

RESEARCH ARTICLE

The sub-lethal effects of repeated freezing in the woolly bear caterpillar *Pyrrharctia isabella*

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SUMMARY

Repeated freeze–thaw cycles are common and are increasing in frequency with climate change in many temperate locations, yet understanding of their impact on freeze-tolerant insects is extremely limited. We investigated the effects of repeated freezing and thawing on the freeze-tolerant final instar caterpillars of the moth *Pyrrharctia isabella* (Lepidoptera: Arctiidae) by subjecting individuals to either a single sustained 35 h freeze or five 7 h freezes. Sub-lethal effects were quantified with changes in three broad groups of measures: (1) cold hardiness, (2) metabolic rate and energy reserves and (3) survival after challenge with fungal spores. Repeated freeze–thaw cycles increased mortality to almost 30% and increased tissue damage in Malpighian tubules and hemocytes. Repeated freezing increased caterpillar glycerol concentration by 0.82 mol l^{-1} . There were no changes in metabolic rate or energy reserves with repeated freezing. For the first time, we report increased survival after immune challenge in caterpillars after freezing and suggest that this may be linked to wounding during freezing. We suggest that little repair of freezing damage is possible in *P. isabella* caterpillars and repeated freeze–thaw cycles may present significant challenges to survival in this species.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/214/7/1205/DC1>

Key words: multiple freeze–thaw, freeze tolerance, *P. isabella*, ecological immunity.

INTRODUCTION

The frequency of extreme events is useful for predicting organismal responses to the abiotic environment (Gaines and Denney, 1993). One physiologically and ecologically relevant event is the freezing transition in insects (Sinclair et al., 2003; Lee, 2010). Freezing occurs when the temperature of an individual, which tracks microhabitat temperature closely in small ectothermic insects, drops below its supercooling point (SCP). The SCP is the lowest temperature an animal reaches before freezing (Lee, 2010). To survive freezing, freeze-tolerant insects often synthesize carbohydrate cryoprotectants and antifreeze proteins, which protect cells and inhibit recrystallization (Bale, 2002; Lee, 2010). Many species also promote ice nucleation at higher temperatures through ice-nucleating agents or selection of more benign overwintering sites (Storey and Storey, 1988; Bale, 2002).

Although the benefits of freeze tolerance also include reduced metabolic and water loss rates during freezing (Irwin and Lee, 2000), there is a potential energetic cost to both preventing and repairing freeze-induced damage (Bale, 2002). Damage from freezing may be a result of mechanical damage to tissues (particularly the nervous system), disruption of cellular membranes and proteins, osmotic shock or hypoxia (for reviews, see Storey and Storey, 1988; Lee, 2010). In addition to physical damage, freeze tolerance may carry energetic costs from the active re-establishment of ion gradients (Churchill and Storey, 1989a), or if cryoprotectants are synthesized from glycogen reserves before or after freezing (Churchill and Storey, 1989a; Churchill and Storey, 1989b; Lee, 2010). Allocation of energy reserves to either prevent or repair cold-induced damage may then consume resources that could otherwise be invested in reproduction.

Modelling of the energetic benefits and costs of freeze-tolerant and freeze-avoidant strategies indicates that freeze tolerance is optimal when there is low cost to prolonged freezing and the freezing event itself is not costly (Voituron et al., 2002). This suggests that understanding the costs of multiple freezing events is an important key to understanding the selection pressures leading to a particular cold-tolerance strategy. In another possible scenario, Sinclair et al. (Sinclair et al., 2003) suggest that freeze tolerance may be favoured in environments where the likelihood of freezing is unpredictable. Nevertheless, the physiological trade-offs that drive the ‘choice’ of the freeze-tolerance strategy remain largely unexplored.

Despite nearly three centuries of research establishing the biochemical and behavioural traits leading to freeze tolerance, the vast majority of research has focused on the effects of a single freeze exposure (for review, see Sømme, 2000; Bale, 2002; Sinclair et al., 2003). Multiple freeze–thaw cycles in a single winter are common in temperate latitudes (Sinclair, 2001; Henry, 2008), and could produce additional stress for individuals that experience low temperatures. In addition, the number of these cycles during a winter in temperate soil is predicted to increase as a result of declining snow cover (Henry, 2008).

There have been few, but contradictory, studies on the effects of multiple freeze–thaw cycles in freeze-tolerant insects. For example, freeze-tolerant larvae of *Eurosta solidaginis* were repeatedly frozen at -16°C , which led to decreased energy availability (reflected in the ATP:AMP ratio) with no recovery of adenylate charge between cold cycles (Churchill and Storey, 1989a). Two studies on freeze-tolerant insects found that, with successive freeze–thaw cycles, some individuals lost their ability to survive freezing, apparently switching

to a strategy of freeze avoidance (Bale et al., 2001; Brown et al., 2004). By contrast, there was no evidence of a switch in strategy after multiple freezes in the freeze-tolerant sub-Antarctic tineid caterpillar *Pringleophaga marioni*, although multiple cold exposures caused a long-term depression of feeding regardless of whether individuals froze, suggesting that chilling injuries may be more important than freezing *per se* in this species (Sinclair and Chown, 2005).

