)			Temperature (b_1)		
			Coefficient ± SE	χ^2	*p*	Coefficient ± SE	χ^2	*p*
T. planipennisi								
Eclosion:								
Warm/long	23	23	−4.871 ± 1.3762B	12.53	<0.001	−0.270 ± 0.0679B	0.07	<0.001
Cool/short	60	60	−0.534 ± 2.123	0.06	0.802	−0.102 ± 0.0821	1.53	0.216
Cool/short[a]	–	–	−7.328 ± 1.349	29.5	<0.001	−0.369 ± 0.0680	29.39	<0.001
S. agrili								
Discoloration:								
Warm/long	44	41	−15.017 ± 2.744a	29.94	<0.001	−0.633 ± 0.115a	30.46	<0.001
One gen. diapause	46	43	−26.520 ± 5.408a	24.05	<0.001	−0.970 ± 0.199a	23.87	<0.001
Two gen. diapause	62	52	−15.651 ± 2.464a	40.35	<0.001	−0.542 ± 0.085a	41.01	<0.001
Eclosion:								
Warm/long	44	44	−27.730±7.380A	14.12	<0.001	−1.186±0.304A	15.22	<0.001

Coefficients followed by the same letter are not significantly different at Bonferroni adjusted $\alpha = 0.05$; lower case letters represent comparisons among rearing treatments based on discoloration; upper case letters represent the comparison between species based on eclosion.
Coefficients are for Eq. (1) in the text.
[a] Model resulting from combined lower lethal temperature and lower lethal time data at 3 d.

with an additional 4 mo. at 4 °C were not different from *S. agrili* or *O. agrili* ($df = 1$; largest $Ka = 1.463$, smallest $p = 0.209$). Supercooling points were not significantly different among runs (Table 1).

3.2. Lower lethal temperature

For *T. planipennisi* from warm/long-photoperiod conditions, a greater proportion of adults failed to eclose as temperatures declined (Table 2; Fig. 3A). A constant, high proportion of *T. planipennisi* from cool/short-photoperiod conditions failed to eclose as adults at all treatment temperatures (Table 2; Fig. 3A). Adult eclosion was not significantly different for *T. planipennisi* from warm/long-photoperiod conditions than from cool/short-photoperiod conditions (Table 2). There was not a significant difference between runs for either rearing treatment (Supplemental Table).

There was significant agreement between the proportion of *S. agrili* that were discolored 3d after cold exposure and the proportion that failed to eclose as adults 30 d after cold exposure (Fig. 4). Discoloration increased with decreasing temperature for *S. agrili* from warm/long-photoperiod conditions and one- or two-generation diapause, but discoloration rates after cold exposure were not significantly different among rearing treatments (Table 2; Fig. 3A). Adult eclosion also decreased with decreasing temperature for *S. agrili* from warm/long conditions (Table 2; Fig. 3B). However, diapausing *S. agrili* did not eclose 16 weeks after being returned to warm/long-photoperiod conditions, so failure of diapausing *S. agrili* to eclose could not be analyzed as a function of cold exposure. Adult eclosion rates were lower for *T. planipennisi* compared with *S. agrili*, when both were reared under warm/long-photoperiod conditions (Table 2). There was not a significant difference between runs for any rearing treatment (Supplemental Table). Temperatures resulting in 50% and 90% mortality from brief cold exposures also provided for *T. planipennisi* and *S. agrili*, e.g., 50% *S. agrili* mortality at −27.4 °C for one generation diapause versus −23.7 °C for warm/long conditions (Table 3).

3.3. Lower lethal time

A smaller proportion of *T. planipennisi* reared under cool/short-photoperiod conditions eclosed as the duration of exposure to constant temperatures between 0 and −15 °C, inclusive, increased. At any length of cold exposure, fewer adults eclosed as the treatment temperature declined (Fig. 5; Table 4). There was not a significant interaction between temperature and exposure time, so a temperature by time interaction term, $t * d$, was not included in the model. No significant difference was found between shipment dates for each rearing treatment (Supplemental Table).

