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RESEARCH ARTICLE

Cold-hardening during long-term acclimation in a freeze-tolerant woolly bear caterpillar, *Pyrrharctia isabella*

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ABSTRACT

The banded woolly bear caterpillar, *Pyrrharctia isabella* (Lepidoptera: Erebidae), overwinters in leaf litter and survives freezing under natural conditions. Following 18 weeks of cold acclimation at 5°C, all caterpillars could survive 1 week of continuous freezing at −20°C or seven cycles of freezing–thawing at −20°C, but none survived freezing at −80°C. Field-collected caterpillars had a temperature of crystallization of −7.7±0.5°C that decreased significantly to −9.5±0.6°C after 12 weeks of acclimation at 5°C. Hemolymph levels of free proline, total amino acids and proteins reached a peak during the first 4 weeks of acclimation; concomitantly, hemolymph osmolality increased markedly during this interval (from 364 to 1282 mosmol kg^{-1}). In contrast, hemolymph pH decreased during the first 4 weeks of acclimation before this trend reversed and pH values gradually returned to initial values. However, pH reached its peak value following 1 week at −20°C, but decreased after longer periods of freezing. During cold acclimation, cholesterol levels decreased in the hemolymph and the membrane fraction of fat body but not in other tissues. Lethal freezing at −80°C reduced cell survival in foregut tissue and caused leakage of free proline, total amino acids and proteins from tissues into the hemolymph. The addition of glycerol to the bathing medium reduced freezing injury in fat body cells, as evidenced by reduced leakage of amino acids and proteins.

KEY WORDS: Cold-hardiness, Cholesterol, Free amino acids, Proline, Glycerol, Cryoprotection

INTRODUCTION

Many temperate and polar insects avoid chilling and freezing injury by enhancing their cold tolerance in advance of winter (Denlinger and Lee, 2010). For freezing-intolerant species, seasonal cold-hardening usually involves a decrease in the temperature of crystallization (T_c) at which ice forms spontaneously within body fluids. Less commonly, species seasonally acclimate by acquiring the capacity to tolerate internal freezing.

During cold-hardening, many insects accumulate substantial amounts of the cryoprotectants glycerol, sorbitol, trehalose, and other polyols and sugars, collectively reaching concentrations of 1 mol l^{-1} or more (Lee, 1991). More recently, attention has focused on a cryoprotective role for small solutes such as proline and other amino acids (Koštál et al., 2011, 2012). Unfortunately, most studies that measure cryoprotectant levels do not include direct tests of whether, and how, these compounds improve survival of specific tissues during freezing and thawing. Adding such experiments should provide a better functional understanding of how cryoprotectants work and differences among tissues in their sensitivity to chilling and freezing.

In addition to thermal hysteresis proteins and glycoproteins, recent work has identified novel classes of glycolipid antifreezes and acetylated triacylglycerols that may play important roles in seasonal cold tolerance and winter survival (Walters et al., 2011; Marshall et al., 2014). Seasonal remodeling of membranes often includes a decrease in saturated lipids that helps maintain membrane fluidity and allows continued function at low temperatures (Crockett, 1998; Yeagle, 1991). In some ectotherms, cold acclimation increases membrane cholesterol levels, which may also function to regulate membrane fluidity at low temperature (Crockett and Hazel, 1995a). However, the role of cholesterol in insect cold tolerance remains relatively unstudied.

Caterpillars of the banded woolly bear, *Pyrrharctia isabella* (Smith 1797) (Lepidoptera: Erebidae), a common North American moth, overwinter in moist leaf litter and survive both moderate supercooling and freezing (Layne et al., 1999). This species is susceptible to inoculative freezing by contact with environmental ice, and substantially elevates hemolymph osmolality and glycerol levels in response to seasonal acclimatization and cold acclimation (Layne et al., 1999; Layne and Kuharsky, 2000; Layne and Peffer, 2006; Marshall and Sinclair, 2011). The elevation of hemolymph osmolality colligatively reduces the freezing point of the hemolymph and, thus, the amount of freezable body water at a given subzero temperature, a crucial factor in determining the lower limit of organismal freeze tolerance (Layne and Blakeley, 2002).

Numerous studies of insect freeze tolerance have examined the effects of low temperature acclimation for relatively short periods of days to weeks; however, few have investigated organismal responses over extended durations comparable to winter. Furthermore, the assessment of cold tolerance is often limited to whether an insect can survive a single low temperature for a fixed time period. While such an assessment is appropriate for many studies, it also has the potential to miss cold injury or acclimatory responses that are manifested only after extended periods of exposure or in response to cycles of freezing and thawing that occur commonly in temperate regions. Recent studies highlight the crucial importance of considering thermal variability and the consequences of repeated cold exposures for understanding low temperature acclimation and seasonal regulation of metabolic rate (Williams et al., 2012; Zhang et al., 2011). Likewise, only assessing organismal survival may miss subtle, sublethal injuries to tissues. Therefore, multiple measures of cold tolerance at both organismal and cellular levels of biological organization may provide a more nuanced understanding of changes during cold acclimation.

Consequently, our objective was to characterize organismal, cellular and biochemical responses to long-term acclimation at 5°C in a freezing-tolerant insect. To assess the effects of acclimation on freeze tolerance, we measured organismal survival after extended periods (1–3 weeks) of continuous freezing and after brief cycles of

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freezing and thawing because caterpillars are likely to experience subzero temperatures for various periods in winter. Special attention was paid to changes in hemolymph osmolality (as a proxy for the accumulation of low-molecular-mass solutes, such as glycerol), pH, and levels of free proline, total amino acids, proteins and cholesterol. In particular, we hypothesized that levels of free proline and total amino acids would increase during cold acclimation and contribute to cryoprotection via colligative and specific (i.e. non-colligative) mechanisms. We also tested the hypothesis that cholesterol levels would increase in membrane lipids to help maintain membrane fluidity and function at low temperature. Next, we examined the effects of nonlethal (−20°C) and lethal (−80°C) freezing on cellular viability and membrane leakage of proline, amino acids and proteins. Lastly, we tested whether glycerol, a major cryoprotectant in this species, protected tissues *in vitro* against freezing injury.

MATERIALS AND METHODS

Insect collection and laboratory acclimation

Approximately 200 woolly bear caterpillars were collected while they were crossing roads in Butler County, OH, from 21 October to 5 November 2001. Body mass was 2.11±0.20 g (mean±s.e.m., N=15) on the day of collection. Some caterpillars were used immediately for experimentation. The rest were placed onto three layers of paper towel in plastic containers with 2-cm thick of moist vermiculite on the bottom, and small holes in the lid, and kept at 22°C for 1 week without food. They were then transferred to a 15°C incubator for another week prior to acclimation to 5°C in a cold room until used. The vermiculite was moistened every 3 days and paper towels were changed every week. Under these conditions, no mortality occurred.

