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ORIGINAL ARTICLE

Sublethal effects of subzero temperatures on the light brown apple moth, *Epiphyas postvittana*: fitness costs in response to partial freezing

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Abstract Population responses to environmental extremes often dictate the bounds to species' distributions. However, population dynamics at, or near, those range limits may also be affected by sublethal effects. We exposed late instars and pupae of an invasive leafroller, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), to cold temperatures and measured the effects of exposure on subsequent survivorship, development, and reproduction. Cold temperature was applied as acute exposure to –10 °C (a low, but not immediately lethal temperature for this species) or the onset of freezing (the peak of the supercooling point exotherm). Survival was defined as the ability to successfully eclose as an adult. We measured immature development times, pupal mass, and adult longevity as proxies of fitness in survivors. Additionally, surviving insects were mated with individuals that had not been exposed to cold to measure fertility. There was no difference between the proportion of larvae or pupae that survived acute exposure to –10 °C and those exposed to the control temperature. Approximately 17% of larvae and 8% of pupae survived brief periods with internal ice formation and continued development to become reproductively viable adults. Importantly, surviving the onset of freezing came with significant fitness costs but not to exposure to –10 °C; most insects that survived partial freezing had lower fertility and shorter adult lifespans than either the –10 °C or control group. These results are discussed within the context of forecasting invasive insect distributions.

Key words fertility; invasive species; partial freeze tolerance; supercooling point

Introduction

In temperate regions, extreme low temperatures can be critical in defining the limits of an insect's geographic distribution (Overgaard *et al.*, 2014). For exotic invasive species, risk-assessment and -management decisions often depend upon accurate characterizations of the effects of low temperature on a given species or population. Mechanistic models that forecast the distribution of exotic invasive species frequently rely on measures of direct mortality from low temperature. For example, distribution forecasts of the invasive moth, *Epiphyas postvittana* (Walker), in North America define the impacts of extreme low temperature using only approximations of direct mortality (e.g., the average supercooling point) (Fowler *et al.*, 2009; Gutierrez *et al.*, 2010). Importantly, though, significant impacts of acute cold exposure on insect population dynamics can also manifest over time as sublethal effects (Baust & Rojas, 1985; Bale, 1987; Lee, 2010).

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The current paradigm for the classification of insect cold tolerance describes the relationship between mortality from internal ice formation and injury from chilling (Lee, 2010; Overgaard & MacMillan, 2017). Most species are freeze-avoidant because they die once ice begins to form within their bodies (alternatively termed freeze-intolerant) or from the deleterious effects of nonfreezing temperatures (alternatively termed chill-intolerant or susceptible). Other species are freeze-tolerant and can survive internal freezing under certain conditions. In addition, intermediate responses exist, such as individuals and/or populations that can tolerate differing degrees and/or periods of ice (Baust & Rojas, 1985; Sinclair, 1999; Hawes & Wharton, 2010). Among these responses are individuals that can survive the onset of internal ice formation but cannot tolerate equilibrium freezing (Hawes & Wharton, 2010). Though the relevance of these individuals outside a laboratory setting remains unclear, examination of their biology could nonetheless provide useful insights into the evolution of low temperature responses (Sinclair, 1999; Voituron *et al.*, 2002).

Insect cold tolerance is often assessed from laboratory studies of survival defined by mortality or lack of coordinated activity, determined within hours or a few days after exposure (e.g., Nedvěd *et al.*, 1998; Koch *et al.*, 2004; Shreve *et al.*, 2007; Bürgi & Mills, 2010). Such designs may fail to capture mortality that occurs in later stages of development, or sublethal injury from cold on development and reproduction that affects population fitness (Baust & Rojas, 1985; Hutchinson & Bale, 1994; Renault *et al.*, 2002; Layne & Peffer, 2006). Increasingly, studies have evaluated sublethal effects of cold on insects, particularly in the context of cold storage of biological control agents and laboratory research stock. Numerous metrics of fitness may be affected by chronic (>24 h) cold exposure, with sublethal effects typically increasing with decreasing temperature and/or increasing time (Chen *et al.*, 2008; Ruan *et al.*, 2012; Mockett & Matsumoto, 2014; reviewed for parasitoids in Hance *et al.*, 2007 and Colinet & Boivin, 2011). Studies of more acute (<24 h), subzero exposure in insects have detected reduced rates of development (McDonald *et al.*, 1997), reduced fertility and reproduction (Coulson & Bale, 1992; Hutchinson & Bale, 1994; Rinehart *et al.*, 2000), shorter adult lifespans (Rinehart *et al.*, 2000), and increased incidence of adult malformations (Turnock *et al.*, 1985), for example. Reduced survivorship to subsequent developmental stages has also been cited as a sublethal effect (e.g., Turnock & Bodnaryk, 1991; Yocum *et al.*, 1994), though this response could arguably be considered a lethal effect. Most studies that evaluate sublethal effects of cold do so for freeze-avoidant insects and, with few exceptions (e.g., Fields & McNeil, 1988; Layne & Blakeley, 2002; Hawes & Wharton, 2011), little is known about the fitness consequences of tolerating internal ice formation.

Epiphyas postvittana is a highly polyphagous moth, attacking numerous trees and crop plants, that is indigenous to southern Australia and was documented in the contiguous United States in 2006 (Brown *et al.*, 2010). Invasion of the USA by *E. postvittana* has potentially significant economic and ecological ramifications (Fowler *et al.*, 2009) and early management responses were controversial (Lindeman, 2013; Liebhold, 2014). Thus, the potential geographic distribution of this insect is of interest. The predominant overwintering stage in the USA is the late instar (Bürgi & Mills, 2010), which slows development but does not enter diapause during winter (Geier & Briese, 1981). However, more than one developmental stage may be present during the winter (Buergi *et al.*, 2011). Bürgi and Mills (2010) classify late instars as "chill susceptible" (*sensu* Bale, 1996) because larvae experience substantial mortality at temperatures above the mean supercooling point. We have previously shown that a small proportion of late instars from a laboratory population survived partial freezing (i.e., acute exposure to the peak of their supercooling exotherm) and continued to develop into reproductively successful adults (Morey *et al.*, 2013, 2016). In a laboratory setting, such tolerance to partial freezing affords an immediate advantage over the chill-susceptible portion of the population when temperatures are cold enough to initiate freezing, but fitness costs to the survivors are currently unknown.