Some *S. agrili* from warm/long-photoperiod conditions, but not from one- or two-generation-diapause conditions, eclosed after exposure to 0 to −15 °C, inclusive, for different lengths of time. The proportion of individuals from warm/long-photoperiod conditions that failed to eclose 30 d after being removed from cold exposure was greater than the proportion that was discolored after 3 d, though there was some agreement between both measures (Fig. 6). The proportion of *S. agrili* reared under warm/long-photoperiod conditions with discoloration increased and eclosion decreased as exposure temperature decreased and exposure duration increased (Table 4; Fig. 7A and B); adult eclosion was also affected by an interaction between temperature and time ($b_3 = -0.009$; SE = 0.001; $p < 0.001$). The proportion of discolored *S. agrili* from one-generation-diapause also increased with increasing exposure duration, but decreasing temperature did not significantly affect discoloration (Table 4; Fig. 7C). Two-generation-diapause discoloration increased with both decreasing temperature and increasing exposure duration (Table 4; Fig. 7D). All *S. agrili* from 1 or 2 generation diapause conditions were discolored 16 weeks after exposure. The proportion of discolored *S. agrili* from warm/long-photoperiod conditions was significantly greater than from two-generation-diapause inducing conditions (Table 4). No significant difference was found between shipment dates for each rearing treatment (Supplemental Table). Exposure times and temperatures resulting in 50% and 90% mortality are provided (Table 5). Half of *T. planipennisi* are predicted to fail to eclose after exposure to 0, −5, −10, and −15 °C for >84, 82, 59, and 36 d, respectively. *Spathius agrili* reared in diapause inducing conditions required >84 d of exposure to 0, −5, −10, or −15 °C to achieve 50% mortality.

Our assessment of the effects of rearing treatment on lower lethal temperature for *T. planipennisi* from cool/short-photoperiod conditions was initially incomplete. The proportion of individuals that eclosed after brief exposures to temperatures between −20 and −35 °C, inclusive, did not change significantly. Eclosion failure was >80% for all temperature treatments (Fig. 3A). However, as measured in our lower lethal time studies, eclosion failure was near zero after exposure to temperatures between 0 and −15 °C, inclusive, even after 3 d (Fig. 5). The combined model suggested