Temperature of crystallization

The T_c was determined as the lowest temperature recorded immediately before the spontaneous release of the latent heat of fusion as an insect freezes (Lee, 1991). Measurement of T_c was carried out by positioning a thermocouple on the surface of the larva in a 50 ml plastic tube suspended in a refrigerated bath (Neslab, model RTE-140, Portsmouth, NH, USA). Temperatures were recorded on a chart recorder connected to a multichannel thermocouple recorder (Omega, model RD3752, Stamford, CT, USA). Caterpillars (N=10–12) were cooled from 22 to −20°C at a rate of 1°C min^{-1}.

Cold-hardiness and freeze tolerance

For the two experiments described in this section, caterpillars were acclimated at 5°C for 18 weeks before use. In one experiment, three groups of five caterpillars were repeatedly exposed to cycles of freezing (22 h at −20°C) and thawing (2 h at 5°C) for 7 days. In a second experiment, four separate groups of 15 caterpillars were frozen at either −20°C for 1, 2 or 3 weeks or −80°C for 1 week only. Each caterpillar was placed in a plastic bag and all bags of caterpillars in a group were kept in a plastic box before being placed directly into freezers at −20±1 or −80±1°C.

After removal from the freezer, caterpillar survival was assessed at 30-min intervals over a 2-h period of thawing at 22°C under a dissecting microscope. Caterpillars that moved or curled in response to light touching were judged as having survived.

Hemolymph collection, osmolality and pH values

Hemolymph was collected individually from 10 larvae with a 100 µl micropipette and centrifuged immediately at 10,000 ***g***, 4°C for 15 min to remove hemocytes. The osmolality of 10 µl hemolymph from each larva was determined using a Wescor 5500 vapor pressure osmometer (Wescor, Logan, UT, USA). Values for hemolymph pH were measured with a surface-touching micro-combination pH electrode (Microelectrodes, Bedford, NH, USA) connected to an ion analyzer EA 920pH meter (Orion Research, Boston, MA, USA). For biochemical assays, the centrifuged hemolymph was diluted (1:1, v/v) with 0.005% 1-phenyl-2-thiourea to prevent oxidation, and stored at −20°C.

Tissue dissection and membrane preparation

Fat body (FB) and midgut (MG) tissues and Malpighian tubules (MT) were dissected in an ice-cold homogenization buffer containing 250 mmol l^{-1} sucrose and 5 mmol l^{-1} imidazole, pH 7.4 (Al-Fifi et al., 1998). Larvae were pinned dorsal-side up in a silicone elastomer (Dow Corning Sylgard 184)-filled Petri dish and opened with a midline incision. Each tissue was gently removed from the body after severing attached trachea, rinsed three times in the homogenization buffer and placed in a 1.5 ml microcentrifuge tube.

Tissues were either immediately homogenized in the same buffer plus 10% dimethyl sulfoxide or stored in 100 µl of the buffer at −20°C overnight. Membrane preparations were conducted with a procedure based on the methods described by Al-Fifi et al. (1998), Crockett and Hazel (1995a), Fogg et al. (1991) and Sørensen (1981, 1993). Briefly, tissues were first homogenized in a glass homogenizer with the homogenization buffer (1 mg tissue in 10 µl buffer), and then processed with an Ultrasonic Processor (Cole-Parmer Instrument Co., Vernon Hill, IL, USA) three times for 10 s with an amplitude setting at 40. All procedures were carried out on ice. The homogenate was centrifuged at 600 ***g***, 4°C in an Eppendorf centrifuge for 10 min to remove nuclei. The supernatant was then centrifuged at 135,000 ***g*** in a Beckman L5-50 B Ultracentrifuge for 20 min at 4°C to yield a supernatant and a membrane fraction. The membrane preparation was suspended by homogenization in an appropriate volume of homogenization buffer containing 10 mmol l^{-1} $MgCl_2$ and 0.025% Triton X-100 for cholesterol and protein assays.

In vitro fat body tissue culture and membrane leakage test

FB was dissected from five to 10 individual caterpillars that had been acclimated at 5°C for 18 weeks, and frozen for 1 week at −20 or −80°C. After dissection and rinsing, the FB was weighed and incubated in a 1.5 ml microcentrifuge tube with 0.5 ml Coast's solution (Coast, 1988) at 10°C for 24 h. Then, free proline, total amino acids and proteins were assayed in both cultured medium and FB homogenate after incubation.

To test whether glycerol can protect the FB from freezing damage, FB was dissected from five 5°C-acclimated insects and incubated individually in 0.5 ml Coast's solution with or without 1 mol l^{-1} glycerol while frozen at −20°C for 1 h followed by 10°C for 24 h. After incubation, free proline, total amino acids and proteins were also assayed in both culture medium and the FB.

Biochemical assays for cholesterol, free amino acids and proteins

The cholesterol was measured in hemolymph and both the supernatant and membrane fractions of FB, MG and MT as described by Yi and Lee (2005). Briefly, in separate 1.5 ml Eppendorf tubes, 1.0 ml of reagent (Cholesterol Assay Kit, Diagnostic Chemicals Limited, Oxford, CT, USA) and 10 µl of sample preparations were mixed. Standard and reagent blanks were prepared using 10 µl of cholesterol calibrator (Sigma Chemical Co., St. Louis, MO, USA) (as standard) or 10 µl of 0.01 mol l^{-1} Tris-HCl buffer, pH 7.2 (as blank). After incubation at room temperature for 20 min, the absorbance (optical density, OD) of the mixture was measured colorimetrically at 505 nm using the reagent blank as a background. Calculation of the cholesterol levels was performed by dividing the OD value of an unknown sample with the OD value of the calibrator and then by multiplying by a factor of 4.6 (concentration of the calibrator in mg 100 ml^{-1}).

The free proline and total amino acids were measured with ninhydrin solution (0.70 mol l^{-1} NaH_2PO_4, 0.30 mol l^{-1} Na_2HPO_4, 0.03 mol l^{-1} ninhydrin and 0.02 mol l^{-1} fructose) according to Cekic and Paulsen (2001) and Rosen (1957). A 0.2 ml sample, 0.8 ml deionized water and 1.0 ml ninhydrin solution were mixed in a Pyrex tube and heated in boiling water for 10 min. After cooling to room temperature, the contents were diluted with 5.0 ml of 10 mmol l^{-1} KIO_3 in 38% ethanol. Absorbance was measured at 440 nm for proline and 570 nm for other amino acids using L-proline and L-leucine as standards, respectively. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

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Protein concentrations in hemolymph, cultured media and tissue homogenates, and membrane preparations were determined using a Bio-Rad standard procedure (Bradford, 1976) with a reduced volume (1.02 ml) with bovine serum albumin as the standard.