The purpose of our study was to examine the sublethal effects of acute low temperature exposure on *E. postvittana*. Late instars and pupae were held at room temperature (~23–25 °C), or gradually cooled to either −10 °C, which represents a subzero temperature that is not immediately lethal to late instars (based on Morey *et al.*, 2013), or until the onset of freezing was detected. Individuals that did not eclose as an adult were considered to have succumbed to direct (i.e., lethal) effects of cold. Potential sublethal effects included impacts to immature development times, pupal mass, adult longevity, and fertility, all components of fitness. We hypothesized that sublethal effects would be present in insects that survived exposure to any subzero temperature, but that the relative degree of sublethal effect(s) would be greatest in those individuals that survived the onset of freezing. We further hypothesized that pupae would experience higher lethal effects from cold exposure, and similar or greater sublethal effects compared to late instars. Late instars are more abundant than other life-stages in overwintering *E. postvittana* populations in California (Buergi *et al.*, 2011), suggesting that they have a greater ability to

tolerate or recover from cold exposure than pupae or other life-stages

Materials and methods

Colony source

Epiphyas postvittana eggs were obtained from a laboratory colony maintained by the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) in Albany, CA, founded from wild California moths in 2007. All subsequent handling and experimentation was conducted in a Biosafety Level 2 Containment Facility in St. Paul, MN (APHIS permit P526P-14-03759). Eggs were surface-sterilized in a 1% bleach solution, and held at 23 ± 2 °C, 60%–65% RH, 14 : 10 (L : D) inside two environmental chambers (Percival Scientific, Perry, IA, USA and Conviron, Winnipeg, Canada) until hatch.

Within 24 h of hatching, *E. postvittana* neonates were placed in groups of 3–4 onto ~2 cm^3 cubes of artificial diet inside lidded containers (29.5 mL P100 soufflés; Solo Cup Co., Lake Forest, IL, USA) and reared until late instars (4th–6th) or pupae in the same chambers and conditions as previously stated. We focused on "late instars" (4th–6th) as a single group because *E. postvittana* has variable instar numbers (Dumbleton, 1932; Danthanarayana, 1975), any of which could be the terminal instar before pupation and could overwinter. The diet used *Phaseolus vulgaris* L (cv. Great Northern) and followed a modified formulation developed by Follet and Lower (2000) for a related tortricid.

Low temperature exposure

The experiment followed an incomplete block design and was repeated twice, once in March 2011 and another in June 2011. A replicate consisted of 10 insects and the total number of replicates within each month ranged from 9–12, depending on the life-stage group. For larval-exposure treatments, instar was confirmed through head capsule measurement (Danthanarayana, 1975). Late instars (4th–6th) were randomly assigned to one of three temperature treatments: cooled to –10 °C (across replicates and time, $n = 88$), cooled to the supercooling point of an individual and held until the peak of the resulting exotherm ($n = 80$), or held at room temperature (i.e., control, $n = 44$). Sample sizes for controls were smaller than treatment groups due to specimen limitation. Though treatment assignment of individuals was random, the relative proportion of specific instars used in a given temperature treatment were approximately equal (Table S1).

Larvae were transferred to individual gelatin capsules (size 4; 14.3 mm length, 5.1 mm diameter), as per Morey *et al.* (2016), and cooled at ~1 °C/min from room temperature to the desired treatment temperature within individual polystyrene cubes inside a −80 °C freezer, as per Carrillo *et al.* (2004). Temperatures were recorded once per second by using the coiled, copper-constantan thermocouple design of Hanson and Venette (2013) connected to a computer through a multichannel data logger (USB-TC, Measurement Computing, Norton, MA, USA). This design protects the larva during cooling, and keeps it in close, indirect contact with the thermocouple measurement junction. Each larva was immediately removed from the freezer and cube once they reached either −10 °C or the peak of their exotherm; the exotherm peak could be observed in real-time as a plateau (typically lasting 15–25 s) following an abrupt spike in temperature. The supercooling point was the lowest temperature reached before the exotherm. We chose the exotherm peak for removal because it was a point that guaranteed some ice formation, but could also be applied most consistently to all individuals. Larvae were then removed from their gelatin capsules with a camel hair brush and gently placed individually on a fresh cube of diet to continue development in a growth chamber (23 ± 2 °C, 60%–65% RH, 14 : 10 L : D). Cooling rates were analyzed post-exposure to ensure that rates were consistent; individuals that deviated more than 0.4 °C from 1 °C/min were not included in the final analysis. Control individuals were left inside gel capsules at room temperature (~23–25 °C) for approximately 1 h while cold-treated individuals were being cooled to their temperature treatments. Controls were removed from the capsules and given fresh diet concomitantly with the cold-treated larvae.

Individuals were monitored daily for pupation, adult eclosion, and/or death. Pupal mass was measured (Mettler Toledo AL54 Analytical Balance, 0.1 mg) 3–5 d after pupation to reduce handling damage. Survival was defined by successful eclosion as an adult. For survivors, total immature duration (hatch to adult eclosion), adult longevity, and fertility (described below) were recorded. Sex of adults was determined from wing coloration and the presence (male) or absence (female) of a forewing costal fold (Brown *et al.*, 2010). All adults were provided with honey-water (10% by weight) within 1 d after eclosion and placed with a mate within 2 d after eclosion. Colony adults used for mating were reared in tandem with the treatment insects, but were not handled until pupation. They were weighed as pupae and mated with an individual from one of the three treatment groups. Mating containers consisted of 0.47 L (16 fluid oz) clear plastic cups with perforated lids to allow air circulation. Cheesecloth was

placed between the lid and the cup opening to deter females from ovipositing on the lids. To supply moths with honey water, a cotton dental roll (3.81 × 0.95 cm) was inserted into a hole cut into the bulb of a 5 mL disposable pipette. The tip of the pipette was also cut so that ~1.9 cm remained above the bulb. The pipette fit snugly into the straw-hole of the lid. The cotton was re-soaked through the top opening of the bulb every other day with honey solution using another pipette. Females oviposited on the sides and bottom of the cup. Fertility was measured as the total number of viable eggs (as evidenced by the presence of a larval head-capsule, or "black-heading," within the egg) produced from a treatment-exposed female mated with an unexposed male, or from an unexposed female mated with treatment-exposed male.

For pupal-exposure treatments, pupae were weighed 3–5 d after pupation and exposed to either –10 °C (across replicates and blocks, $n = 75$), the onset of freezing ($n = 76$), or used as control ($n = 38$) as described above for larvae. For survivors, immature duration, adult longevity, and fertility were measured as described above.

Analysis

Statistical analyses were conducted with SAS® software 9.4 (SAS Institute Inc., Cary, NC, USA) with generalized linear mixed models (Proc GLIMMIX). The larval- and pupal-exposure assays were conducted separately and, therefore, analyzed separately. Raw means and standard errors are presented in the figures. For some measures, the final sample sizes used in analysis varied from the original sizes. Mechanical or user error prevented some or all of the data for a given individual from being used. For example, if an individual survived treatment to eclose but was inadvertently killed during adult processing, data on their larval and pupal development, and cold tolerance, were included, but reproductive data was not.