Previous experimental designs have failed to control for the total amount of time spent frozen, which may drive the accumulation of injuries (Churchill and Storey, 1989a; Bale et al., 2001; Brown et al., 2004; Sinclair and Chown, 2005). In addition, the sub-lethal effects of multiple freeze exposures have not been well elucidated (but see Churchill and Storey, 1989a; Sinclair and Chown, 2005), because binary responses (survival or emergence *versus* death) in the short term do not account for sub-lethal effects (Layne and Peffer, 2006). These shortcomings limit the capacity to predict potential climate change impacts in insects that overwinter in temperate zones.

The arctiid moth *Pyrrharctia isabella* Smith (Lepidoptera: Arctiidae) overwinters as a freeze-tolerant final instar caterpillar under leaf litter in the USA and southern Canada (Goettel and Philogene, 1978; Layne et al., 1999). During the fall, *P. isabella* move to diapause locations and cease feeding (Goettel and Philogene, 1978; Layne et al., 1999). At this time they accumulate large quantities of glycerol as a cryoprotectant and their winter-acclimatized SCP varies between -6 and -8°C (Layne et al., 1999). The overwintering microhabitat of *P. isabella*, its susceptibility to inoculative freezing (Layne et al., 1999) and its relatively high SCP suggests that individuals of this species would likely experience repeated freezing events through the winter in southern Ontario.

We used *P. isabella* to investigate the sub-lethal effects of multiple freeze–thaw cycles on three sub-lethal measures likely to affect post-winter fitness. First, multiple freeze–thaw cycles could affect aspects of caterpillar overwintering and freeze tolerance such as SCP, cryoprotectant concentration and survival, as caterpillars physiologically adjust to freezing events. Second, repeatedly frozen caterpillars could use more energy reserves, which would be predicted to impact future fitness because overwintering energy reserves are directly tied to spring reproductive ability in univoltine insects (e.g. Irwin and Lee, 2003). Third, given that immune function is costly to many organisms (Zuk and Stoehr, 2002), immune function could be compromised if energy is prioritized to cryoprotectant compounds or repairing freeze-induced damage and away from immune system components.

We tested three competing hypotheses. First, if there is no cost of freezing *per se* (Sinclair and Chown, 2005), then fitness will not differ between individuals exposed to multiple freeze–thaw cycles and those exposed to a single sustained freezing event. Second, if there is a cost to the freezing and/or thawing processes (Churchill and Storey, 1989a), then repeated freezing will reduce fitness compared with the sustained freezing and control treatments. Finally, if the lowered metabolic rate experienced by frozen animals results in increased fitness and overwinter energy use is a prime determinant of subsequent fitness (Irwin and Lee, 2000), then caterpillars that experienced sustained freezing and caterpillars that experienced multiple freeze–thaw cycles would have similar fitness, and both would have higher fitness than control individuals.

MATERIALS AND METHODS

Animal collection and maintenance

Pyrrharctia isabella caterpillars were collected from fields and pathways in London, ON, Canada ($43^{\circ}00'N$, $81^{\circ}15'W$) and Niagara on the Lake, ON, Canada ($43^{\circ}04'N$, $79^{\circ}04'W$) during September

and October 2007, 2008 and 2009. Caterpillars were maintained individually in plastic containers containing pinto bean diet (Goettel and Philogene, 1978) until feeding ceased (mid-November) and food was removed. Incubator temperatures followed weekly climate maximum and minimums for London, ON, obtained from Environment Canada (www.weatheroffice.gc.ca) until 0°C was reached in the incubators. Relative humidity was maintained at 70% by the addition of a tray of salt solution in the incubator. Caterpillars were then maintained at 0°C in constant darkness, and experiments were conducted after at least 3 weeks at 0°C . These methods were employed each year with the following modifications: in 2007–2008, caterpillars were maintained on grass until feeding ceased, and in 2009–2010, temperatures were set to fluctuate daily from 0 to 2°C (12h:12h light:dark).

Freeze treatments

In all experiments, caterpillars were assigned to one of three treatment groups: maintained at 0°C (control), frozen for five bouts of 7 h ($5 \times 7\text{h}$) or frozen for a single bout of 35 h ($1 \times 35\text{h}$). Caterpillars in the $5 \times 7\text{h}$ freeze–thaw treatment were frozen every fifth day. Due to interannual variability in SCP, to ensure that all caterpillars froze the cold exposure temperature used varied between years: -12°C in 2007–2008 and 2009–2010, and -14°C in 2008–2009. To reduce the effect of unequal freezing time between the $1 \times 35\text{h}$ and $5 \times 7\text{h}$ freeze groups due to cooling rate, caterpillars were cooled at $0.5^{\circ}\text{C min}^{-1}$ in all years. Freeze treatments were performed by placing caterpillars in 35 ml plastic vials in contact with the tip of a 36 AWG (American wire gauge) copper-constantan thermocouple (Omega, Laval, PQ, Canada) held in place with a foam stopper. These vials were placed into wells in an aluminum block cooled by either methanol or propylene glycol circulated by either a programmable Proline 3530C refrigerated bath (Lauda, Wurzburg, Germany) or a programmable VWR Signature 1157P refrigerated circulator (VWR, Mississauga, ON, Canada).

Thermocouples were connected to Picotech TC-08 thermocouple interfaces and temperatures were recorded using PicoLog software for Windows (Pico Technology, Cambridge, UK). Freezing was detected and SCP was determined from the exotherm (Lee, 2010). After freezing, caterpillars were warmed to 0°C at $0.5^{\circ}\text{C min}^{-1}$, and returned to their incubator at 0°C . SCPs in repeatedly frozen caterpillars were compared between 2008–2009 and 2009–2010, as well as within each year for effects of freezing interval using a repeated measures two-way ANOVA conducted in R (R Development Core Team, 2010).