Cell viability of foregut

Freeze tolerance of foregut (FG) cells was tested and compared among the 5°C-acclimated (18 weeks), −20°C-treated (1 week) and −80°C-treated (1 week) caterpillars. The viability of FG cells was assessed using a LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA) as we have described previously (Mugnano et al., 1998; Yi and Lee, 2003). Immediately after dissection, FG was cut into ∼2×2 mm square pieces and placed on a microscope slide containing 2 µl SYBR-14 dye mixed in 100 µl Coast's solution and incubated in a Petri dish at 22°C for 15 min, followed by the addition of 4 µl propidium iodide in 100 µl Coast's solution, and incubated for another 15 min. After the second incubation, the slide was covered with a cover slip and cells were examined with fluorescence microscopy (Olympus BH-2) (Davis and Lee, 2001; Yi and Lee, 2003). Live cells with intact cell membranes fluoresced bright green while cells with damaged membranes fluoresced red. For each of five individuals, 200 cells were counted and the mean rate of survival per individual was reported.

Cell viability was also tested in the FG after *in vitro* culture. FG tissues from five 5°C-acclimated (18 weeks) caterpillars were individually incubated in 0.5 ml Coast's solution with or without 1 mol l^{-1} glycerol at −20°C for 1 h followed by 10°C for 24 h. Vital dye assay procedure for the FG after incubation was the same as described above.

Data analysis

All data are expressed as means±s.e.m. Sample means were analyzed by ANOVA using SigmaPlot 12.5 (Systat Software, Richmond, CA, USA), followed by either Bonferroni–Dunn or Tukey's *post hoc* tests for multiple comparisons (Statview 5.1, SAS Institute, Cary, NC, USA). To evaluate cellular viability in the live/dead vital dye assays, we used R for one-way ANCOVA analysis (Marshall and Sinclair, 2011; Marshall et al., 2014). A value of $P<0.05$ between groups was considered a statistically significant difference.

RESULTS

Effects of cold acclimation at 5°C and freezing at −20°C

Newly field-collected caterpillars had a T_c of −7.7±0.5°C (N=15). This value decreased significantly to −9.5±0.6°C after acclimation at 5°C for 12 weeks ($P<0.05$). There was no further change of the T_c value from 12 to 18 weeks at 5°C.

After 18 weeks of acclimation at 5°C, all caterpillars survived and were highly tolerant of freezing at −20°C (Fig. 1). Recovery of motility was faster for caterpillars that had experienced fewer cycles of freezing (Fig. 1A). All caterpillars frozen for one or two cycles recovered within 0.5 h of thawing; however, 2 h were required for all individuals to recover after seven freeze–thaw cycles. No caterpillars that were frozen continuously for 1, 2 or 3 weeks recovered their motility within 30 min (Fig. 1B). After allowing 2 h of recovery time, the proportion of motile caterpillars was 100, 73 and 67% for caterpillars frozen for 1, 2 and 3 weeks, respectively; between 1 and 2 weeks of continuous freezing at −20°C, there was a significant decrease ($P<0.05$) in the survival rate, as judged by the return of motility (Fig. 1B). While motility was useful as a short-term assessment of survival and is appropriate for our study, it should be noted that it is not an ecologically rigorous measure of freeze tolerance.

Hemolymph pH and osmolality values in caterpillars changed significantly during acclimation at 5°C and freezing at −20°C. During acclimation at 15°C, hemolymph pH of newly collected caterpillars remained near 6.92 for 1 week (Fig. 2A). However, this value declined significantly (P=0.01, N=15) to 6.76 following 24 h at 5°C and continued to decrease for the next 4 weeks, reaching a minimum value of 6.58. From 4 to 18 weeks at 5°C, the pH

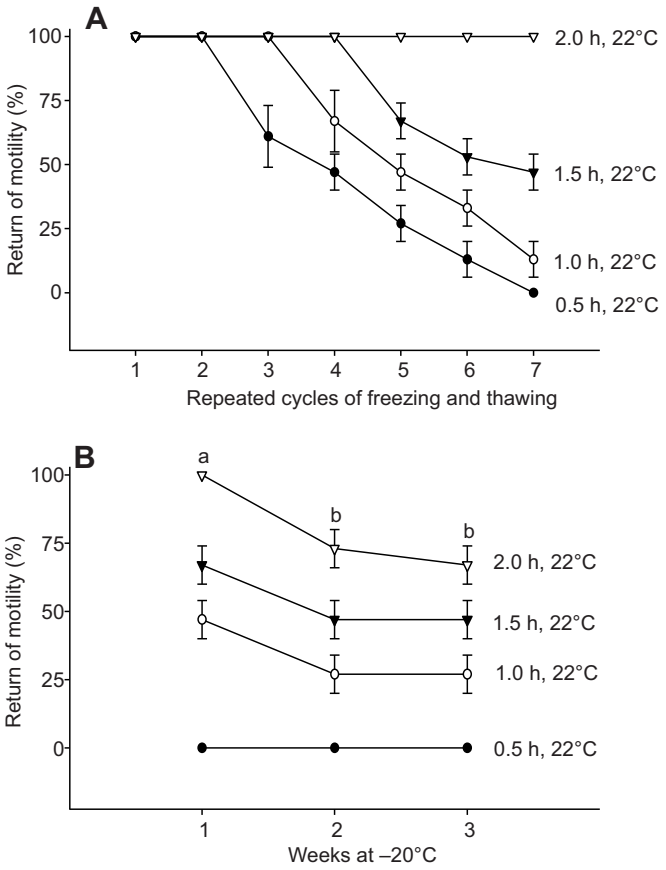

Fig. 1. Effects of multiple freeze–thaw cycles and prolonged freezing on survival of the banded woolly bear caterpillar. Survival (expressed as return of motility) of caterpillars after experiencing (A) repeated bouts of freezing (22 h at −20°C) and thawing (2 h at 22°C) or (B) prolonged freezing at −20°C for 1, 2, or 3 weeks. Following field collection, the caterpillars were acclimated in the laboratory (at 22°C for 1 week, then 15°C for 1 week, and finally 5°C for 18 weeks). Each point represents a mean±s.e.m. from three replicates of five caterpillars. Mean values that do not share common letters are significantly different from each other at $P<0.05$.

gradually increased to 6.91, a value similar to that of the newly collected caterpillars. Interestingly, the value further increased to 7.27 following 1 week of freezing at −20°C, and then declined abruptly to 7.00 and 6.58 after 2 and 3 weeks of freezing, respectively. The initial osmolality of the hemolymph was 364±12 mosmol kg^{-1}. Transfer to 5°C triggered a significant elevation in the osmolality, increasing by 44% within 24 h and continuing to increase to 1282±63 mosmol kg^{-1} after 4 weeks of acclimation to 5°C (Fig. 2B). The rapid increase in osmolality was inversely related to decreases in pH during this interval (Fig. 2A). After this period, the osmolality stabilized at ∼1250 mosmol kg^{-1} until transfer to −20°C, when the level decreased to 1023±34 mosmol kg^{-1} after 3 weeks.