Survival after partial freezing was compared across temperature treatments, within each stage of exposure, using a binomial distribution and logit link. Temperature ($n = 3$) and experiment month ($n = 2$) were treated as fixed effects, and replicate ($n = 22$ for larval-exposure, $n = 19$ for pupal-exposure across months) was treated as a random effect. Sex was not included in these analyses because the sex of individuals that died before adult eclosion were not determined. Maximum likelihood estimates were produced using the Laplace method, as recommended by Capanu *et al.* (2013) for binary outcomes in mixed models. Degrees of freedom were estimated by the containment method to be compatible with Laplace estimation (SAS/STAT® 9.2 User's Guide, 2009) and differences in the least squares means were separated using Tukey–Kramer groupings to maintain an overall $\alpha = 0.05$.

To examine the potential relationship between the temperature at which freezing begins and the likelihood of surviving partial freezing, the individual state (dead or alive) and month were treated as explanatory variables of individual supercooling points, and replicate was included as a random effect. Degrees of freedom were estimated by the Kenwood–Roger method, as recommended for unbalanced data by Spilke *et al.* (2005), and Tukey–Kramer groupings compared the supercooling points of individuals that died to those that survived.

For all developmental and reproductive measures, only survivors (i.e., those that survived to adult eclosion) were included in the analyses. Each mixed-effects model used temperature and month as fixed effects and replicate as a random effect. *Epiphyas postvittana* is known to be sexually dimorphic in some developmental traits (Danthanarayana, 1975), so sex was also included in these analyses as a (fixed) covariate. Nonsignificant interactions ($P > 0.05$) between fixed effects were removed from the final models.

Total immature developmental times and adult longevities were analyzed using a gamma distribution and log link, as recommended by Manly (1989) for life-stage durations. Degrees of freedom were approximated by the Kenwood–Roger method and Tukey–Kramer groupings compared differences in the least squares means. Pupal masses (larval-exposures only) were ln-transformed for analysis to achieve equal variances (Brown–Forsythe test; SAS/STAT® 9.22 User's Guide); a Gaussian distribution was used with Kenwood–Roger degrees of freedom estimation, and Tukey–Kramer comparisons were made as before. Fertility counts were analyzed using a negative binomial distribution and log link, with quadrature likelihood approximation, as recommended by Stroup (2015).

Results

Lethal effects of cold exposure

Temperature treatment affected the proportion of late instars ($F_{2, 41} = 32.67$, $P < 0.0001$) that survived to adult eclosion. The proportion of late instars that survived to adulthood was not different among individuals exposed to −10 °C (0.97 ± 0.02; mean ± SEM) or room temperature (0.98 ± 0.02), but a significantly smaller proportion (0.17 ± 0.04) survived exposure to the onset of freezing (Fig. 1). Similarly, the proportion of treated pupae that survived to adulthood was affected by temperature treatment ($F_{2, 36} = 29.37$, $P < 0.0001$). The proportion of

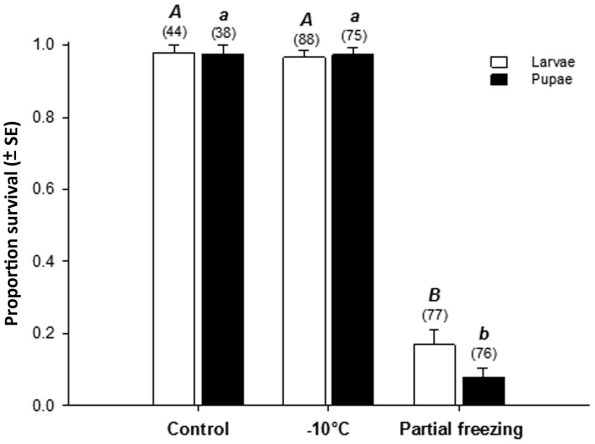

Fig. 1 Proportion of *E. postvittana* that survived to adult eclosion following acute low temperature exposure as late instars (4th–6th) or pupae to –10 °C, partial freezing (the peak of the supercooling point exotherm), or control conditions (~23–25 °C). Numbers in parentheses indicate sample size across all replicates. Different letters indicate significant differences across temperature treatments, within a stage ($P < 0.05$).

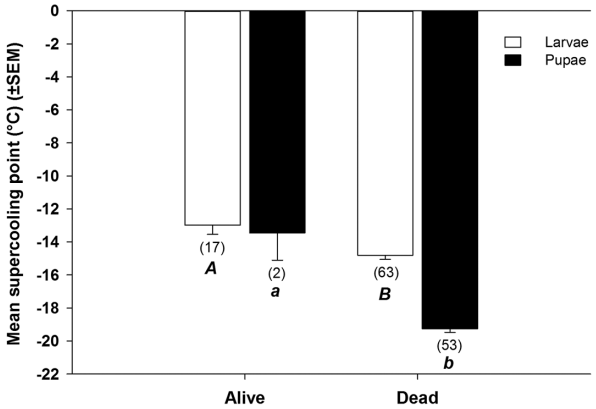

Fig. 2 Relationship between survival or mortality after partial freezing and mean supercooling points in *E. postvittana* instars (4th–6th) and pupae. Survival was defined by successful adult eclosion. Different letters indicate significant differences across temperature treatments, within a stage ($P < 0.05$). Numbers in parentheses indicate the sample size across all replicates.

survivors was not different among individuals exposed to −10 °C (0.97 ± 0.02) and controls held at room temperature (0.97 ± 0.03), but a significantly smaller proportion of pupae (0.08 ± 0.03) survived exposure to the onset of freezing (Fig. 1). The month of the experiment did not affect the survival of either late instars ($F_{1, 41} = 0.78$, $P = 0.38$) or pupae ($F_{1, 36} = 1.99$, $P = 0.17$).

The overall mean supercooling point for late instars was −14.5 °C (± 0.3 SEM). However, the larvae that survived exposure to partial freezing had significantly ($F_{1, 67.8} = 5.4$, $P = 0.023$) higher mean supercooling points (−13.0 ± 0.6 °C) than those that died (–14.8 ± 0.3 °C) (Fig. 2). Experiment month did not affect survival ($F_{1, 18.4} = 0.46$, $P = 0.51$), nor did the interaction of main effects ($P > 0.05$).

For pupae, the overall mean supercooling point was −19.0 °C (± 0.3). As with larvae, pupae that survived exposure to partial freezing had significantly ($F_{1,52} = 16.99$, $P = 0.0001$) higher mean supercooling points (−13.5 ± 1.7 °C) than those that died (–19.3 ± 0.2 °C) (Fig. 2). Experiment month affected supercooling ($F_{1, 52} = 4.32$, $P = 0.043$), with the average supercooling points measured in the first month (−17.0 ± 0.6 °C) being lower than the second (–14.0 ± 1.2 °C). No interactions were significant ($P > 0.05$).