Survival

Survival was defined as a curling defensive response to stimulus from a pointed probe 24 h after removal from the freeze treatment. Control caterpillars were set aside when the $5 \times 7\text{h}$ treatments were started and then survival was tracked during the duration of the experiments (25 days). Survival was compared between caterpillars in the three treatment groups (control, $1 \times 35\text{h}$ and $5 \times 7\text{h}$) using a generalized linear model with binomial error and logit link in R.

Metabolic rate

Metabolic rate measurements were conducted during the winter of 2007–2008. Eighteen hours after thawing, caterpillars from each treatment ($N=5$ per treatment) were placed a glass respirometry chamber suspended in a propylene glycol VWR refrigerated bath set to 5°C and allowed to acclimate for 6 h before metabolic rate measurements (approximated from CO_2 production) commenced. CO_2 production by caterpillars was then measured using a flow-

through respirometry system with dried CO₂-free air flowing at 12 ml min⁻¹ (Sable Systems International, Las Vegas, NV, USA) connected to a Li-Cor 7000 CO₂ differential infrared gas analyzer (Li-Cor Biosciences, Lincoln, NB, USA) for a continuous recording period of 6 h [method modified from Williams et al. (Williams et al., 2010)]. Data extraction was conducted as in Williams et al. (Williams et al., 2010). For CO₂ production traces that exhibited discontinuous gas exchange, data from an entire cycle were selected. For traces that exhibited continuous gas exchange, a section of data that exhibited no upward or downward trends, with no detectable caterpillar movement, was analyzed. The rate of CO₂ production was compared among groups using a nonparametric Kruskal–Wallis test conducted in SPSS Statistics 17.0 (IBM, Somers, NY, USA), with caterpillar mass immediately before respirometry as a covariate.

Quantification of metabolic reserves and cryoprotectants

Twenty-four hours after cold exposure, 10 caterpillars from each treatment group in winter 2008–2009 were flash-frozen in liquid nitrogen and stored at –80°C. Caterpillars were then weighed (fresh mass), dried at 60°C for 1 week and then weighed again (dry mass). Water mass was calculated as the difference between wet mass and dry mass. Whole caterpillars were homogenized in 10 ml 0.05% Tween 20, then diluted 10× in additional 0.05% Tween 20. Subsamples of 1 ml (in duplicate for each caterpillar) were centrifuged at 16,000g for 1 min, and the supernatant was removed and stored at –20°C. Total protein, glucose, glycogen, glycerol and glycerol from triglyceride in the supernatant were assayed in triplicate using spectrophotometric assays as described previously (Gefen et al., 2006). Differences in energy reserve mass, cryoprotectant concentration and protein mass among treatment groups were investigated using analysis of covariance (ANCOVA), with dry mass as a covariate, in R.

Immune challenge

Immune challenge experiments were conducted during the fall of 2009. A fungal challenge was chosen because entomopathogenic fungi are common in the overwintering habitat of *P. isabella* (Bidochka et al., 1998) and we had previously noted fungal infection in laboratory-maintained caterpillars (data not shown). Cultures of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae; strain no. 2575, USDA, Ithaca, NY, USA), a broadly entomopathogenic fungus (Bidochka et al., 1998), were obtained from Michael Bidochka (Brock University, St Catharine's, ON, Canada). Spores were plated onto potato-dextrose agar plates and cultured at 25°C in darkness for 14 days. After sporulation, plates were frozen at –20°C until use. To obtain standardized spore suspensions, spores were suspended in a 0.01% solution of Triton-X in distilled water. Spore concentration was determined using an improved Neubauer counting chamber (CA Hauser & Son, Philadelphia, PA, USA), and the suspension was diluted to 1×10⁶ spores ml⁻¹.

Twenty-four hours after thawing, 5 µl of spore suspension was injected into the ventral surface of caterpillars between the first and second pair of prolegs, immediately below a proleg to avoid piercing the gut. A 20 µl Hamilton syringe with a 30.5-gauge needle was used for all injections. Control caterpillars received a sham injection of 5 µl 0.01% Triton-X. The needle and syringe were sterilized with bleach between injections, and the order of injections was randomized. Caterpillars were then placed on moistened vermiculite in Parafilm[®]-sealed 100 ml plastic vials. Vials were placed into an incubator at 26°C. Containers were opened to check caterpillar survival and allow for fresh air circulation daily. Mortality

was compared between control and spore-injected caterpillars within each freeze treatment group after 8 days using Fisher's exact test in SigmaStat (Systat Software Inc., San Jose, CA, USA).

Tissue damage

Five caterpillars from each group were dissected 24 h after their final cold exposure in 2009–2010. First, a 10 µl sample of hemolymph was obtained by pricking the caterpillar on the ventral surface between the first and second thoracic segment with an insect pin, and then sampling from the beaded hemolymph with a pipette. The caterpillar was then dissected from the ventral surface, and a small portion of Malpighian tubules and the fat body was removed from the abdomen.