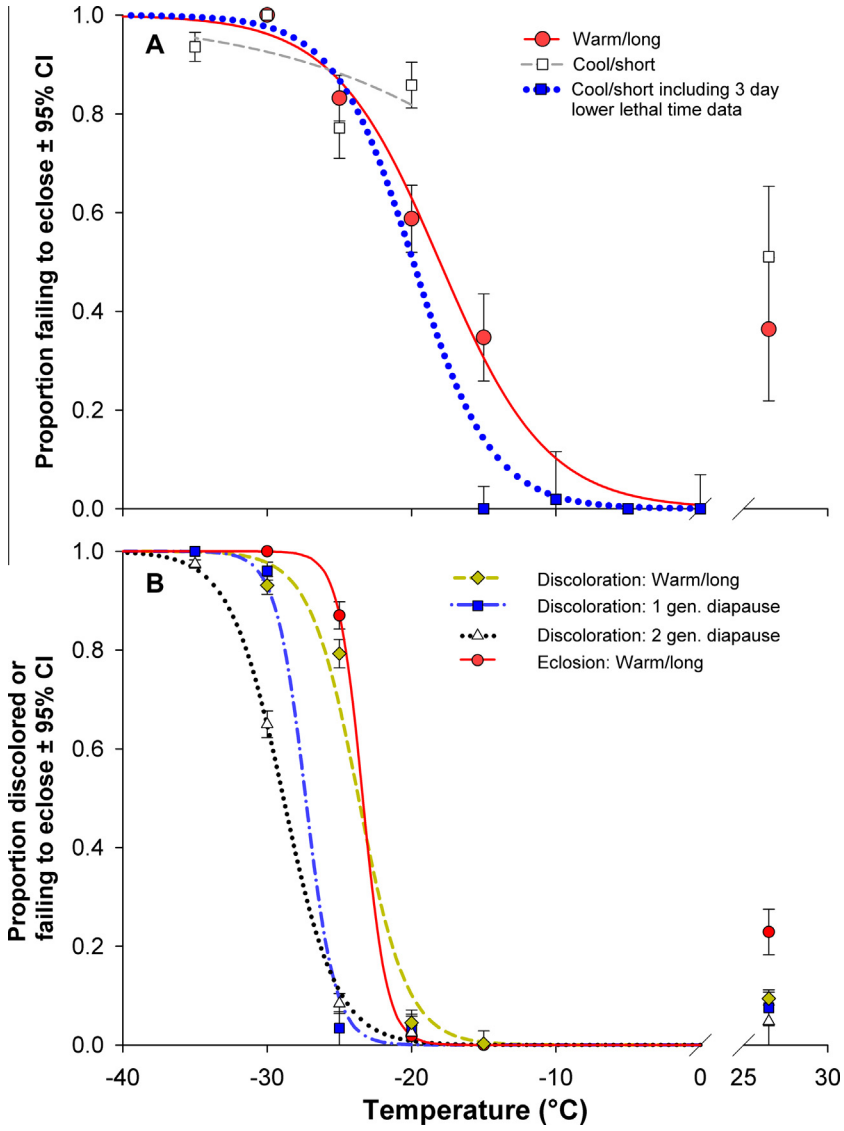

Fig. 3. Observed (symbols) and predicted (lines) proportion of emerald ash borer parasitoids that were discolored or failed to eclose after brief exposure to low temperatures: (A) *Tetrastichus planipennisi* adult eclosion. (B) *Spathius agrili* discoloration and adult eclosion. Observations at 26.5 °C were used to adjust proportions at other temperatures.

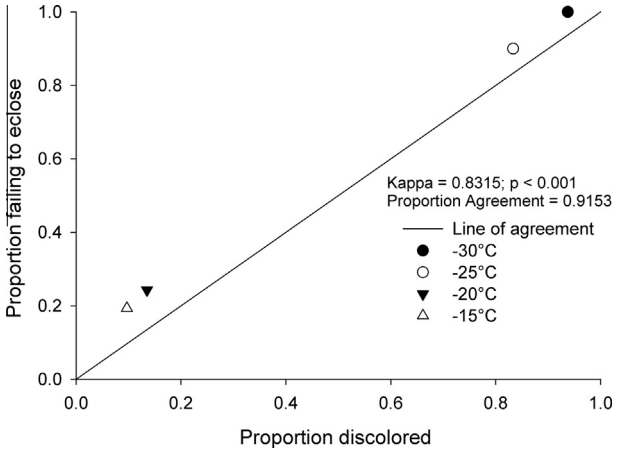

Fig. 4. Agreement between the proportion (not Abbott corrected) of *Spathius agrili* from normal rearing conditions that were discolored and those that failed to eclose in lower lethal temperature studies.

that the proportion of individuals that failed to eclose increased with brief exposure to colder temperatures, and eclosion rates were not significantly different than rates observed for *T. planipennisi* from warm/long-photoperiod conditions (Table 4; Fig. 3A).

4. Discussion

4.1. Previous research

Other researchers have reported some supercooling points and lower lethal time estimates for *T. planipennisi* and *S. agrili*, but measures of cold tolerance for *O. agrili* have not been published to date. Wu et al. (2007) reported that supercooling points of *T. planipennisi* ranged from −13.5 to −28.1 °C and *S. agrili* ranged from −19.9 to −28.4 °C. Our supercooling points of unacclimated individuals closely match these findings (Fig. 2). Lelito (*unpublished data*) also stored *T. planipennisi* larvae and *S. agrili* pre-pupae from warm/long-photoperiod rearing conditions inside ash bolts for extended periods of time at 4 °C and observed 15% *T. planipennisi* mortality at 185 d of exposure and 25% *S. agrili* mortality at 255 d of exposure. If the lower lethal time model from this work is extrapolated

Table 3
Lower lethal temperature for 50% (LT_{50}) and 90% mortality (LT_{90}) with 95% confidence intervals.

Species & rearing	LT_{50}	95% C.I.	LT_{90}	95% C.I.
T. planipennisi				
Eclosion:				
Warm/long	−18.0	−15.7 to −20.4	−26.2	−22.3 to −30.0
Cool/short	–	–	–	–
Cool/short[a]	−19.9	−18.1 to −21.7	−25.8	−22.8 to −28.8
S. agrili				
Discoloration:				
Warm/long	−23.7	−22.7 to −24.7	−27.2	−25.6 to −28.8
One gen. diapause	−27.3	−26.3 to −28.3	−29.6	−28.2 to −31.0
Two gen. diapause	−28.9	−27.8 to −29.8	−32.9	−31.1 to −34.7
Eclosion:				
Warm/long	−23.4	−22.3 to −24.5	−25.2	−24.1 to −26.4

*Values not shown since logistic regression parameters were not significant.
[a] Model resulting from combined lower lethal temperature and lower lethal time data at 3 d.

to 4 °C, 92% of *S. agrili* and >99% of *T. planipennisi* would be expected to fail to eclose. This mismatch between the projected and observed values suggests that our projections of cold injury over time should not be extrapolated outside the temperature (0 to −15 °C) and exposure time (3–84 d) ranges that were measured. Gould et al. (2011) also performed a lower lethal time study for non-diapausing *S. agrili* pupae at 10 °C and found that 41% eclosed upon return to normal rearing conditions after 3 months of exposure, but only 25% of females laid eggs.