Pupal mass (larval-exposed group only)

Temperature treatment did not affect the mass of pupae ($F_{2, 121.7} = 0.50$, $P = 0.61$) that survived after exposure as late instars. Females had significantly ($F_{1, 130.9} = 385.55$, $P < 0.0001$) greater mass (47.9 ± 0.8 mg) than males (26.6 ± 0.3 mg) across temperature treatments. The interaction between temperature and sex was also significant ($F_{2, 131.5} = 3.36$, $P = 0.04$), with control pupae being largest among females and smallest among males. However, the difference between treatment means within a sex was <4 mg (Fig. 3A). There was no effect of experiment month on pupal mass ($F_{1, 18.4} = 0.33$, $P = 0.57$).

Immature developmental times

For individuals that survived exposure as late instars, temperature treatment did not affect the total time spent developing (egg hatch to adult eclosion) ($F_{2, 119.8} = 1.80$, $P = 0.17$). On average, females took longer (32.8 ± 0.3 d) to develop than males (31.1 ± 0.2 d) across temperature treatments ($F_{1, 125.9} = 31.39$, $P < 0.0001$). Though males that survived the onset of freezing appeared to have longer development times, this treatment had small relative samples sizes, and the temperature by sex interaction was not significant ($F_{2, 123.8} = 2.91$, $P = 0.06$) (Fig. 3B). Experiment month did not affect development time in this group ($F_{1, 19.8} = 4.09$, $P = 0.06$).

Similarly, temperature treatment did not affect the total development time of survivors exposed as pupae ($F_{2, 94.1} = 0.57$, $P = 0.57$). However, in this group, surviving females and males did not differ in development time ($F_{1, 96.8} = 1.44$, $P = 0.23$), which averaged ~30 d for all. Of note, the sex ratio in all treatment groups was highly

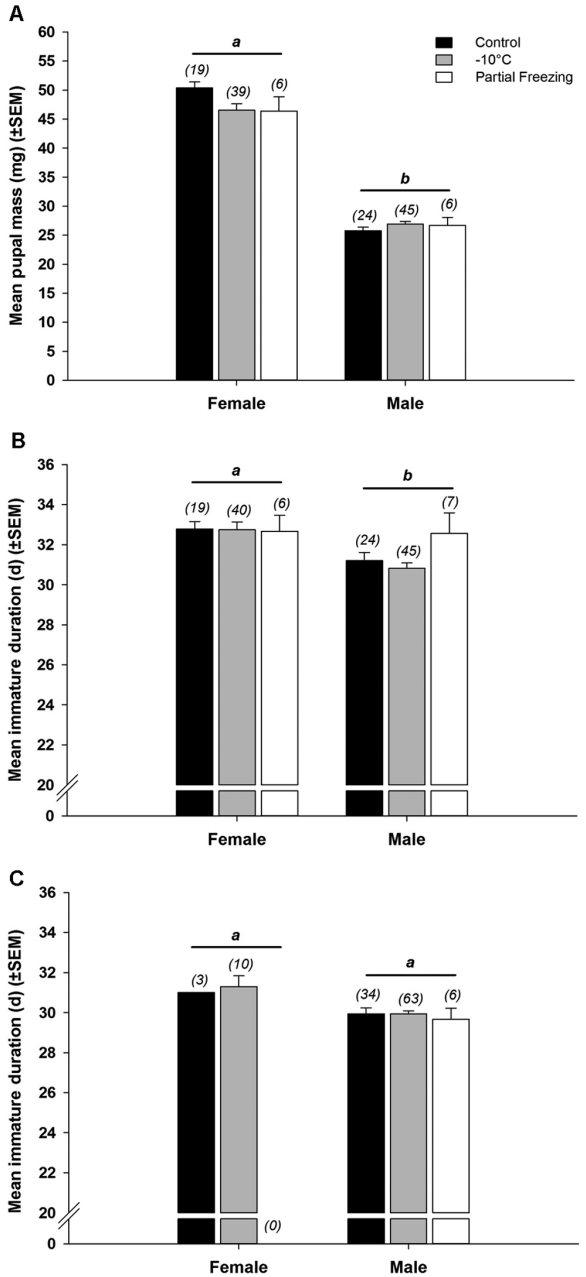

Fig. 3 Effects of temperature on developmental traits of *E. postvittana* following acute exposure to –10 °C, partial freezing (the peak of the supercooling point exotherm), or control conditions (~23–25 °C). Insects were exposed as late instars (4th–6th) (A and B) or pupae (C). Pupal mass (A) and mean immature developmental duration (hatch to adult eclosion) (B and C) were measured following temperature exposure. Raw means are presented for surviving females and males. Numbers in parentheses indicate sample size across all replicates. Different letters indicate significant differences of sex across temperature groups ($P < 0.05$). There were no main effects of temperature on the above measures.

skewed toward males, and the partial freezing treatment had no surviving females (Fig. 3C). Experiment month did not affect developmental duration ($F_{1, 16.9} = 2.26$, $P = 0.15$).

Adult longevity

Temperature affected adult longevity for larval-exposed groups ($F_{2, 134} = 12.88$, $P < 0.0001$). Adult longevity was the same for those exposed to −10 °C (22.1 ± 0.6 d) and the control (22.5 ± 1.1 d), whereas individuals exposed to partial freezing lived significantly fewer days as adults (15.1 ± 1.2 d) (Fig. 4A). Adult longevity did not vary by sex ($F_{1, 134} = 0.01$, $P = 0.93$). There was a main effect of experiment month for this group ($F_{1, 134} = 10.26$, $P = 0.0017$). No interactions were significant ($P > 0.05$). However, the interaction of sex with temperature was marginally insignificant ($F_{2, 132} = 2.86$, $P = 0.061$), and there is qualitative indication that partial freezing may more severely reduce male longevity compared to females (Fig. 4A).

Similarly, temperature affected adult longevity for pupal-exposed groups ($F_{2, 94.5} = 19.90$, $P < 0.0001$). Adult longevity was the same for those exposed to −10 °C (21.9 ± 0.7 d) and the control (22.8 ± 1.0 d), whereas individuals exposed to partial freezing lived significantly fewer days as adults (10.8 ± 3.9 d) (Fig. 4B). Sex ($F_{1, 107} = 0.52$, $P = 0.47$) and experiment month ($F_{1, 17.6} = 1.08$, $P = 0.31$) did not affect adult longevity for this group, and there were no significant interactions ($P > 0.05$).