Live/dead staining was conducted with modifications to the method of Yi and Lee (Yi and Lee, 2003). Tissue and hemolymph samples were incubated at room temperature for 10 min in 30 µl of a 1:10 solution of SYBR 14 (in DNA-bound complex $\lambda_{\text{max ex}}=475$ nm, $\lambda_{\text{max em}}=516$ nm) in Coast's solution on a glass slide (Coast, 1988; Yi and Lee, 2003). Then, 30 µl of a 1:20 solution of propidium iodide (in DNA-bound complex $\lambda_{\text{max ex}}=540$ nm, $\lambda_{\text{max em}}=617$ nm; both dyes obtained from the Live/Dead Sperm Viability Kit, Invitrogen Canada Inc., Burlington, ON, Canada) in Coast's solution was added and the cells or tissues were incubated for a further 10 min. Slides were then visualized under 50× magnification using an Axio Observer Z1 microscope (Carl Zeiss MicroImaging GmbH, Goettingen, Germany) using green fluorescent protein (excitation wavelengths=430–510 nm, emission wavelengths=474–575 nm) and Rhodamine (excitation wavelengths=534–568 nm, emission wavelengths=575–640 nm) filters for the SYBR 14 and propidium iodide dyes, respectively. SYBR 14 dyes live cells and fluoresces green whereas propidium iodide stains dead cells and fluoresces red.

Image analysis was conducted with ImageJ software (Abramoff et al., 2004). Contrast of images was enhanced and standardized by setting the percentage of saturated pixels to 0.4% and equalizing and normalizing the histogram. The threshold for each image was set at a level that minimized background noise, and only the area that was identifiably part of the tissue in question was included. Finally, the area of the image covered by fluorescence was calculated separately for red (propidium iodide) and green (SYBR 14) channels. One-way ANCOVA with total fluorescing area as a covariate was used to compare amounts of damaged tissue between freeze treatment groups for each tissue in R.

RESULTS

Survival

In every year, caterpillars that were repeatedly frozen had higher mortality than control caterpillars and caterpillars that had a single prolonged freeze exposure. Although the difference was not statistically significant in every year (supplementary material Table S1), when pooled across years within treatment groups, mortality was significantly higher in caterpillars repeatedly frozen at 29.7% compared with caterpillars that experienced a single prolonged freeze exposure and control caterpillars at 10.3 and 13.1%, respectively (Wald $\chi^2=12.685$, d.f.=2, $P=0.002$, $N=69$ –111 in each group; Fig. 1). Cumulative survival in caterpillars repeatedly frozen decreased linearly with increased freeze–thaw cycles ($R^2=0.981$, $P<0.001$, $N=5$, total caterpillars tested=111; Fig. 2).

Supercooling points

Caterpillars that were frozen for the first time had SCPs that ranged from –8.2 to –12.05°C in 2008–2009 and from –3.79 to –12.11°C

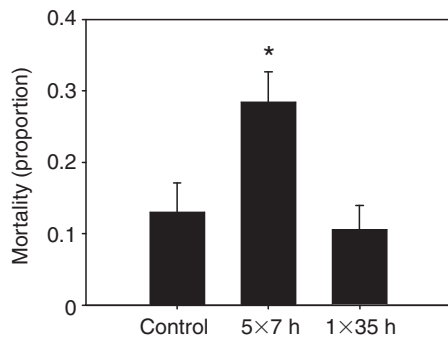


Fig. 1. Mortality of *Pyrrharctia isabella* caterpillars 24 h after freeze treatments pooled over three winters of experiments. Mortality in caterpillars repeatedly exposed to freeze events was significantly higher than that in caterpillars in the other treatment groups (control caterpillars were followed from the first freeze treatment in the 5×7 h freeze group to 24 h after final freeze; Wald $\chi^2=12.685$, d.f.=2, $P=0.002$, $N=69-111$ in each group). Control, maintained at 0°C; 5×7 h, five 7 h freezes at -12°C with 5 days between each freeze at 0°C; 1×35 h, a single 35 h freeze at -12°C. Error bars indicate s.e.m. of the proportion, asterisk indicates a significant difference between caterpillars with multiple freeze-thaw exposures and other groups.

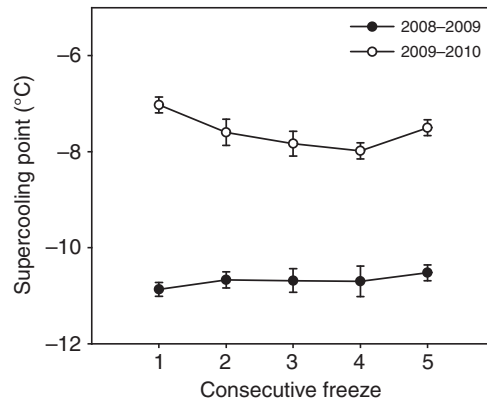


Fig. 3. Supercooling points (SCPs; means \pm s.e.m.) of freeze-tolerant *P. isabella* caterpillars. SCPs declined by only -0.5°C in 2009-2010 after repeated freeze-thaw cycles between -12 and 0°C. In 2008-2009, SCPs changed very slightly with increased freeze-thaw cycles between -14 and 0°C. All caterpillars belonged to the 5×7 h treatment group.

in 2009-2010. SCPs in 2008-2009 ($-10.7 \pm 0.2^\circ\text{C}$) were significantly lower than in 2009-2010 ($-7.6 \pm 0.2^\circ\text{C}$, $F_{1,538}=207.82$, $P<0.001$; Fig. 3). Within the winters of 2008-2009, mean SCPs insignificantly increased by $\sim 0.2^\circ\text{C}$ (from -10.5 ± 0.2 to $-10.7 \pm 0.3^\circ\text{C}$) over five freeze-thaw cycles. By contrast, in 2009-2010 SCPs declined by 0.5°C (from -7.1 ± 0.2 to $-7.6 \pm 0.2^\circ\text{C}$) after 5 freeze-thaw cycles ($F_{1,538}=4.83$, $P=0.028$).