Within the hemolymph and various other tissues, patterns of cholesterol levels varied during temperature acclimation. The initial value for hemolymph cholesterol was 1.60 mmol l^{-1} (Fig. 3). However, after 6 weeks of acclimation at 5°C, levels decreased by 34% to ∼1.0 mmol l^{-1}. The concentration further declined to 0.61 mmol l^{-1} when the insects were frozen at −20°C for 1 week, but rose to 0.79 and 1.37 mmol l^{-1} after 2 and 3 weeks at −20°C, respectively. In the FB and MG, a higher ratio of cholesterol to protein content (nmol mg^{-1}) was measured in the membrane fractions than in the supernatants (Fig. 3). In the FB, membrane cholesterol levels

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declined during both 5°C acclimation and during freezing at −20°C (P<0.05). Cholesterol levels in the MG and MT remained relatively constant in both membrane and supernatant fractions.

We examined free proline, total amino acid and protein concentrations in the hemolymph from caterpillars that were newly collected, acclimated to 5°C or frozen at −20°C. Proline was a major component of the hemolymph, accounting for 38–45% of the total free amino acids in the caterpillars (Fig. 4A). Compared with newly collected caterpillars, both total free amino acid and proline were significantly higher (by 38%) following 4 weeks of 5°C acclimation, and remained relatively constant throughout the remaining period of 5°C acclimation (18 weeks) and after 2 weeks of freezing at −20°C. However, a dramatic decrease (20 and 28%, respectively) occurred in both levels between 2 and 3 weeks of freezing. In contrast, protein concentration in the hemolymph (Fig. 4B) showed a different pattern, as newly collected caterpillars (201±14 g l^{-1}) and those frozen for 3 weeks (195±6 g l^{-1}) had higher levels than 5°C-acclimated caterpillars and those frozen for 1 or 2 weeks.

Effects of freezing at nonlethal (−20°C) and lethal (−80°C) temperatures

Although all caterpillars readily tolerated repeated freeze–thaw cycles and 1 week of freezing at −20°C (Fig. 1), none survived freezing at −80°C for 1 week. To compare organismal levels of cold tolerance with survival at the cellular level, we used a two-step vital fluorescent dye procedure used previously for assessing the cold tolerance of a variety of insect tissues (Yi and Lee, 2003). After screening a number of tissues in the caterpillars, we chose the foregut because it has relatively large cells that are readily counted

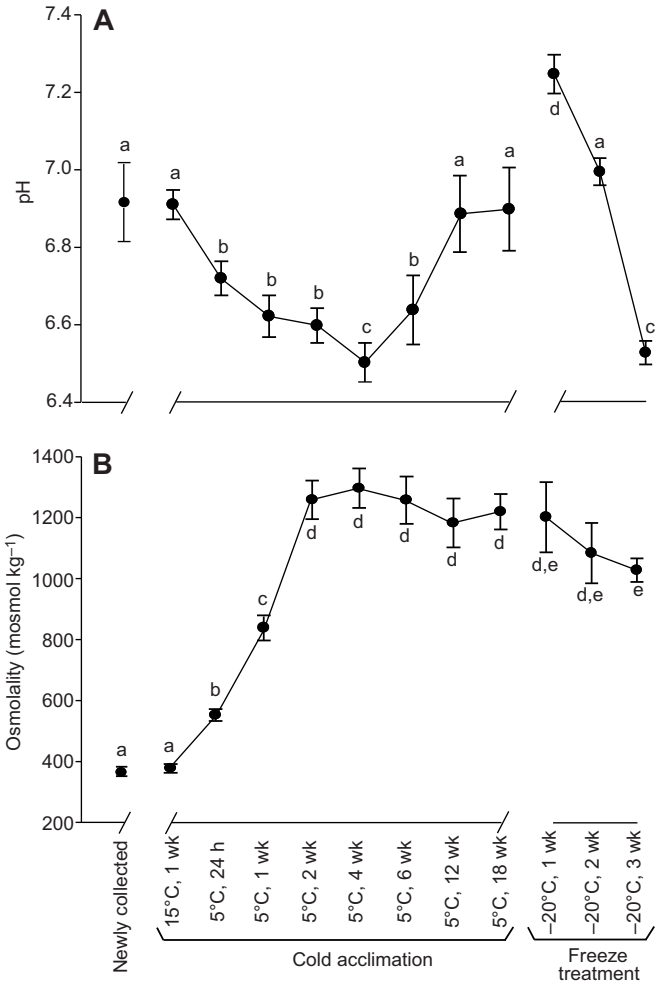

Fig. 2. Changes in hemolymph pH values and osmolality of the caterpillar. (A) Hemolymph pH values and (B) osmolality of newly collected, 15°C- and 5°C-acclimated, and −20°C-treated caterpillars. Data are presented as means± s.e.m. (N=10). Statistical significance is indicated as stated in Fig. 1.