Fertility

For larval-exposed groups, temperature had a significant impact on the number of viable eggs produced when an exposed and unexposed individual were mated ($F_{2, 90} = 3.31$, $P = 0.041$), irrespective of the sex of the exposed individual ($F_{1, 90} = 1.25$, $P = 0.27$). Pairs with one mate exposed to partial freezing had the lowest average fertility (383.7 ± 76.0 eggs). Pairs with one mate that was exposed to −10 °C produced 577.8 ± 37.3 viable eggs, which was not significantly different than the average fertility of control pairs (741.5 ± 38.9 eggs) (Fig. 4C). The production of viable eggs was not affected by the experiment month ($F_{1, 90} = 2.39$, $P = 0.13$), nor any interactions between main effects ($P > 0.05$).

Conversely, temperature did not affect the number of viable eggs produced for pupal-exposed groups ($F_{2, 66} = 2.62$, $P = 0.08$). Pairs with one mate exposed to partial freezing had the lowest average fertility (198.8 ± 162.6 eggs), followed by pairs with one mate exposed to

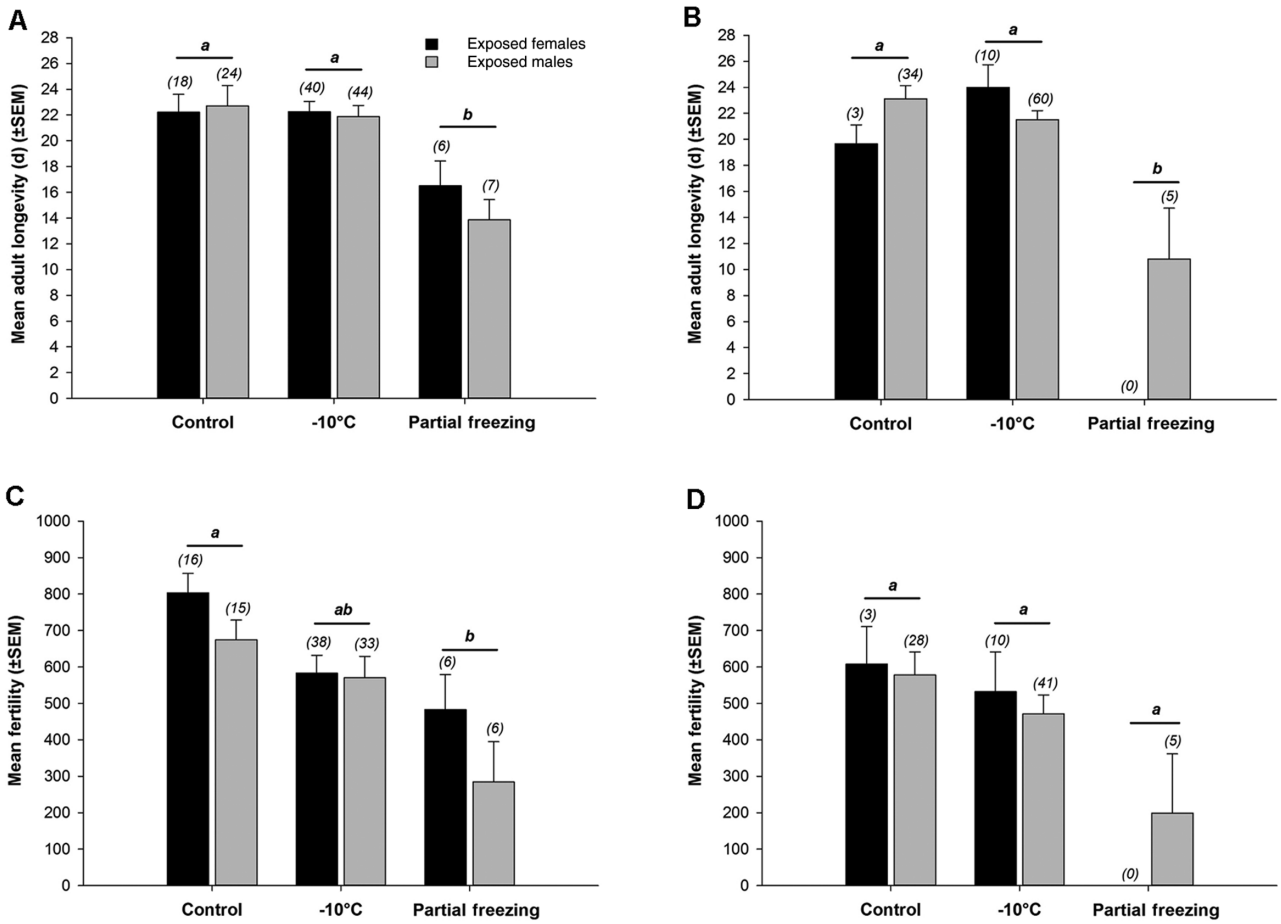

Fig. 4 Effects of temperature on reproductive traits of *E. postvittana* following acute exposure to –10 °C, partial freezing (the peak of the supercooling point exotherm), or control conditions (~23–25 °C). Insects were exposed as late instars (4th–6th) (A and C) or pupae (B and D). Adult longevity (A and C) and fertility (B and D) of survivors were measured following temperature exposure. Fertility was measured from the single pair mating of a treated female with an untreated male (black bars), or an untreated female with a treated male (gray bars). Raw means are presented. Numbers in parentheses indicate sample size across all replicates for a given bar. Different letters indicate significant differences across temperature groups; there was no effect ($P > 0.05$) of the sex of the exposed individual on the above measures.

−10 °C (483.63 ± 45.9 eggs), and then control pairs (581.12 ± 57.2 eggs) (Fig. 4D). The production of viable eggs was not affected by the experiment month ($F_{1, 66} = 1.18, P = 0.28$), the sex of the exposed individual ($F_{1, 66} = 0.10, P = 0.75$), nor any interactions between main effects ($P > 0.05$).