Metabolic rate

Discontinuous gas exchange was exhibited in six out of 15 caterpillars (Williams et al., 2010). Neither log nor square-root transformation improved normality of CO_2 production rate data, so a nonparametric Kruskal-Wallis test was used to compare CO_2 production rates between treatment groups. Caterpillars that experienced multiple freeze-thaw events did not have an increased metabolic rate 24 h after their freeze compared with caterpillars that experienced a single sustained freezing event (Kruskal-Wallis test, $H=0.033$, d.f.=2, $P=0.981$, $N=5$ in each group; Fig. 4), nor was there a trend in metabolic rate with successive freezing events (data not shown).

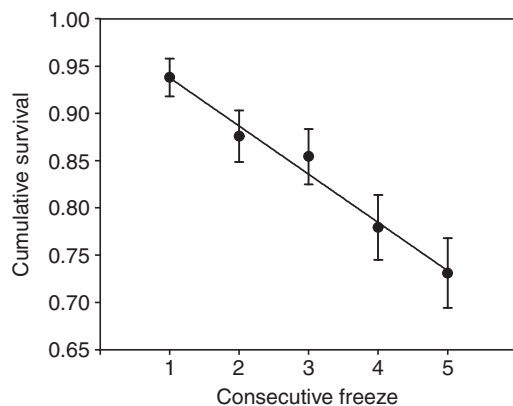


Fig. 2. Cumulative survival in freeze-tolerant *P. isabella* caterpillars after repeated freeze-thaw cycles between -12 and 0°C (5×7 h treatment). Survival declined linearly with each consecutive freeze ($R^2=0.982$, $P<0.001$, $N=5$, total caterpillars tested=111). Error bars are s.e.m. of the proportion.

Energy reserves and cryoprotectants

Repeated freezing increased caterpillar glycerol concentration by 0.82 mol l^{-1} compared with control caterpillars, whereas caterpillars that had experienced a sustained freeze had an intermediate concentration of glycerol (Table 1). Other than this change in glycerol content, there were generally few differences in energy reserves or cryoprotectant concentrations between caterpillars in different freeze treatment groups (Table 1). Triglyceride stores did not differ between caterpillars in different freeze treatments, although there was a trend towards decreased quantities in caterpillars that had experienced repeated freezing. Repeated or prolonged freezing did not change caterpillar water content, protein content or glucose mass. There was a near-significant effect of the interaction between dry mass and freeze treatment group on glycogen mass ($F_{2,29}=3.39$, $P=0.051$); however, when the residuals of a regression of glycogen mass on dry mass were examined, there was no difference between caterpillars in different freeze treatment groups ($F_{2,29}=0.258$, $P=0.774$).

Immune challenge

Caterpillars injected with *M. anisopliae* spores died ~ 5 days after injection, and white mycelia were observed on their ventral surface.

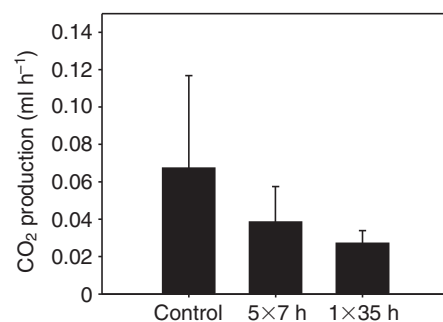


Fig. 4. CO_2 production rate (ml min^{-1} , means \pm s.e.m.) of *P. isabella* caterpillars at 5°C. Rates did not differ between treatment groups 24 h after freezing (Kruskal-Wallis test, $H=0.033$, d.f.=2, $P=0.981$, $N=5$ in each group).

Table 1. Energy reserves, cryoprotectant concentration and protein mass of *Pyrrarctia isabella* caterpillars in three treatment groups: maintained at 0°C (control), frozen for a single bout of 35 h at -12°C (1×35 h) and frozen for five bouts of 7 h at -12°C (5×7 h)

Measure	Control	5×7 h	1×35 h	F	P
Protein (mg mg ⁻¹ dry tissue)	0.0783±0.0093	0.0675±0.0093	0.0733±0.0076	0.332	0.720
Glucose (mg mg ⁻¹ dry tissue)	0.014±0.006	0.016±0.002	0.015±0.002	1.08	0.355
Glycogen (mg mg ⁻¹ dry tissue)	0.002±0.0004	0.003±0.0013	0.003±0.0009	0.258	0.774
Free glycerol (mol l ⁻¹)	1.8257±0.2659 ^a	2.6434±0.2281 ^b	2.5159±0.2271 ^{a,b}	4.548	0.021
Glycerol from triglyceride (μmol mg ⁻¹ dry tissue)	0.5968±0.0845	0.4542±0.0875	0.4981±0.0894	0.125	0.883
Caterpillar dry mass (mg)	255.166±17.467	309.626±12.552	293.449±18.542	0.756	0.480
Caterpillar water mass (mg)	286.188±35.544	283.304±27.008	252.433±24.436	0.433	0.653

Treatment groups were compared using an ANCOVA with dry mass as a covariate (except for dry mass, which was tested using one-way ANOVA). Values are means ± s.e.m.; N=10 per group. Values with the same superscripted letters are not significantly different (Tukey's *post hoc* test).