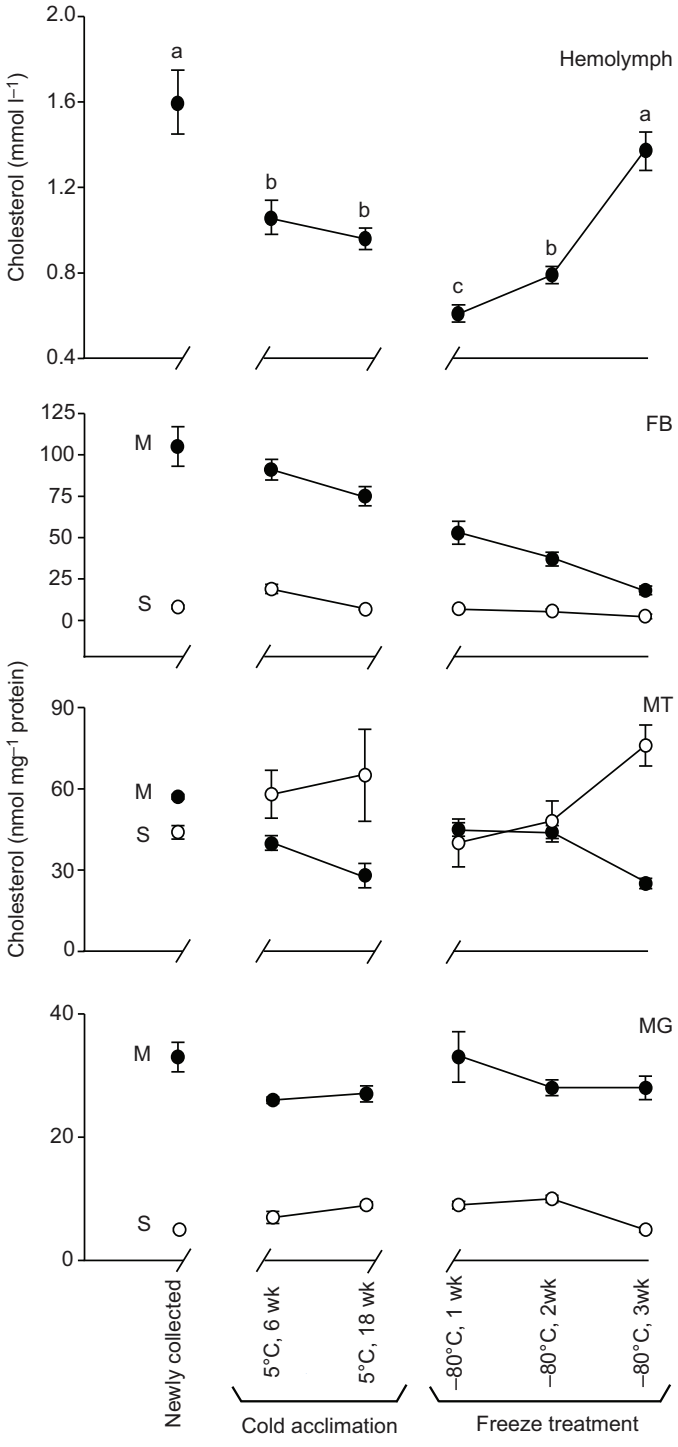

Fig. 3. Effects of cold acclimation and freeze treatment on cholesterol levels. Cholesterol concentration in hemolymph and tissue homogenates including supernatant (S, open symbols) and membrane fractions (M, solid symbols) of fat body (FB), Malpighian tubules (MT) and midgut (MG) of newly collected, 5°C-acclimated and −20°C-treated caterpillars. Data are presented as means±s.e.m. (N=10). Statistical significance is indicated as stated in Fig. 1.

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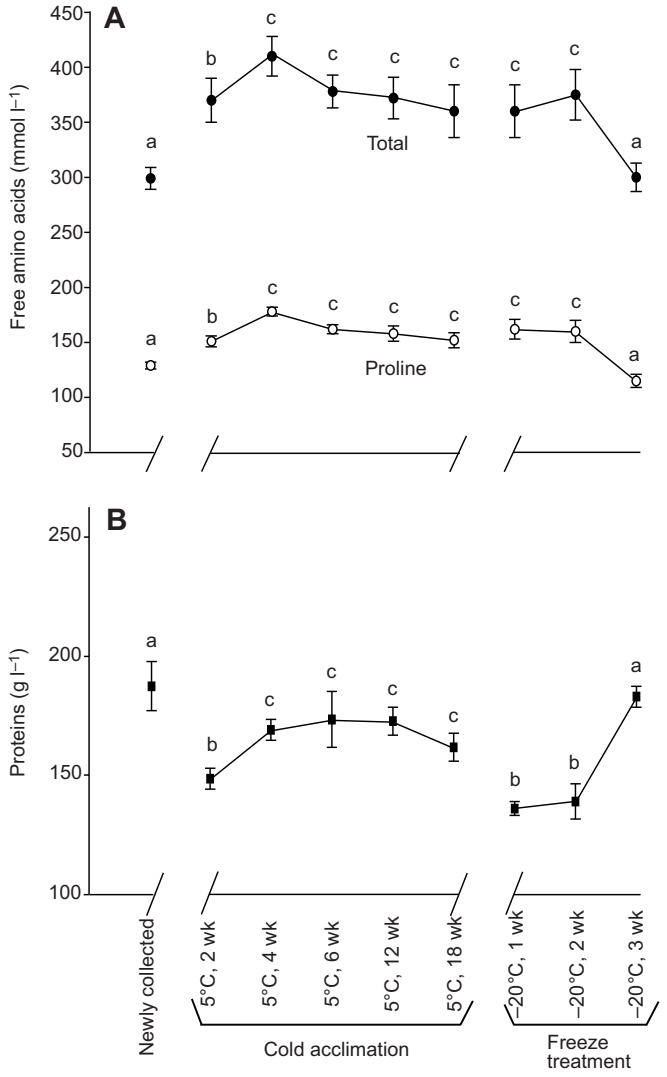

Fig. 4. Effects of cold acclimation and freeze treatment on concentrations of free amino acids and proteins in hemolymph. (A) Free proline and total amino acids, and (B) protein levels in the hemolymph of newly collected, 5°C-acclimated and −20°C-treated caterpillars. Data are presented as means± s.e.m. (*N*=10). Statistical significance is indicated as stated in Fig. 1.

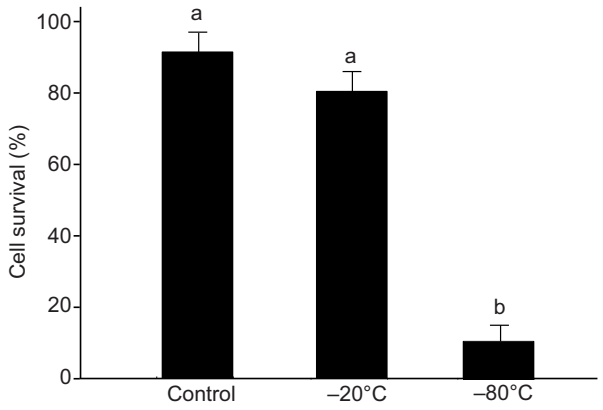

Fig. 5. Cellular viability of the foregut epithelium from 5°C-acclimated (18 weeks) control caterpillars, and from caterpillars frozen for 1 week at −20 or −80°C. For each of five individuals, 200 cells were counted and the mean rate of survival per individual was reported. Statistical significance is indicated as stated in Fig. 1.

and clearly react to the fluorescent dyes as either green (live) or red (dead). The survival rate (91.5±5.5%) of foregut cells in 5°C-acclimated (18 weeks) caterpillars did not differ from that of caterpillars frozen for 1 week at −20°C (80.5±5.5%); however, few cells survived freezing for 1 week at −80°C (12.5±4.5%; Fig. 5). Thus, organismal tolerance of freezing closely matched that of the foregut cells.

Because freezing injury is commonly manifested by damage to the cell membrane that results in leakage of cytoplasmic contents, we also monitored the effects of nonlethal and lethal freezing on the composition of hemolymph and FB. Hemolymph levels of free proline, total amino acids and proteins were indistinguishable between the control and −20°C groups; however, lethal freezing at −80°C significantly increased levels for all three constituents (Fig. 6A). To determine whether *in vivo* freezing damaged cell membranes, we carefully dissected FB from caterpillars in the control group (5°C acclimated for 18 weeks), and from those frozen for 1 week at either −20 or −80°C and then held *in vitro* for 24 h at 10°C. Free proline, total amino acids and proteins were significantly higher in the control and −20°C groups compared with the −80°C treatment (Fig. 6B), suggesting that these compounds had leaked from damaged cells into the hemolymph.