4.2. Meaning of cold tolerance measures

Supercooling points, lower lethal temperature, and lower lethal time address different mechanisms by which low temperatures might cause injury. Supercooling points indicate the temperatures at which insects freeze. However, not all insects that freeze will die because some can survive freezing, while others die before they freeze (Sømme, 1982). If the frozen insect does not survive freezing during a supercooling point measurement, it may be either freeze-intolerant or chill-intolerant, but these alternatives cannot be distinguished until the temperature that caused mortality is known (Renault et al., 2002). As freezing is not always an indicator of mortality, supercooling point measurements alone are not fully sufficient to measure cold tolerance, but instead must be used in conjunction with measures of mortality (Renault et al., 2002). Taken collectively, our results suggest that *S. agrili* and *O. agrili* are freeze-intolerant, while *T. planipennisi* seems chill-intolerant.

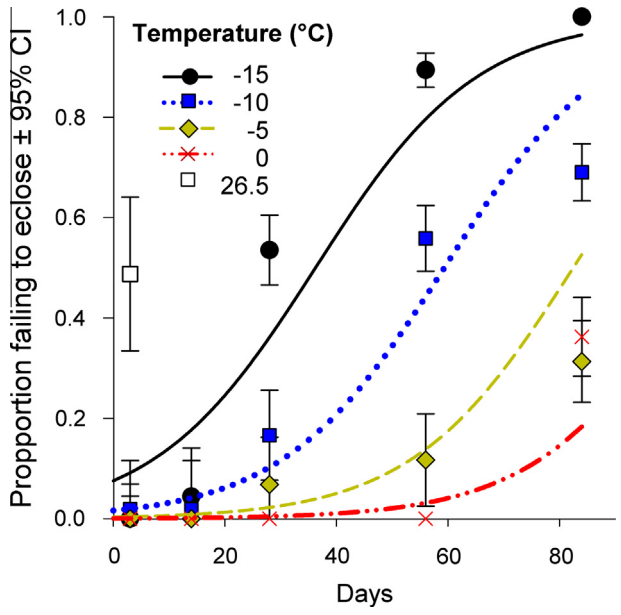

Fig. 5. Mortality of *Tetrastichus planipennisi* due to length of exposure at constant temperature. Observed (symbols) and predicted (lines) proportion of *T. planipennisi* that failed to eclose over time at constant temperatures. Observations at 26.5 °C were used to adjust proportions at other temperatures and times.

Observations of individuals after they have frozen, either during supercooling point measurements or in some lower-lethal temperature treatments, allow one to discern whether individuals have some degree of freeze tolerance. After freezing, *T. planipennisi* larvae and most *S. agrili* pre-pupae turned black or brown; none eclosed. Thus, these observations suggest *T. planipennisi* is not freeze-tolerant. However, we had difficulty breaking diapause in *S. agrili*. If eclosion is used as the measure of mortality, *S. agrili* is also not freeze-tolerant. If discoloration is the metric, a fraction (<~10%) of the diapausing *S. agrili* population might be considered freeze-tolerant. Discoloration could not be measured for *O. agrili* because the chorion of the emerald ash borer egg obscured view of the developing parasitoid, but one *O. agrili* did eclose after freezing. This lone survivor may indicate at least some *O. agrili* are freeze-tolerant. Interestingly, this individual had a supercooling point of −29.8 °C, while freeze-tolerant individuals typically have supercooling points near -10 °C (Turnock and Fields, 2005). There are exceptions to this trend among parasitoids (Salt 1959; Sømme 1964; Baust 1973; Ring 1982).

Lower lethal temperature studies showed that *T. planipennisi* and *S. agrili* were injured after brief exposures to temperatures below −20 °C. Such brief cold exposures might occur on the coldest day of the year in some locations. Median and 90th percentile supercooling points can also be compared with 50% and 90% mortality in the lower lethal temperature experiment to further assess freeze-tolerance (Fig 2; Table 3). For *S. agrili* from two-generation diapause conditions, median and 90th percentile supercooling points occurred within the 95% confidence interval for 50% and 90% mortality, respectively. This result indicates that *S. agrili* can be freeze-intolerant, but some degree of chill-intolerance also occurs among those reared under warm/long and one-generation diapause conditions. Meanwhile, *T. planipennisi* appears to be chill-intolerant as 50% mortality occurs at temperatures that are warmer than the median supercooling point. However, temperatures that result in 90% of *T. planipennisi* freezing and dying, respectively, appear to be relatively similar.