Discussion

As noted by Hawes and Wharton (2011), the interplay of fitness with insect low temperature responses "has received wide recognition, but relatively less actual examination." Our work expands the otherwise limited body of empirical data that documents fitness costs associated with different low temperature exposures. In our study, *E. postvittana* responded to subzero temperatures in two ways. First, with respect to direct lethality, most individuals withstood acute exposure to −10 °C, but died sometime between exposure to this temperature and the onset of freezing, on average −14.5 °C for larvae and −19.0 °C for pupae. Second, a small proportion of individuals survived the onset of internal ice formation (i.e., the peak of their supercooling point exotherm) (Fig. 1), a response that could be considered "partial freeze tolerance" (e.g., Sinclair, 1999). This apparent mixed response to cold confirms previous observations for late instars (Morey *et al.*, 2013, 2016), which we show here also occurs in pupae, albeit to a lesser extent than late

instars. For both life-stages, the individuals that survived partial freezing had relatively higher supercooling points than those that died (Fig. 2). On average, pupae supercooled ~4.5 °C lower than larvae, which may explain why fewer pupae survived partial freezing compared to larvae; species that tolerate freezing typically have supercooling points at higher temperatures than species that cannot tolerate freezing (Sømme, 1982). The mean supercooling point for late instars (−14.5 ± 0.3 °C) is similar to those previously observed by Bürgi and Mills (2010) for unacclimated late instars. Their study measured supercooling points for 4th, 5th, and 6th instars, separately. Our treatment groups were dominated by 6th instars (Table S1), and likely explains why our aggregate mean most closely matches their reported means for unacclimated 5th and 6th instars (approx. −14.25 to −14.5 °C), which they found to be statistically equivalent (Bürgi & Mills, 2010).

Acute exposure to a low, but not immediately lethal subzero temperature (−10 °C) did not appear to affect *E. postvittana* late instars or pupae differently from control individuals, either in mortality (Fig. 1) or sublethal effects (Figs. 3 and 4). The expected sexual dimorphism of *E. postvittana* in developmental parameters (Fig. 3) (Danthanarayana, 1975) was generally seen, but the relative differences did not change with temperature. The mean fertility of individuals exposed to −10 °C, as larvae (Fig. 4C) or pupae (Fig. 4D), suggest a possible reduction in fertility that is intermediate to control and partially frozen individuals, but this was not statistically supported. Other studies involving freeze-avoidant species have observed negative effects on subsequent development and/or reproduction following exposure to nonfreezing, subzero temperatures (Turnock *et al.*, 1985; Hutchinson & Bale, 1994; Colinet & Boivin, 2011). Aside from relatively small sample sizes affecting detectable statistical significance, a possible explanation for the lack of sublethal effects seen at –10 °C is that our design involved only brief exposure. Previous work has shown that *E. postvittana* late instars held at −10.5 °C did not begin to experience mortality until after 90 min (Bürgi & Mills, 2010). So, while brief, subzero exposures are capable of causing injury in some insects (Chen *et al.*, 1987; Lee, 2010), deleterious effects of −10 °C may only manifest with prolonged exposures in this species.

In contrast to −10 °C exposure, survival after partial freezing elicited fitness costs compared to the control groups. These costs appeared in the reproductive measures of fitness, including shorter adult lifespans for insects exposed as late instars or pupae (Figs. 4A, B), and a decrease in the production of viable offspring for those exposed as late instars (Fig. 4C). Given the very low survival of pupae exposed to partial freezing, caution is warranted in drawing strong conclusions about potential differences in impact between sexes, and across temperature treatments, for that life-stage. No female pupae survived exposure to the onset of freezing, but the sex ratio of the control groups indicates we may have begun with a highly male-biased population of pupae. The lack of statistical difference in fertility of freezing-exposed pupae compared to the other temperature treatments may also be due to the small sample size in this group (Fig. 4D). We structured our mating design to test for potential maternal- (Watson & Hoffmann, 1996) or paternal-specific (Costanzo *et al.*, 2015) effects of cold exposure on fertility, but the sex of the exposed individual did not appear to differentially impact fertility for either larval- or pupal-exposure groups. The fertility resulting from the mating of two cold-exposed individuals was not measured here; though, it is likely the effect would be equal to or more pronounced than the response seen with one cold-exposed mate.

The sublethal consequences of freezing on insects are much less explored than those for nonfreezing exposures, with measures of reproductive costs to freezing being particularly sparse in the literature (Hawes & Wharton, 2011). While our study was not designed to quantify the amount of ice formation for each individual, the same relative exposure was achieved for all samples and we can be sure that freezing was initiated, but had not necessarily progressed to a point appropriate to assessing freeze tolerance (*sensu* Bale, 1996; Hawes, 2014). Demonstrating the presence of fitness costs from partial freezing may, nonetheless, offer interesting insights into the mechanisms required for the evolution of freeze tolerance (Voituron *et al.*, 2002). Moreover, our findings underscore the importance of considering sublethal measures in cold tolerance studies, generally. Even when assessments of survival are based on more ecologically meaningful timescales (e.g., successful progression to the reproductive stage), the effect of cold on survival alone may not reflect the ultimate fitness of an individual or population (Renault, 2011).

Our laboratory findings offer interesting insights to justify and guide future work to explore these phenomena with a more ecologically informative design. Further studies are needed to assess the relevance and extent of our results, particularly regarding partial freeze tolerance, for *E. postvittana* in the field. For example, characterizing the physiology of individuals that survived exposure to the onset of freezing, such as the extent of ice formation and water content, could help illuminate the mechanisms behind the differences we saw in survival after partial freezing. The insects in our study were not acclimated

prior to exposure, and while previous work has shown that acclimation did not enhance supercooling ability in this species (Bürgi & Mills, 2010), measures of survival and fitness may still be impacted by acclimation. Investigating the impacts of cooling rates, photoperiods, and temperature exposures that reflect natural fluctuations on the low temperature responses used in this study would be an important next step. Supercooling points can also be affected by the presence of gut nucleators (Sømme, 1982), the latter of which were likely present in varying amounts in our larvae because they were not starved prior to testing. However, our replicated and randomized design helps to control for variation in feeding status. More importantly, overwintering *E. postvittana* may continue to feed and develop (Geier & Briese, 1980; Buergi *et al.*, 2011), making natural variation in gut contents of field individuals likely.

Current risk maps that exist for *E. postvittana* (e.g., Fowler *et al.*, 2009; Gutierrez *et al.*, 2010; Lozier & Mills, 2011) acknowledge the importance of cold in shaping this species' potential U.S. range. However, the parameter(s) used to describe cold tolerance reflect short-term assessments of lethal responses to low temperatures and do not account for downstream attrition of fitness, including potential trade-offs in populations with variable responses to cold. If our findings from an artificial environment in the laboratory are also reflective of variation in, and effects of, extreme low temperature tolerance in a natural *E. postvittana* population, even low levels of putative partial freeze tolerance may have implications for expanding the distribution limits of invading populations over time (Morey *et al.*, 2013). This may be especially true for species that have a broad host range; diet can impact cold tolerance response, as we have previously seen in *E. postvittana* (Morey *et al.*, 2016). However, we show here that tolerance to initial ice formation is not without consequence for organismal fitness. A more cold tolerant population may enable expansions of the invading population front during periods of cold stress (e.g., winter), but this potential could be limited by trade-offs in overall fitness (e.g., Blows & Hoffmann, 1993; Watson & Hoffmann, 1996), particularly if there is significant gene flow among populations during periods of noncold stress (Jenkins & Hoffmann, 1999).