Cryoprotection by glycerol

Because overwintering caterpillars accumulate high levels of glycerol (Layne and Blakeley, 2002), which likely accounted for the increases in hemolymph osmolality shown in Fig. 2B, we directly tested the effect of glycerol in preventing cryoinjury at the cellular level using foregut epithelia. Excised foreguts were individually held in 0.5 ml of Coast's solution with or without 1 mol l^{-1} glycerol for 1 h at −20°C followed by 24 h at 10°C before viability was assessed using the vital dye assay. Few cells (26.8±8.1%) could survive even 1 h of freezing *in vitro* at this temperature. In contrast, addition of glycerol significantly (P<0.05) enhanced cold tolerance, as evidenced by a survival rate of 68.9±5.8%.

Cryoprotection by glycerol was also investigated by testing its effect on leakage across the cell membrane (Fig. 7). FBs isolated from caterpillars acclimated at 5°C for 18 weeks were cultured in Coast's solution, with or without 1 mol l^{-1} glycerol, and either placed directly at 10°C for 24 h (control) or frozen at −20°C for 1 h prior to a 24-h incubation at 10°C. Following incubation, free proline, total amino acids and proteins in the culture medium were measured. The addition of glycerol at 5°C had no effect on leakage for any constituent. When compared with the unfrozen controls at 5°C, significantly more free proline (47%, Fig. 7A), total amino acids (67%, Fig. 7B) and proteins (33%, Fig. 7C) were released to the medium by the freezing-treated tissues. The addition of 1 mol l^{-1} glycerol to the medium before freezing significantly reduced leakage of free proline, total amino acids and proteins compared with the control (Coast's only) treatment (Fig. 7).

DISCUSSION

Our purpose in this study was to characterize the effects of long-term cold acclimation at multiple levels of biological organization in a freezing-tolerant insect. At the organismal level we measured the capacity to tolerate freezing for extended periods of up to 3 weeks. We also determined their tolerance of brief cycles of freezing and thawing that often occur within their hibernacula throughout the winter. To better understand the role of underlying mechanisms influencing seasonal cold-hardening, we concurrently

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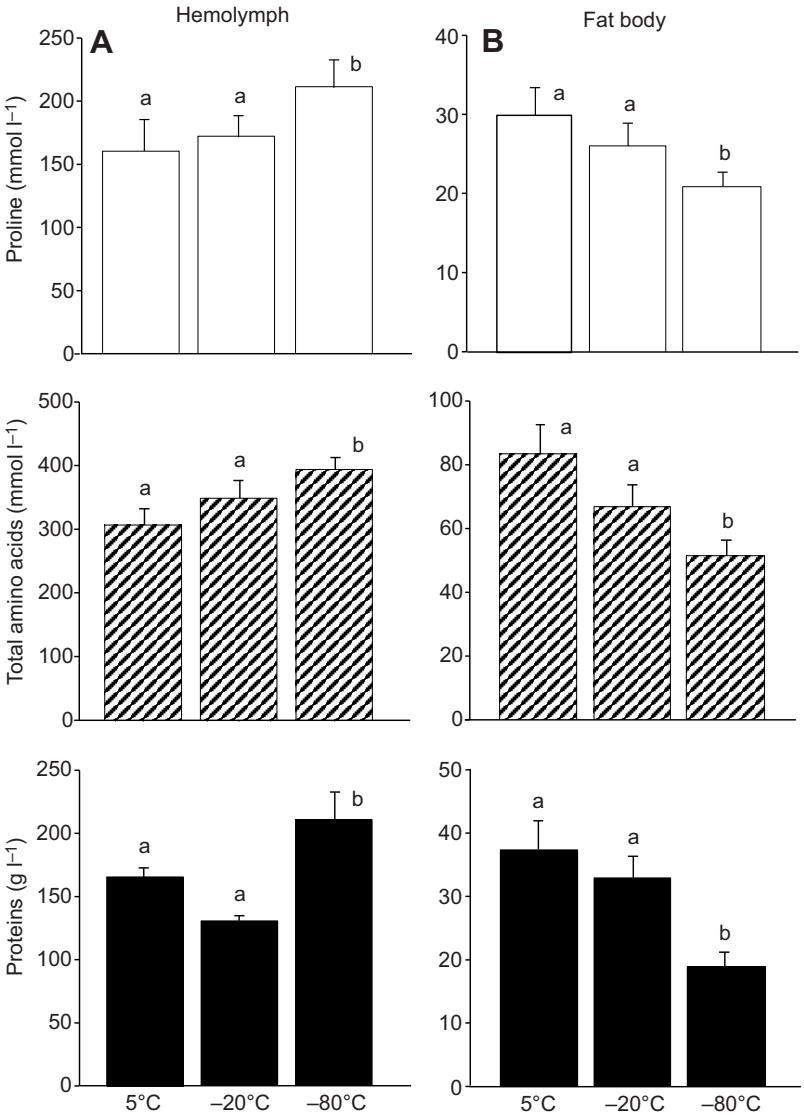

Fig. 6. Leakage of amino acids and proteins from freeze-damaged fat body tissues into the hemolymph. Comparison of free proline, total amino acid and protein concentrations in the (A) hemolymph and (B) fat body from caterpillars acclimated for 18 weeks at 5°C, or frozen at −20 or −80°C. Data are presented as means± s.e.m. (*N*=10). Statistical significance indicated as stated in Fig. 1.

monitored a relatively large number of cellular and biochemical parameters known (or likely) to be associated with freezing tolerance. We used changes in hemolymph osmolality as a proxy for the accumulation of cryoprotectants, such as glycerol, that are well known to promote freeze tolerance in this species and many other insects (Lee, 2010). At the same time, we measured other parameters (pH, and levels of free proline, total amino acids, protein and cholesterol) that are likely to be important but are often not measured or not measured in the same study as other parameters. To gain a better understanding of the consequence of freezing to nonlethal (−20°C) and lethal (−80°C) temperatures, we tested for leakage of proline, amino acids and proteins across cell membranes *in vivo* and *in vitro*. Finally, we tested whether glycerol protected fat body against freezing injury.

Freeze tolerance after 18 weeks of cold acclimation

Previous studies of *P. isabella* characterized the cold tolerance of woolly bear caterpillars at various temperatures and durations of exposure. Layne and Peffer (2006) reported that nearly all caterpillars survived 6 weeks of freezing at −4.5°C. A similar rate of survival was found after 1 week of freezing at −10°C (Layne and Blakeley, 2002). In another study, all caterpillars survived 1–2 weeks of freezing at −14°C but not 3–6 weeks, nor did they survive freezing at −30°C (Boardman et al., 2011). We found that after 18 weeks of cold acclimation, all caterpillars exhibited motility (a measure of short-term survival that is unlikely to be an ecologically rigorous assessment of freeze tolerance) after being frozen for 7 days at −20°C; however, significantly fewer caterpillars (~70%) survived freezing for 2 or 3 weeks (Fig. 1B) and, unsurprisingly, none survived freezing at −80°C, a temperature they would not naturally experience.