Measures of cold tolerance such as supercooling points and lower lethal temperature that use brief exposures do not account for cold stress that may have accrued from previous cold events and may underestimate injury when an insect experiences repeated low temperatures. Lower lethal time models can be used to forecast cold injury over an entire winter. However, these models often as-

Table 4
Results of lower lethal time studies for *Spathius agrili* and *Tetrastichus planipennisi* from different rearing conditions: estimated logistic-regression parameters to characterize discoloration or failure of adults to eclose as a function of exposure to low temperatures over time.

Species & rearing	Intercept (b_0)			Temperature (b_1)			Days (b_2)		
	Coefficient ± SE	χ^2	p	Coefficient ± SE	χ^2	p	Coefficient ± SE	χ^2	p
T. planipennisi									
Eclosion:									
Cool/short	−7.286 ± 0.747	95.08	<0.001	−0.319 ± 0.042	56.48	<0.001	0.069 ± 0.008	78.24	<0.001
S. agrili									
Discoloration:									
Warm/long	−5.283 ± 0.458a	133.00	<0.001	−0.126 ± 0.025a	26.15	<0.001	0.059 ± 0.005a	114.44	<0.001
One gen. diapause	−4.173 ± 0.376	102.81	<0.001	–	–	–	0.041 ± 0.006	53.36	<0.001
Two gen. diapause	−4.087 ± 0.391a	109.37	<0.001	−0.091 ± 0.026a	11.04	<0.001	0.037 ± 0.005b	28.82	<0.001
Eclosion:									
Warm/long	−4.103 ± 0.464	48.48	<0.001	0.065 ± 0.009	21.74	<0.001	−0.198 ± 0.0424	57.00	<0.001

Coefficients followed by the same letter are not significantly different at Bonferroni adjusted $\alpha = 0.05$.
Coefficients are for Eq. (3) in the text.

sume that an insect experiences a constant temperature, which is reasonable for many subterranean insects or insects that persist beneath a snowpack where temperatures changes can be buffered reviewed by Sinclair et al. (2003). Daily variations in winter temperature can affect survival rates where many freeze-intolerant and chill-intolerant individuals have lower injury under fluctuating thermal regimes when compared to constant temperatures (Colinet and Hance, 2010; Colinet et al., 2011). However, repeated freeze–thaw cycles can also increase mortality for freeze-tolerant individuals (Marshall and Sinclair, 2011, 2012). Therefore, the effect of fluctuating winter temperatures may be quite important in this system. However, properly assessing ecologically relevant temperature regimes could prove difficult given the current cost of rearing the parasitoids and the sample sizes required to assess multiple temperature and fluctuation combinations for multiple lengths of time.

4.3. Assessing mortality

Our primary interest during lower lethal temperature or lethal–time experiments was to measure the effect of cold exposure on insect mortality. Discoloration and failure to eclose represent mortality. Eclosion of adults seemed to be obvious evidence of survival and failure to eclose as evidence of death, but it was later learned that diapausing *S. agrili* require up to 26 weeks at 4 °C to break diapause (Lelito, *unpublished data*). Adult emergence was unable to be induced for individuals exposed to these conditions even after being placed in warm/long-photoperiod conditions. Individuals that failed to eclose also were discolored, so the possibility that pre-pupae were healthy but had not yet broken diapause can be ruled out. However, discoloration and adult eclosion failure rates for *S. agrili* from warm/long-photoperiod conditions were similar. Diapausing and nondiapausing individuals that froze when supercooling points were measured also had obvious discoloration. Thus, discoloration is likely to be a useful indicator of mortality when death is caused by freezing. However, discoloration after cold exposure during lower lethal time studies only weakly agreed with adult eclosion. Many individuals that had normal coloration still failed to eclose and no indication was apparent that these pre-pupae entered diapause due to the cold exposure treatment. All pre-pupae were discolored by 30 d after cold exposure if they did not eclose. These findings may indicate differences between acute and chronic cold injury in *S. agrili* (Sinclair and Roberts, 2005). Acute injury may occur from cell lysis during freezing to cause the visible discoloration of the pre-pupae. Chronic injury may be due to cold stress where the insect uses resources to survive cold, but reaches the point where it is unable to continue development.