Injection with the spores greatly increased mortality in caterpillars relative to sham injections in both the control and 1×35 h freeze treatment groups (Fisher's exact test, $P < 0.03$ in both cases, $N = 17-20$ in each group). However, repeatedly frozen caterpillars had similarly low mortality whether injected with spores or sham solution (Fisher's exact test, $P = 1.00$, $N = 28$ in each group; Fig. 5).

Tissue damage

Repeated freeze-thaw cycles caused increased damage to both Malpighian tubules ($F_{2,9} = 7.05$, $P = 0.014$; Fig. 6) and hemocytes ($F_{2,9} = 11.18$, $P = 0.004$) compared with a single sustained freeze in caterpillars. Viability of fat body cells did not differ significantly among caterpillars in different freeze treatments ($F_{2,9} = 0.09$, $P = 0.933$; Fig. 6).

DISCUSSION

We found that there was a fitness cost to freeze-thaw events in overwintering freeze-tolerant *P. isabella* caterpillars that was independent of the amount of time spent frozen. Individuals that had experienced multiple freeze-thaw events had higher mortality and more damaged cells in their Malpighian tubules and hemolymph than individuals that experienced either no freezing events (control) or a single freezing event. These fitness consequences also appeared to be independent of energetics: there was neither an increase in metabolic rate nor changes in energy reserves observed in individuals

that experienced multiple freezing events. Because we controlled for the amount of time spent frozen, in contrast to Sinclair and Chown (Sinclair and Chown, 2005), our results indicate that the impact of freezing on freeze-tolerant organisms like *P. isabella* depended on the number of freeze-thaw transitions that the animal experiences rather than the cumulative amount of time spent in the frozen state.

We also found that cumulative survival in *P. isabella* decreased linearly with increasing freeze-thaw cycles, indicating that the proportion of caterpillars that die increases with each successive freezing exposure (Fig. 2). Thus, the probability of being killed by freezing increased with the number of freezing exposures *P. isabella* experienced. This implies that, rather than a constant threat of mortality, each freezing event causes caterpillars to accumulate injuries that are not fully repaired and consequently increases the likelihood of being killed by subsequent freezing events. Similarly, in the fly *Delia radicum* and the moth *Mamestra configurata*, repair from extreme cold stress does not take place even with 4 weeks recovery time (Turnock et al., 1983; Turnock et al., 1985).

Similar to previous studies (Bale et al., 2001; Brown et al., 2004), we observed a statistically significant decrease in SCP with increased freeze-thaw cycles in one year (Fig. 3). However, this change was very small (0.5°C) and likely does not have any ecological significance. After five freeze-thaw cycles, glycerol concentration increased by 0.82 mol l⁻¹, which would be expected to decrease SCP by ~1°C (Zachariassen, 1985). Because SCP declined by only 0.5°C, we suggest that SCP in this species is defended by the presence of ice nucleators. Although survival was reduced in caterpillars that experienced multiple freeze-thaw cycles, this appears not to be a result of the loss of freeze tolerance, as reported by Bale et al. (Bale et al., 2001) and Brown et al. (Brown et al., 2004), as many individuals were still able to survive freezing, but rather a result of accumulated freeze-thaw damage.

There was no significant difference in energy reserves or metabolic rate between any of the experimental groups 24 h after a freeze (Fig. 4, Table 1). We interpret this to indicate that the response to being frozen does not demand a sustained investment of energy in *P. isabella*. There has been much speculation about the energetic costs of repair of freezing-induced damage and this has been used to conclude that reduced metabolic rate in the 4 h post-freezing indicates that repair has not occurred (Sinclair et al., 2004), and (conversely) that elevated metabolism post-freezing indicates the cost of repair (Block et al., 1998). We similarly observed a non-significant trend towards depression in metabolic rate 24 h after a freezing event (Fig. 4). However, we also observed residual tissue damage after 24 h. As we do not know how long (if ever) that damage takes to be repaired, we cannot draw a conclusion about the relationship between metabolic rate and tissue damage, although

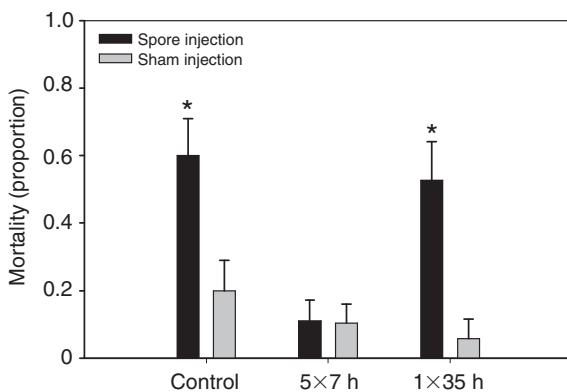


Fig. 5. Mortality of *P. isabella* caterpillars after injection with *Metarhizium anisopliae* spores. Injection of spore solution caused significantly increased mortality in control caterpillars and caterpillars frozen once at -12°C for 35 h; however, caterpillars that experienced five repeated 7 h freezes at -12°C had similarly low mortality whether injected with spore solution or a sham solution. Asterisks indicate significant differences in mortality between injections within each freeze treatment. Error bars are s.e.m. of the proportion.