Caterpillars hibernate beneath leaf litter, which is sometimes covered by snow, where they experience repeated cycles of freezing and thawing (Layne et al., 1999). Following long-term acclimation, all caterpillars regained motility after seven cycles of freezing and thawing (Fig. 1A). The capacity to survive frequent freezing and thawing is likely to be especially important as these cycles and accompanying temperature excursions are expected to be exacerbated by climate change (Marshall and Sinclair, 2012; Williams et al., 2015).

An important caveat with respect to our results, and to the data from many other studies of this type, concerns the criterion used for assessing survival after freezing (Lee, 1991). We judged that caterpillars had survived if they regained motility within 2 h of

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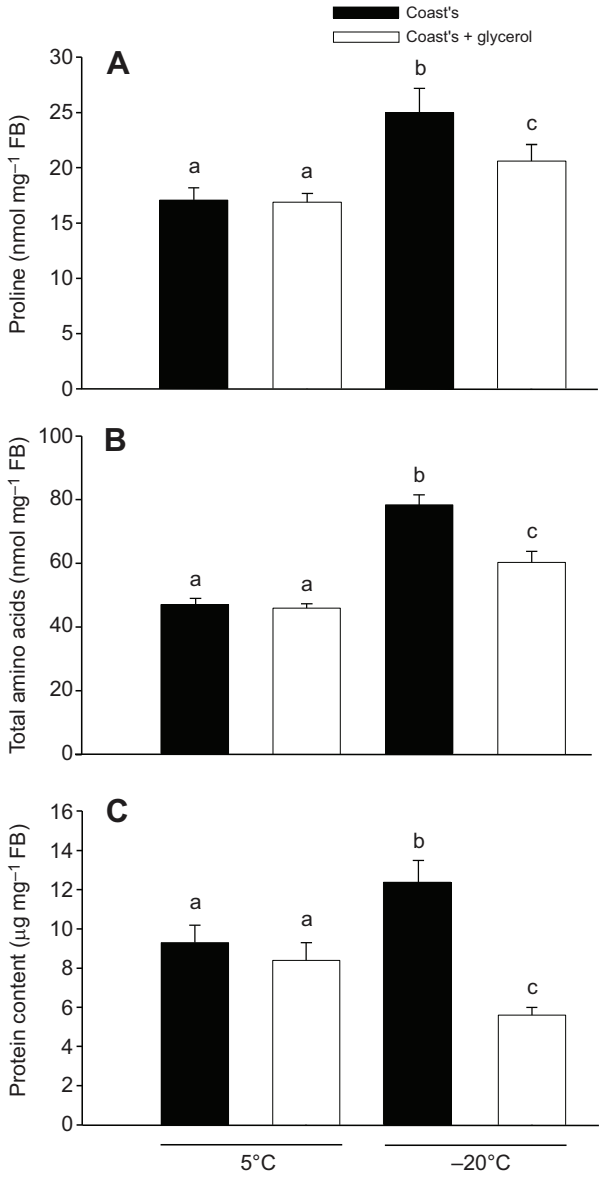

Fig. 7. Cryoprotection by glycerol in the culturing medium of the fat body cells. Cryoprotective effect of glycerol on cell leakage of (A) free proline, (B) total amino acids and (C) proteins using fat body frozen *in vitro* from 5°C-acclimated (18 weeks) caterpillars. For each treatment, fat body samples from five insects were incubated individually in 0.5 ml of Coast's solution with or without 1 mol l^{-1} glycerol. Statistical significance indicated as stated in Fig. 1.

thawing. Clearly, this criterion does not reflect differences in sublethal effects of freezing. For example, after two freeze–thaw cycles, all caterpillars recovered motility within 30 min of transfer to 22°C (Fig. 1A). In contrast, although all caterpillars frozen for seven cycles recovered motility within 2 h, none recovered within 30 min, and only 50% within 1.5 h. Marshall and Sinclair (2011) also found that survival of *P. isabella* caterpillars decreased, and tissue damage in hemocytes and Malpighian tubules increased, with multiple cycles of freezing and thawing. Interestingly, these authors reported that repeated cycles of freezing appeared to upregulate the immune system, a response that may be important in promoting winter survival. Numerous studies of freeze tolerance have observed delayed mortality or an inability to successfully molt to the next life stage (Bennett and Lee, 1997; Layne and Blakeley, 2002; Marshall and Sinclair, 2011), which may be due to various causes including reduced energy charge (Churchill and Storey, 1989), tissue damage due to freezing (Sinclair and Chown, 2005; Marshall and Sinclair, 2011), inability to regain ion homeostasis (Boardman et al., 2011) and excessive oxidative stress (Doelling et al., 2014).

Changes in hemolymph osmolality, pH, amino acids and proteins

In addition to polyhydric alcohols and sugars, insects accumulate proteins and free amino acids such as proline and alanine during cold-hardening (Storey et al., 1981). Besides their colligative role in cryoprotection, certain low molecular weight solutes, such as trehalose and proline, also function to stabilize and protect membrane structure during freezing and thawing (Crowe et al., 1983; Rudolph and Crowe, 1985). In a previous study, Layne and colleagues (1999) reported that the hemolymph osmolality of field-collected *P. isabella* caterpillars throughout autumn increased approximately two-fold, reaching ∼720 mosmol kg^{-1}, with 26% of the osmolality accounted for by glycerol. In another study, hemolymph osmolality increased by 45% (from 445 to 647 mosmol kg^{-1}) after only 4 days at 5°C, following field collection in late October, and reached a high of 747 mosmol kg^{-1} after 28 days of observation (Layne and Kuharsky, 2000). Repeated cycles of freezing or continuous freezing at −12°C substantially increased the glycerol levels of *P. isabella* caterpillars (Marshall and Sinclair, 2011).

Here, we found a similar increase: hemolymph osmolality increased by 44% from 389 to 559 mosmol kg^{-1} after 24 h at 5°C, and continued to increase to 1282 mosmol kg^{-1} (another 129% increase) after 4 weeks of 5°C acclimation (Fig. 2B). A striking amount of this increase was due to the accumulation of proline and other free amino acids, accounting for ∼16% of the increase in hemolymph osmolality (Fig. 4A). Recent reports highlight the importance of proline for insect freezing tolerance. Proline is a crucial metabolite, accumulated to levels of 147 mmol l^{-1} during cold-hardening in the drosophilid fly *Chymomyza costata*, which can survive freezing in liquid nitrogen (Koštál et al., 2011). Remarkably, the addition of proline to the diet of a chilling-intolerant fly confers the capacity to survive freezing (Koštál et al., 2012).