4.4. Rearing condition effects

Rearing conditions affected the cold tolerance of *S. agrili* and *T. planipennisi*. Supercooling points were warmest for individuals from warm/long-photoperiod conditions. For *T. planipennisi*, supercooling points were unaffected by rearing under cool/short-photoperiod conditions; supercooling points were not lowered until individuals from cool/short-photoperiod were held at 4 °C for four months. For *S. agrili*, supercooling points were lowered when exposed to diapause-inducing conditions, but were not lowered further when exposed to other rearing treatments that were intended to induce cold acclimation. Based on supercooling points, it appears that cold tolerance of *S. agrili* changes more quickly than *T. planipennisi* when environmental conditions changed. Changes in supercooling points due to rearing conditions were typically small (e.g., 1.3 °C between the two cold acclimated *T. planipennisi* groups), but such small changes in cold tolerance can translate into substantial differences in potential overwintering distributions (Morey et al., 2013).

Lower lethal temperature models did not differ for *T. planipennisi* from warm/long-photoperiod conditions or cool/short-photoperiod conditions. This result suggests that the cold tolerance of *T. planipennisi* as measured by lower lethal temperature is not affected by exposure to cool/short-photoperiod conditions, a result similar to that observed with supercooling points. These two results suggest that likelihood of an individual surviv-

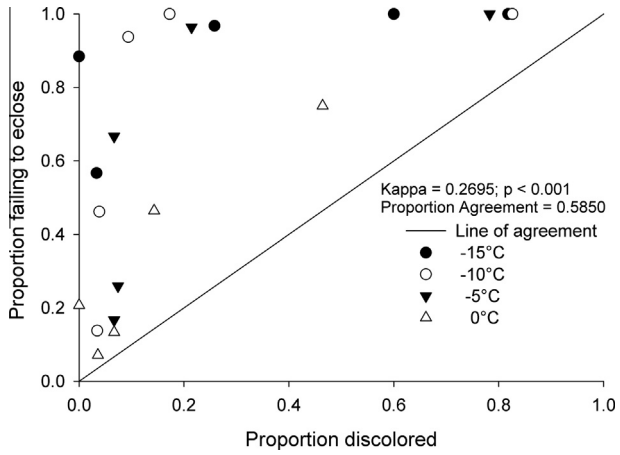

Fig. 6. Agreement between the proportion (not Abbott corrected) of *Spathius agrili* from normal rearing conditions that were discolored and those that failed to eclose in lower lethal time studies. Multiple points per temperature are independent observations through time.

ing the winter would not change if *T. planipennisi* were released in the spring or fall. However, releasing *T. planipennisi* in the spring could allow populations to build during the growing season and increase the likelihood that a fraction of the population will survive the winter.

An effect of rearing treatment on discoloration rates of *S. agrili* was not detected in lower lethal temperature studies, an interesting finding given that rearing treatments significantly affected supercooling points. This outcome suggests that while these insects lower supercooling points as rearing conditions more closely mimic fall than summer, there is not an associated increase in resilience to chilling.

In lower lethal time studies, *S. agrili* from two-generation-diapause conditions survived longer at a given temperature from 0 to −15 °C, inclusive, than *S. agrili* from warm/long-photoperiod or one-generation-diapause conditions. Because diapause induction appears to increase *S. agrili* cold tolerance, releasing *S. agrili* earlier in the year so that it has time to complete at least two generations may improve its ability to overwinter after release.

4.5. Differences in cold tolerance between species

Another objective of this study was to determine if any of the parasitoid species was more cold tolerant than the others. Lower supercooling points for *O. agrili* might be expected because the insect must survive on the surface of a tree, unlike the larval parasitoids that may be slightly buffered from extreme cold temperatures by the insulating effects of bark (Vermunt et al., 2012a). *Oobius agrili* may be the most cold tolerant of the three species based on supercooling points, but mortality measures are needed to further understand *O. agrili* cold tolerance.

Significant differences were not expected in overwintering mortality for *T. planipennisi* and *S. agrili* based on similar supercooling points. However, after brief cold exposure, a higher proportion of *S. agrili* eclosed than *T. planipennisi* when both species were reared under warm/long-photoperiod conditions. This result suggests *T. planipennisi* may have higher overwintering mortality than *S. agrili*. These findings should be interpreted with caution as late-instar *T. planipennisi* are susceptible to handling stress, which may have occurred during shipping, when parasitoids were removed from ash bolts, or when individuals were prepared for experiments (Lelito, *unpublished data*). Handling stress is common among parasitoids prior to pupation (Colinet and Boivin, 2011). Since eclosion was near 50% in the control groups it seems factors other than cold exposure were causing mortality. However, handling stress was less of an issue for *S. agrili* because discoloration of controls was typically <10% and the percentage of individuals from warm/long-photoperiod conditions that failed to eclose was only about 20%. If the larvae were already stressed, the results from this study may underestimate the level of eclosion that might occur in the field after cold exposure.

