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RESEARCH ARTICLE

Survival and energetic costs of repeated cold exposure in the Antarctic midge, *Belgica antarctica*: a comparison between frozen and supercooled larvae

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SUMMARY

In this study, we examined the effects of repeated cold exposure (RCE) on the survival, energy content and stress protein expression of larvae of the Antarctic midge, *Belgica antarctica* (Diptera: Chironomidae). Additionally, we compared results between larvae that were frozen at –5°C in the presence of water during RCE and those that were supercooled at –5°C in a dry environment. Although >95% of larvae survived a single 12 h bout of freezing at –5°C, after five cycles of RCE survival of frozen larvae dropped below 70%. Meanwhile, the survival of control and supercooled larvae was unchanged, remaining around 90% for the duration of the study. At the tissue level, frozen larvae had higher rates of cell mortality in the midgut than control and supercooled larvae. Furthermore, larvae that were frozen during RCE experienced a dramatic reduction in energy reserves; after five cycles, frozen larvae had 25% less lipid, 30% less glycogen and nearly 40% less trehalose than supercooled larvae. Finally, larvae that were frozen during RCE had higher expression of *hsp70* than those that were supercooled, indicating a higher degree of protein damage in the frozen group. Results were similar between larvae that had accumulated 60 h of freezing at –5°C over five cycles of RCE and those that were frozen continuously for 60 h, suggesting that the total time spent frozen determines the physiological response. Our results suggest that it is preferable, both from a survival and energetic standpoint, for larvae to seek dry microhabitats where they can avoid inoculative freezing and remain unfrozen during RCE.

Key words: Antarctic midge, energy reserves, freeze-tolerance, heat shock protein, repeated cold exposure.

INTRODUCTION

The Antarctic midge, *Belgica antarctica* Jacobs 1900, is the southernmost insect (Sugg et al., 1983) and is found exclusively on the west coast of the Antarctic Peninsula and its islands. The midge has a 2 year life cycle, during which larvae feed primarily on the algae *Prasiola crispa*, mosses such as *Drepanocladus uncinatus*, microorganisms and detritus in nutrient-enriched substrate near seal colonies and bird nesting sites (Convey and Block, 1996). Adults emerge during a brief period in early summer, mate, oviposit and die within 10 days. *B. antarctica* is apterous, a common adaption in wind-swept polar areas. This species has a sporadic, albeit locally abundant, distribution on the Antarctic Peninsula.

Not surprisingly, given its habitat, larvae of *B. antarctica* are extremely tolerant of a number of environmental stresses; larvae can survive freezing to –15°C, loss of 70% of body water by desiccation, anoxia, long periods of immersion in freshwater and saltwater, and a broad range of pH conditions (Baust and Lee, 1987). Although air temperatures on the Antarctica Peninsula reach winter lows of –40°C, the microhabitat of *B. antarctica* is thermally buffered by snow and ice, so that the microhabitat temperature rarely dips below –5°C (Baust and Lee, 1981; Elnitsky et al., 2008). Larvae remain freeze tolerant year-round and can increase their freeze tolerance by undergoing rapid cold hardening (Lee et al., 2006). Furthermore, under laboratory conditions, *B. antarctica* larvae are capable of cryoprotective dehydration, in which gradual chilling to –3°C in the presence of ice causes larvae to lose 40% of their body water and remain unfrozen at subzero temperatures (Elnitsky et al., 2008).

In general, all insects can be classified as either freeze tolerant or freeze avoiding (Lee, 2010). Whereas freeze-tolerant insects can survive internal ice formation, freeze-avoiding insects must remain supercooled at subzero temperatures. However, the evolutionary forces that drive insects to adopt a particular strategy remain unclear. Freeze tolerance tends to predominate in the Southern Hemisphere, where higher climatic variability requires insects to be able to survive internal ice nucleation when a sudden cold snap arrives (Sinclair et al., 2003; Sinclair and Chown, 2005a). Also, the water balance characteristics of a species tend to drive its cold-tolerance strategy; high transcuticular water permeability is often associated with freeze tolerance, as freezing is a means of conserving body water in the winter (Zachariassen et al., 2008). In the case of *B. antarctica*, larvae are clearly freeze tolerant (Baust and Edwards, 1979), but during gradual chilling some larvae can avoid inoculative freezing and undergo cryoprotective dehydration, particularly when the soil moisture content is low (Elnitsky et al., 2008). These larvae remain unfrozen at subzero temperatures for 14 days and nearly all survive, suggesting that cryoprotective dehydration is a viable means of long-term cold survival. Additionally, provided they avoid contact with environmental ice, larvae could remain supercooled during a sudden, acute exposure to cold, as the minimum microhabitat temperature (i.e. approximately –5°C) rarely drops below the supercooling point (approximately –7°C). However, it has not been empirically

addressed whether freezing or supercooling is the preferred means of acute low temperature survival.