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Disclosure

The authors declare no conflict of interest, including specific financial interests, relationships, and affiliations relevant to the subject of the manuscript.

References

Bale, J.S. (1987) Insect cold hardiness: freezing and supercooling—an ecophysiological perspective. *Journal of Insect Physiology*, 33, 899–908.

Bale, J.S. (1996) Insect cold hardiness: a matter of life and death. *European Journal of Entomology*, 93, 369–382.

Baust, J.G. and Rojas, R.R. (1985) Insect cold hardiness: facts and fancy. *Journal of Insect Physiology*, 31, 755–759.

Blows, M.W. and Hoffmann, A.A. (1993) The genetics of central and marginal populations of *Drosophila serrata*. 1. Genetic variation for stress resistance and species borders. *Evolution*, 47, 1255–1270.

Brown, J.W., Epstein, M.E., Gilligan, T.M., Passoa, S.C. and Powell, J.A. (2010) Biology, identification, and history of the light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae: Archipini) in California. *American Entomologist*, 56, 34–43.

Buergi, L.P., Roltsch, W. and Mills, N.J. (2011) Abundance, age structure, and voltinism of light brown apple moth populations in California. *Environmental Entomology*, 40, 1370–1377.

Bürgi, L.P. and Mills, N.J. (2010) Cold tolerance of the overwintering larval instars of light brown apple moth *Epiphyas postvittana*. *Journal of Insect Physiology*, 56, 1645–1650.

Capanu, M., Gonen, M. and Begg, C.B. (2013) An assessment of estimation methods for generalized linear mixed models with binary outcomes. *Statistics in Medicine*, 32, 4550–4566.

Carrillo, M.A., Kaliyan, N., Cannon, C.A., Morey, R. and Wilcke, W. (2004) A simple method to adjust cooling rates for supercooling point determination. *CryoLetters*, 25, 155–160.

Chen, C.P., Denlinger, D.L. and Lee, R.E. (1987) Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Physiological Zoology*, 60, 297–304.

Chen, W.L., Leopold, R.A. and Boetel, M.A. (2008) Cold storage of adult *Gonatocerus ashmeadi* (Hymenoptera:

Mymaridae) and effects on maternal and progeny fitness. *Journal of Economic Entomology*, 101, 1760–1770.

Colinet, H. and Boivin, G. (2011) Insect parasitoids cold storage: a comprehensive review of factors of variability and consequences. *Biological Control*, 58, 83–95.

Costanzo, J.P., Irwin, J.T. and Lee, R.E.J. (2015) Freezing impairment of male reproductive behaviors of the freeze-tolerant wood frog, *Rana sylvatica*. *Physiological Zoology*, 70, 158–166.

Coulson, S.C. and Bale, J.S. (1992) Effect of rapid cold hardening on reproduction and survival of offspring in the housefly *Musca domestica*. *Journal of Insect Physiology*, 38, 421–424.

Danthanarayana, W. (1975) The bionomics, distribution and host range of the light brown apple moth, *Epiphyas postvittana* (Walk.) (Tortricidae). *Australian Journal of Zoology*, 23, 419–437.

Dumbleton, L.J. (1932) The apple leaf roller (*Tortrix postvittana* Walker). *New Zealand Journal of Science and Technology*, 14, 83–92.

Fields, P.G. and McNeil, J.N. (1988) The cold-hardiness of *Ctenucha virginica* (Lepidoptera: Arctiidae) larvae, a freezing-tolerant species. *Journal of Insect Physiology*, 34, 269–277.

Follett, P.A. and Lower, R.A. (2000) Irradiation to ensure quarantine security for *Cryptophlebia* spp. (Lepidoptera: Tortricidae) in Sapindaceous fruits from Hawaii. *Journal of Economic Entomology*, 93, 1848–1854.

Fowler, G., Garrett, L., Neeley, A., Magarey, R., Borchert, D. and Spears, B. (2009) *Economic Analysis: Risk to U.S. Apple, Grape, Orange, and Pear Production from the Light Brown Apple Moth, Epiphyas postvittana (Walker)*. Raleigh, NC.

Geier, P.W. and Briese, D.T. (1980) The light-brown apple moth, *Epiphyas postvittana* (Walker): 4. Studies on population dynamics and injuriousness to apples in the Australian Capital Territory. *Australian Journal of Ecology*, 5, 63–93.

Geier, P.W. and Briese, D.T. (1981) The light-brown apple moth, *Epiphyas postvittana* (Walker): a native leafroller fostered by European settlement. *The Ecology of Pests* (eds. R.L. Kitching & R.E. Jones), pp. 131–155. C.S.I.R.O, Melbourne, Australia.

Gutierrez, A.P., Mills, N.J. and Ponti, L. (2010) Limits to the potential distribution of light brown apple moth in Arizona–California based on climate suitability and host plant availability. *Biological Invasions*, 12, 3319–3331.

Hance, T., van Baaren, J., Vernon, P. and Boivin, G. (2007) Impact of extreme temperatures on parasitoids in a climate change perspective. *Annual Review of Entomology*, 52, 107–126.

Hanson, A.A. and Venette, R.C. (2013) Thermocouple design for measuring temperatures of small insects. *CryoLetters*, 34, 261–266.

Hawes, T.C. (2014) Extracellular ice phase transitions in insects. *CryoLetters*, 35, 395–399.

Hawes, T.C. and Wharton, D.A. (2010) Tolerance of freezing in caterpillars of the New Zealand magpie moth (*Nyctemera annulata*). *Physiological Entomology*, 35, 296–300.

Hawes, T.C. and Wharton, D.A. (2011) Freeze fitness in alpine tiger moth caterpillars and their parasitoids. *Oecologia*, 167, 39–48.

Hutchinson, L.A. and Bale, J.S. (1994) Effects of sublethal cold stress on the aphid *Rhopalosiphum padi*. *Journal of Applied Ecology*, 31, 102–108.

Jenkins, N. and Hoffmann, A. (1999) Limits to the southern border of *Drosophila serrata*: cold resistance, heritable variation, and trade-offs. *Evolution*, 53, 1823–1834.

Koch, R.L., Carrillo, M.A., Venette, R.C., Cannon, C.A. and Hutchison, W.D. (2004) Cold hardiness of the multicolored Asian lady beetle (Coleoptera: Coccinellidae). *Environmental Entomology*, 33, 815–822.

Layne, J.R. and Blakeley, D.L. (2002) Effect of freeze temperature on ice formation and long-term survival of the woolly bear caterpillar (*Pyrrharctia isabella*). *Journal of Insect Physiology*, 48, 1133–1137.