At the time these studies were conducted, *T. planipennisi* had only been described overwintering as late-instars (Bauer et al., 2008). However, recent findings suggest *T. planipennisi* can be found overwintering as early-instars in the host or as late-instars, pupae, or pharate adults outside the host, (Duan et al., 2013; L. Bauer, *personal comm.*). These additional stages may be of interest for cold tolerance research in case a different stage is more cold-

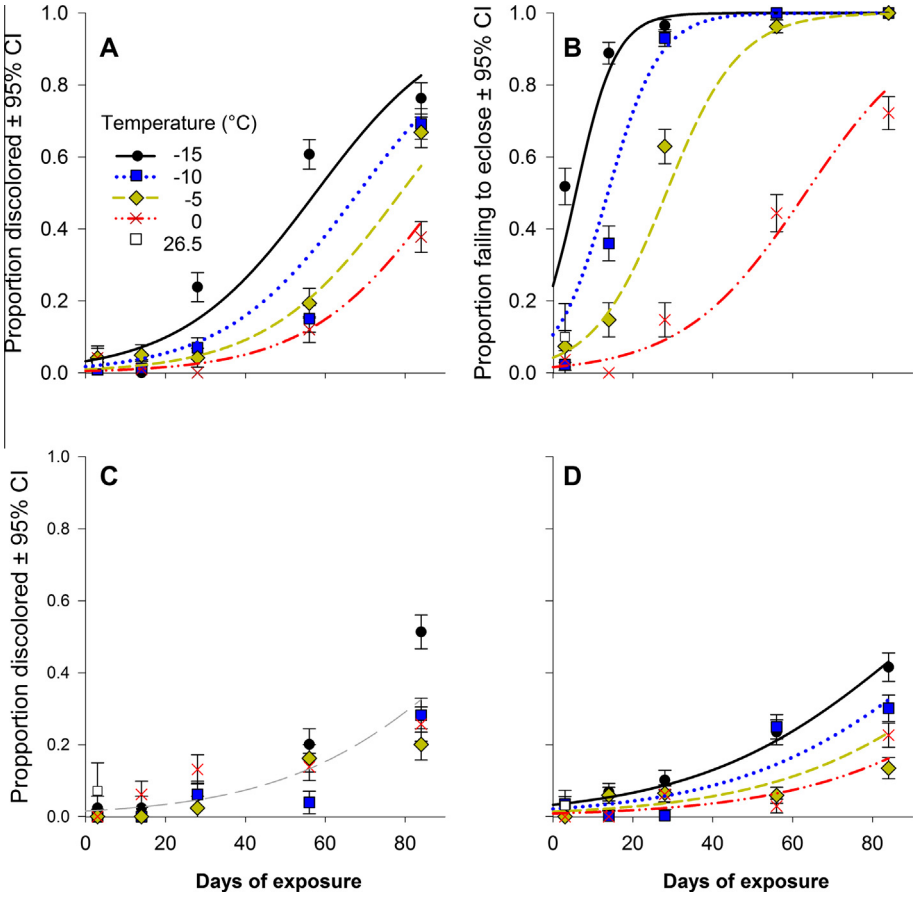

Fig. 7. Mortality of *Spathius agrili* due to length of exposure at constant temperature. Observed (symbols) and predicted (lines) mortality of *S. agrili* from different rearing conditions over time at constant temperatures: (A) warm/long-photoperiod conditions, (C) *S. agrili* one generation diapause (hashed line represents predicted mortality as a function of time; temperature did not have a significant effect on mortality), (D) *S. agrili* two generation diapause.

Table 5
Days of cold exposure resulting in 50% (LT_{50}) and 90% (LT_{90}) mortality with 95% confidence intervals for *T. planipennisi* and *S. agrili* at 0, −5, −10, and −15 °C.