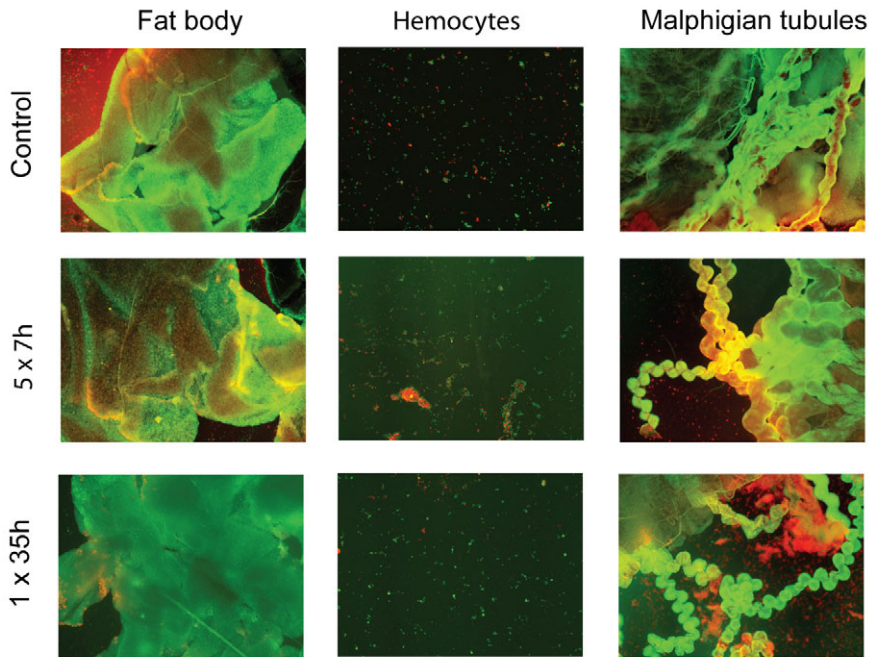
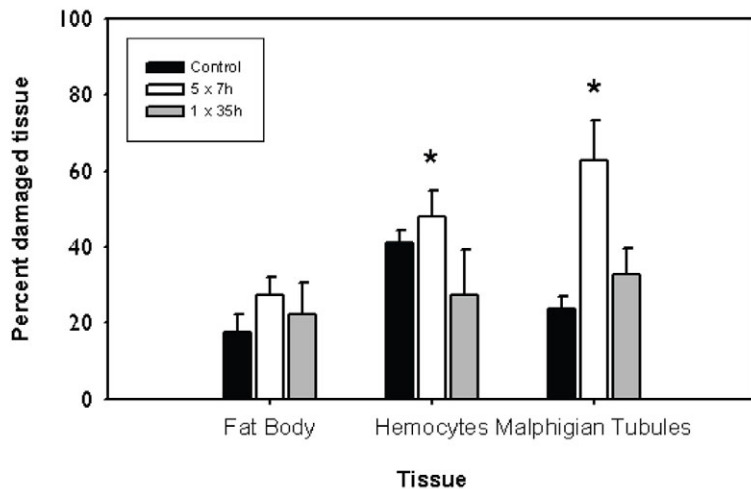


Fig. 6. Representative images of fat body (A,D,G), hemocytes (B,E,H) and Malpighian tubules (C,F,I) of a *P. isabella* caterpillar 24 h after a 35 h freeze at -12°C (D,E,F), five 7 h freezes at -12°C (G,H,I) or maintained at 0°C (A,B,C). Images were manipulated as described in the text with colour channels merged to present localization of tissue damage. Images are at $50\times$ total magnification, with green fluorescence indicative of staining by SYBR-14 (live cells) and red fluorescence indicative of staining by propidium iodide (cells with compromised cellular membranes). (J) Amount of tissue damage in the Malpighian tubules and hemocytes, calculated as area fluorescing red due to propidium iodide staining divided by total fluorescing area (means \pm s.e.m., $N=5$ caterpillars in each group). Tissue damage differed in *P. isabella* individuals between control, 5×7 h and 1×35 h treatments. The percent damaged area of the fat body was similar between groups. Asterisks indicate significant differences between groups within tissues with *post hoc* testing in an ANCOVA with total fluorescing area as a covariate.



the relationship could be resolved using the imaging approach we described (see Materials and methods) to track a time series of tissue viability through the process of recovery from freezing.

We found that *P. isabella* caterpillars collected in southern Ontario constitutively contained almost 10 times the previously reported concentration of glycerol (Layne et al., 1999; Layne and Kuharsky, 2000). As some broadly distributed freeze-tolerant insects vary their cold tolerance (and glycerol concentration) along latitudinal gradients (e.g. *E. solidaginis*) (Williams and Lee, 2008), this large difference between glycerol concentrations may be at least partially due to greater cold hardiness in our more northerly population. Another possible explanation is an effect of our overwintering treatment, because dry conditions can increase glycerol concentration in *P. isabella* up to fivefold (Layne and Kuharsky, 2000).

In the present study, repeatedly frozen caterpillars increased their glycerol concentration by 0.82 mol l^{-1} relative to controls, whereas there was a non-significant increase after a single prolonged exposure (Table 1). Given that water content did not differ between experimental groups, and that feeding does not occur during winter, this indicates that *P. isabella* synthesize glycerol in response to a

freezing event. Churchill and Storey found that, with an increased number of freeze–thaw cycles, pre-pupae of the freeze-tolerant gall fly *Eurosta solidaginis* almost quadrupled their concentration of sorbitol and concomitantly decreased their glycogen content (Churchill and Storey, 1989a). Similarly, both freeze-tolerant frogs (*Rana sylvatica*) and earthworms (*Dendrobaena octaedra*) mobilize glucose as a cryoprotectant during freezing (Storey and Storey, 1996; Calderon et al., 2009).