Few studies have investigated the effect of low temperature on pH in insects. In the freeze-tolerant gall fly, larvae exposure to subzero temperatures caused an increase in intracellular pH; however, it did not affect the extracellular pH, which remained between 6.1 and 6.3 (Storey et al., 1984). In the present study, hemolymph pH values decreased steadily from nearly neutral (6.92) to acidic (6.58) during the first 4 weeks of 5°C acclimation, before reversing this pattern and returning to 6.91 after 18 weeks of acclimation (Fig. 2A). After 4 weeks of acclimation, pH values began to increase at the same time as hemolymph osmolality (Fig. 2B) and the accumulation of free amino acids and proteins (Fig. 4) reached a plateau, perhaps signifying a coordinated shift in metabolism. A variety of factors are potentially involved in the observed pH changes, including direct temperature effects related to alphastat regulation, and accumulation of lactate or other anaerobic compounds associated with metabolic depression and freezing (Storey et al., 1984; Harrison, 1988).

When caterpillars were frozen at −20°C for 1 week, the pH increased significantly from 6.91 to 7.27, but longer-term freezing resulted in a marked reduction in values that reached an acidity of 6.58 (Fig. 2A). This decrease may be associated with the production of lactate during anaerobic glycolysis, as occurs in frozen larvae of *Eurosta solidaginis* (Storey and Storey, 1985). This decrease in pH may also be associated with increased sublethal injury due to freezing (Fig. 1), as suggested by Layne and Kennedy (2002).

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Cholesterol's role in cold resistance and its liberation to the hemolymph by freezing

Cholesterol is an important membrane lipid and a precursor for the synthesis of steroid hormones in insects (Waterman, 1995). Because insects cannot synthesize cholesterol, it must be obtained in the diet. Evidence from diverse sources suggests that cholesterol promotes retention of membrane fluidity at low temperature (Crockett, 1998; Yeagle, 1991). High cholesterol levels stabilize membranes during cooling; human sperm, which naturally contain high levels of cholesterol, are highly resistant to cold shock (Drobnis et al., 1993). In addition, cold acclimation increases cholesterol levels in the brush border membranes of trout intestinal epithelia (Crockett and Hazel, 1995a), and adding cholesterol to the diet of *Drosophila melanogaster* decreases susceptibility to cold-shock injury (Shreve et al., 2007).

In the present study, hemolymph cholesterol levels decreased significantly throughout 5°C acclimation and after 1 week of freezing at −20°C, but returned to levels similar to those in newly collected caterpillars when freezing was extended to 2 or 3 weeks (Fig. 3). Concomitantly, the ratio of cholesterol to protein in the membrane fraction of fat body decreased more than 6.5-fold (from 105 to 16 nmol mg^{-1} protein). These results contrast with data from a similar study of seasonal acclimatization in a freeze-tolerant gall fly larvae (*E. solidaginis*) in which hemolymph cholesterol increased four-fold during cold-hardening from September to November, and freezing at −20°C or −80°C decreased cholesterol levels in the hemolymph (Yi and Lee, 2005). Thus, contrary to our prediction that cholesterol levels would increase during cold acclimation, patterns of cholesterol regulation varied markedly among tissues and no simple pattern emerged (Fig. 3). Similar variability is often found among tissues, and between species, of ectotherms during low-temperature acclimation and freezing (Crockett and Hazel, 1995b).

Freezing injury and cryoprotection

Damage to cell membranes is a primary cause of freezing injury, often detectable by the release of intracellular solutes including amino acids (Siminovitch et al., 1964). To test whether freezing causes leakage of cytoplasmic solutes, we examined free proline and total amino acid as well as protein levels in both hemolymph (*in vivo*) and cultured medium (*in vitro*) of fat bodies from woolly bear caterpillars frozen at −20 and −80°C. All caterpillars survived freezing at −20°C, while none survived freezing at −80°C; corresponding rates of survival were observed at the cellular level (Fig. 5). Lethal freezing at −80°C resulted in significantly higher levels of proline, amino acids and proteins in the hemolymph than at −20°C, suggesting that the release of these compounds was due to cryoinjury (Fig. 6A). A reciprocal pattern was observed in fat body cells frozen at −80°C; these cells had significantly lower levels of proline, amino acids and proteins than those frozen at −20°C (Fig. 6B).

Cytoplasmic leakage of protein due to freezing injury may also have occurred during freezing for 3 weeks at −20°C. Hemolymph levels of proline, amino acids and proteins were similar after 1 or 2 weeks of freezing at −20°C (Fig. 4). However, protein levels increased significantly between 2 and 3 weeks of −20°C freezing (Fig. 4B) at the same time caterpillar survival rates decreased to 67% (Fig. 1B). Hemolymph pH also declined abruptly after 2 and 3 weeks of freezing (Fig. 2A), which is consistent with progressive injury due to freezing. Sublethal freezing damage can accumulate gradually over days to weeks, even though injury is not obvious at the organismal level in this and other species (Layne and Blakeley, 2002; Marshall and Sinclair, 2010). In contrast, free amino acid levels did not rise in the hemolymph but decreased significantly, even though one might expect small amino acids to leak from damaged cells more readily than proteins.

Because large amounts of glycerol are produced during cold-hardening in *P. isabella* caterpillars (Layne and Blakeley, 2002; Marshall and Sinclair, 2011), we tested whether this common cryoprotectant protected fat body cells against freezing injury. When glycerol was added to the bathing medium of fat body cells frozen at −20°C, leakage of amino acids and proteins was significantly reduced compared with controls (Fig. 7). A similar cryoprotective effect occurs in fat body cells of *E. solidaginis* (Lee et al., 1993) and in the freezing-intolerant flesh fly *Sarcophaga crassipalpis* (Davis and Lee, 2001).

In conclusion, after long-term acclimation at 5°C, caterpillars exhibited high levels of recovery at −20°C after 1 week; however, extending the duration freezing by 2 or 3 weeks negatively affected recovery (Fig. 1B). A similar increasing pattern of impairment was evident when caterpillars were subjected to repeated cycles of freezing and thawing (Fig. 1A). Taken together, these results suggest that although cold-hardy insects can tolerate long periods of freezing and repeated freezing and thawing, dramatic and unpredictable variations in winter conditions and temperatures caused by anthropogenic climate change may exceed survival limits for some species (see review by Williams et al., 2015).

Early in the cold acclimation regimen, increases in free proline, total amino acids and proteins contributed to an overall elevation and plateau in hemolymph osmolality. In contrast, hemolymph pH values and cholesterol levels in different tissues exhibited distinct trends during acclimation and in response to extended periods of freezing. Future study of the factors regulating these changes is likely to provide a better understanding of freeze tolerance in this species and other wintering insects.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.-X.Y. and R.E.L. conceived and designed the study. S.-X.Y. collected and analyzed the data. S.-X.Y. and R.E.L. interpreted the data and wrote the manuscript.

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