Species	Rearing	Mortality measure	Exposure temperature (°C)	LT_{50}	95% C.I.	LT_{90}	95% C.I.
T. planipennisi	Cool/short	Adult eclosion	0	>84	–	>84	–
			−5	>84	–	>84	–
			−10	56	36–76	>84	–
			−15	33	19–45	51	38–63
S. agrili	Warm/long	Discoloration	0	>84	–	>84	–
			−5	70	47–92	>84	–
			−10	73	48–98	>84	–
			−15	56	39–72	>84	–
	Warm/long	Adult eclosion	0	61	41–79	>84	–
			−5	26	16–35	44	34–53
			−10	17	10–23	26	19–32
			−15	2	0–7	17	10–22

Confidence intervals not calculated for LT50s or LT90s beyond 84 days as this was the longest exposure time in the experiment.

tolerant or if the host larva affects overwintering survival of early instar parasitoids that are still within a live host (Colinet and Boivin, 2011).

4.6. Applications of models

Ultimately, the cold tolerance of these parasitoids could be considered by natural resource managers before selecting one or more parasitoids species to control emerald ash borer in an area. The suitability of the climate in areas where these species are introduced may affect both the likelihood of establishment and parasitism rates reviewed in Hajek (2004). If overwintering mortality is high for an *A. planipennis* parasitoid, the species may not be able to establish or reach population densities that would be sufficient to control *A. planipennis*. Therefore, understanding the cold tolerance of each species is an important consideration before starting a release program, particularly in northern latitudes. Forecasts for *S. agrili* from diapause-inducing conditions and *T. planipennisi* from warm/long conditions could be based initially on lower lethal temperature models and the minimum winter temperature recorded at a site. The amount of time spent at cold temperatures could be used to refine estimates of mortality at a location. Interpreting these measures of cold tolerance also requires accounting for relevant ecological conditions (Turnock and Fields, 2005). Daily minimum under-bark temperatures of *Fraxinus* spp. are typically warmer than the minimum air temperature. Using air temperature to predict mortality of *T. planipennisi* and *S. agrili* may overestimate mortality, so temperatures underneath the bark should be used to predict overwintering survival (Vermunt et al., 2012a). Large daily temperature fluctuations may also occur due to solar heating of a tree (Vermunt et al., 2012b). These warmer daytime temperatures may lead to the repair of sublethal cold injury in these parasitoids reviewed in Turnock and Fields (2005). However, assessing the effect of varying temperature on survival and sub-lethal effects is beyond the scope of this work and would an avenue for future research.

The cold tolerance of *A. planipennis* and these parasitoids can also be compared to determine how overwintering temperature may affect control of *A. planipennis* the following year. Crosthwaite et al. (2011) measured supercooling points and lower lethal temperature for *A. planipennis* and determined that freezing was lethal; and mean supercooling points of overwintering pre-pupae ranged between −28 and −30 °C. Discoloration of *S. agrili* during lower lethal temperature studies also appears to increase substantially within this temperature range. *T. planipennisi* had similar supercooling points to *A. planipennis*, but appeared to have additional pre-freeze mortality. These findings suggest *S. agrili* may have similar overwintering mortality as *A. planipennis* in a given location where both species are present, but *T. planipennisi* can experience higher overwintering mortality than *A. planipennis*. While supercooling points are similar for *O. agrili* and *A. planipennis*, *Oobius agrili* mortality needs to be measured at cold temperatures before determining the similarity in cold tolerance between the two species. Even though *S. agrili* appear likely to survive winters where *A. planipennis* is present, further research is needed to determine the minimum winter survivorship required for populations of each species to persist year-round. Nevertheless, our current results will improve efficiency in selecting parasitoids for a release site.

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Appendix A.

We adjusted estimates of mortality in response to cold exposure to account for mortality in control (i.e., room temperature) treatments, following methods developed by Rosenheim and Hoy (1989). Adjustments were based on:

$$Adj.Mortality = \frac{(1 - M_{treatment})}{\frac{(1-M_{control})}{(1-g)}}$$

where

$$g = \frac{Var(M_{control}) * t_{dist}^2}{(1 - M_{control})^2 * n_{control}}$$

Mortality in a treatment group of interest is adjusted using the observed mortality in that treatment ($M_{treatment}$) and control group ($M_{control}$). $M_{treatment}$ and $M_{control}$ can vary between 0 and 1, inclusive, but $M_{treatment}$ must be greater than or equal to $M_{control}$. The variance of the control group, $Var(M_{control}) = 4(M_{control} * (1 - M_{control})/n)$, and ($t_{dist}$) is the critical *t*-value chosen from the t-distribution based on the of the lesser sample size of the treatment or control group.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2013.08.015.

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