Contrary to expectations, we observed no change in glycogen content corresponding to the increase in glycerol. However, evidence that glycogen is the direct source of all glycerol produced during cold hardening is mixed. For example, during prolonged cold exposure in larvae of the moth *Epiblema scudderiana*, the amount of glycogen initially dips as glycerol is synthesized, and then rebounds to control values over the course of 2 days even though glycerol concentration remains high (Churchill and Storey, 1989b). Similarly, although the rice stem borer *Chilo suppressalis* and the eastern spruce budworm *Choristoneura fumiferana* increase glycerol concentration during seasonal cold hardening, in both cases the amount of carbon in glycerol produced greatly outstrips the quantity

of carbon depleted in glycogen (Han and Bauce, 1995; Li et al., 2002). Given that glycogen content in overwintering lepidopteran larvae is often very low (Han and Bauce, 1995; Li et al., 2002), we suspect that glycerol was synthesized from other sources. Although we found no significant difference between freeze treatment groups in glycerol released from triglycerides, there was a large trend, and we suggest that either the glycerol backbone or the free fatty acids produced could be the source. Another potential glycerol source is phospholipids, which can be an important energy source during starvation (for a review, see Fast, 1964).

Caterpillars that had experienced multiple freeze–thaw cycles were more likely to survive an immune challenge. To our knowledge, this is the first report of enhancement of the immune response in an insect as a result of freezing, and adds to the limited knowledge of the interactions between ecological processes and immune function (Rolff and Siva-Jothy, 2003). Although research on the effects of temperature extremes on invertebrate immune function is limited, mild cold stress has been shown to improve survival of fungal infection in *Drosophila melanogaster* (LeBourg et al., 2009) whereas snails given repeated high temperature shocks have shown decreased immune function (Seppälä and Jokela, 2010). In the present study, rather than trading off allocation to immune function for repairing freeze damage, *P. isabella* caterpillars increased their immune response to a fungal challenge after multiple freeze–thaw cycles. We did not investigate the mechanisms underlying this improvement in immune function; however, we note that repeated freezing damaged Malpighian tubules and hemocytes (Fig. 6). This damage could induce a wounding response, leading to increased immune function through the phenoloxidase pathway (Marmaras et al., 1996; Cerenius and Söderhäll, 2004). Alternatively, this increased immune function could be a result of a generalized stress response – for example, the heat shock protein Hsp70 is upregulated in many insect species after cold shock (Sinclair et al., 2007) or as part of seasonal hardening (Rinehart et al., 2007), and is also important in the immune responses in vertebrates (Pockley, 2003), suggesting a link between cold stress and immune responses in insects. Given that the spores of the species of fungus we used are found broadly and abundantly throughout Ontario (at up to 70% of sites sampled) (Bidochka et al., 1998), and that entomopathogenic fungi in general are common (91% of sites sampled) (Bidochka et al., 1998), we suggest that overwintering immune function is an important yet neglected part of insect overwintering ecology.

Interestingly, the increased tissue damage in caterpillars subjected to multiple freeze events occurred in the Malpighian tubules and hemocytes, but not in the fat body. Malpighian tubules and hemocytes are also among the most freeze-sensitive tissues in *E. solidaginis* (Yi and Lee, 2003). In contrast to our results, Yi and Lee also found that *E. solidaginis* fat body cells are severely damaged during prolonged freezes (Yi and Lee, 2003). However, unlike in other freeze-tolerant insects, *E. solidaginis* (whole organism) and their fat body cells freeze at similar temperatures (Bennett and Lee, 1997). *Pyrrharctia isabella* fat body cells do not freeze at -10°C (Layne and Blakeley, 2002), and the authors suggested that freezing of the fat body is unrelated to survival in this species. Given the lack of increased tissue damage to fat body cells in the present study, as well as the low water content of the fat body, it seems unlikely that *P. isabella* fat body cells freeze during a whole-organism freezing event.

In summary, we found that multiple freeze–thaw events led to increased damage to the Malpighian tubules and hemocytes of *P. isabella* and increased mortality compared with single prolonged

freezes. We suggest that the stress of freeze–thaw events outweighs the stress of prolonged freezing; however, repeated freezing also appears to upregulate immune function. These effects indicate the potential for important trade-offs in overwintering insects in the field. Given that the response to multiple freezing events increased immune function enough to completely match survival of infected caterpillars with that of uninfected caterpillars (reduced mortality fivefold), yet only tripled freezing mortality, the advantages of increased immune function due to repeated freezing may interact with the increased risk of freezing mortality in a field context. Taken together, the results of our study demonstrate that important sublethal effects of repeated freezing are not predictable from the effects of single freezing event, and we suggest that these differential responses to multiple freezing events will become increasingly important in a changing climate.

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LIST OF ABBREVIATIONS

$\lambda_{\text{max,em}}$	wavelength of light at which peak emission occurs in a particular substance
$\lambda_{\text{max,ex}}$	wavelength of light at which peak excitation occurs in a particular substance
AMP	adenosine monophosphate
ATP	adenosine triphosphate
SCP	supercooling point (the lowest temperature an animal reaches before freezing occurs)

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