Layne, J.R. and Peffer, B.J. (2006) The influence of freeze duration on postfreeze recovery by caterpillars of *Pyrrharctia isabella* (Lepidoptera: Arctiidae): when is survival enough to qualify as recovery? *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, 305A, 570–575.

Lee, R.E. (2010) A primer on insect cold-tolerance. *Low Temperature Biology of Insects* (eds. D.L. Denlinger & R.E. Lee), pp. 3–25. Cambridge University Press, New York.

Liebhold, A.M. (2014) Responses to "Clear, present, significant, and imminent danger: questions for the California light brown apple moth (*Epiphyas postvittana*) technical working group". *American Entomologist*, 60, 244–248.

Lindeman, N. (2013) Subjectivized knowledge and grassroots advocacy: an analysis of an environmental controversy in Northern California. *Journal of Business and Technical Communication*, 27, 62–90.

Lozier, J.D. and Mills, N.J. (2011) Predicting the potential invasive range of light brown apple moth (*Epiphyas postvittana*) using biologically informed and correlative species distribution models. *Biological Invasions*, 13, 2409–2421.

Manly, B.F.J. (1989) A review of methods for the analysis of stage-frequency data. *Estimation and anlaysis of insect populations: Proceedings of a Conference held in Laramie, Wyoming, January 25–29, 1988* (eds. L.L. McDonald, B.F.J. Manly, J.A. Lockwood & J.A. Logan), pp. 3–70. Springer-Verlag, Berlin.

McDonald, J.R., Bale, J.S. and Walters, K.F.A. (1997) Effects of sub-lethal cold stress on the western flower thrips, *Frankliniella occidentalis. Annals of Applied Biology*, 131, 189–195.

Mockett, R.J. and Matsumoto, Y. (2014) Effect of prolonged coldness on survival and fertility of *Drosophila melanogaster*. *PLoS ONE*, 9, e92228.

Morey, A.C., Venette, R.C. and Hutchison, W.D. (2013) Could natural selection change the geographic range limits of light brown apple moth (Lepidoptera, Tortricidae) in North America? *NeoBiota*, 18, 151–156.

Morey, A.C., Venette, R.C., Nystrom Santacruz, E.C., Mosca, L.A. and Hutchison, W.D. (2016) Host-mediated shift in the cold tolerance of an invasive insect. *Ecology and Evolution*, 6, 8267–8275.

Nedvěd, O., Lavy, D. and Verhoef, H.A. (1998) Modelling the time-temperature relationship in cold injury and effect of high-temperature interruptions on survival in a chill-sensitive collembolan. *Functional Ecology*, 12, 816–824.

Overgaard, J., Kearney, M.R. and Hoffmann, A.A. (2014) Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species. *Global Change Biology*, 20, 1738–1750.

Overgaard, J. and MacMillan, H.A. (2017) The integrative physiology of insect chill tolerance. *Annual Review of Physiology*, 79, 187–208.

Renault, D., Salin, C., Vannier, G. and Vernon, P. (2002) Survival at low temperatures in insects: what is the ecological significance of the supercooling point? *CryoLetters*, 23, 217–228.

Renault, D. (2011) Long-term after-effects of cold exposure in adult *Alphitobius diaperinus* (Tenebrionidae): The need to link survival ability with subsequent reproductive success. *Ecological Entomology*, 36, 36–42.

Rinehart, J.P., Yocum, G.D. and Denlinger, D.L. (2000) Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sarcophaga crassipalpis*. *Physiological Entomology*, 25, 330–336.

Ruan, C.C., Du, W.M., Wang, X.M., Zhang, J.J. and Zang, L. (2012) Effect of long-term cold storage on the fitness of prewintering *Harmonia axyridis* (Pallas). *BioControl*, 57, 95–102.

SAS/STAT® 9.2 User's Guide (2009) *SAS Institue Inc*., Cary, NC.

Shreve, S.M., Yi, S.X. and Lee, R.E. (2007) Increased dietary cholesterol enhances cold tolerance in *Drosophila melanogaster*. *CryoLetters*, 28, 33–37.

Sinclair, B.J. (1999) Insect cold tolerance: How many kinds of frozen? *European Journal of Entomology*, 96, 157–164.

Sømme, L. (1982) Supercooling and winter survival in terrestrial arthropods. *Comparative Biochemistry and Physiology*, 73A, 519–543.

Spilke, J., Piepho, H.P. and Hu, X. (2005) Analysis of unbalanced data by mixed linear models using the mixed procedure of the SAS system. *Journal of Agronomy and Crop Science*, 191, 47–54.

Stroup, W.W. (2015) Rethinking the analysis of non-normal data in plant and soil science. *Agronomy Journal*, 107, 811–827.

Turnock, W.J., Jones, T.H. and Reader, P.M. (1985) Effects of cold stress on the survival and development of *Delia radicum* (Diptera: Anthomyiidae) in England. *Oecologia*, 67, 506–510.

Turnock, W.J. and Bodnaryk, R.P. (1991) Latent cold injury and its conditional expression in the bertha armyworm, *Mamestra configurata* (Noctuidae: Lepidoptera). *CryoLetters*, 12, 377–384.

Voituron, Y., Mouquet, N., de Mazancourt, C. and Clobert, J. (2002) To freeze or not to freeze? An evolutionary perspective on the cold-hardiness strategies of overwintering ectotherms. *The American Naturalist*, 160, 255–270.

Watson, M.J.O. and Hoffmann, A.A. (1996) Acclimation, cross-generation effects, and the response to selection for increased cold resistance in *Drosophila*. *Evolution*, 50, 1182–1192.

Yocum, G.D., Zoarek, J., Joplin, K.H., Lee, R.E., Smith, D.C., Manter, K.D. *et al*. (1994) Alteration of the eclosion rhythm and eclosion behavior in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress. *Journal of Insect Physiology*, 40, 13–21.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1: The proportion of the three *E. postvittana* instars used in each temperature treatment that comprise the late instar exposure group. Proportions are of the total sample size (n), across all replicates, for each treatment. Instars were grouped by headcapsule measurements (Danthanarayana, 1975).

Table S2: Final model output for each measure of *E. postvittana,* following exposure as late instars. Output is from the Type III tests of fixed effects for each mixed effects model, where replicate ($n = 22$) was included as a random effect in all models. Each model began fully crossed, with interactions eliminated until all interactions were significant ($\alpha = 0.05$) or only main effects remained.

Table S3: Final model output for each measure of *E. postvittana,* following exposure as pupae. Output is from the Type III tests of fixed effects for each mixed effects model, where replicate ($n = 19$) was included as a random effect in all models. Each model began fully crossed, with interactions eliminated until all interactions were significant ($\alpha = 0.05$) or only main effect remained.