Moreover, although previous studies on stress tolerance in *B. antarctica* examined a single stress exposure (e.g. Baust and Lee, 1987; Benoit et al., 2007), in nature larvae are exposed to repeated bouts of environmental stress. In particular, during late summer and early winter, microhabitat temperatures fluctuate numerous times across the larvae's freezing point (Elnitsky et al., 2008). In recent years, several studies have addressed the effects of repeated cold exposure (RCE) in insects. Typically, for freeze-avoiding insects, RCE allows for better survival in comparison to a single long-term exposure, because insects can repair damage, such as disruption of ion homeostasis, accrued during the cold exposure (Kostal et al., 2007). However, in the few studies that have looked at RCE in freeze-tolerant insects, the opposite appears to be true. In the freeze-tolerant hoverfly *Syrphus ribesii*, most larvae can only survive a single freezing event, and switch to a freeze-avoiding strategy thereafter (Brown et al., 2004). Similarly, in the freeze-tolerant caterpillar *Pringleophaga marioni*, repeated freeze–thaw cycles are deleterious, perhaps because of accumulated damage to sensitive tissues such as the midgut epithelium (Sinclair and Chown, 2005b). In addition to being ecologically relevant, studies of RCE can also reveal novel adaptations for cold survival. For example, in the parasitic wasp *Aphidius colimani*, the proteome of wasps exposed to RCE was distinct from those exposed to a single bout of low temperature (Colinet et al., 2007).

In this study we examined the effects of RCE in *B. antarctica* larvae. Furthermore, results were compared between larvae that were frozen and those that were supercooled, by exposing them to cold in the presence and absence of water, respectively. In particular, we focused on both the survival costs of RCE as well as other physiological consequences, including tissue damage, energy reserve depletion and stress protein expression. Insects have a suite of physiological adaptations to cold (Clark and Worland, 2008), but these adaptations are energetically expensive. In particular, recovery from cold exposure can be costly; therefore, measuring key energy reserves in response to cold is an indirect measure of both the amount of damage accumulated and the fitness costs of that damage, as energy is diverted from growth and reproduction (Marshall and Sinclair, 2010). Additionally, we measured the expression of heat shock protein 70 (*hsp70*) as a biomarker of cellular stress during RCE. Heat shock proteins, as well as other stress proteins, are essential for repairing misfolded proteins during the recovery phase (Kostal and Tollarova-Borovanska, 2009). This is the first study to examine multiple stress exposure and the consequences of freezing *versus* supercooling in *B. antarctica*.

MATERIALS AND METHODS

Experimental animals

In January 2010, larvae were collected on Cormorant Island, Humble Island and Norsel Point near Palmer Station on the Antarctic Peninsula (64°46′S, 64°04′W). For experiments conducted at Palmer Station, larvae were immediately hand-picked from the substrate in ice water and placed on wet filter paper at 4°C overnight prior to the experiments. Additionally, samples of substrate were shipped chilled (~0°C for 7 days) to Ohio State University and stored at 4°C for additional experiments.

Experimental conditions

For the RCE treatments, larvae were exposed to one to five diurnal cycles of 12 h at –5°C followed by 12 h at 4°C, 100% relative humidity (RH). Additionally, to compare the effects of RCE to a single prolonged exposure to cold, separate groups of larvae were exposed to –5°C for 60 h, because after five cycles of RCE, larvae have accumulated 60 h at –5°C. These treatment groups were also given 12 h of recovery at 4°C, 100% RH. Meanwhile, control animals were held at 4°C, 100% RH for the duration of the study. Larvae in the 'frozen' groups were submerged in ~50 µl of double-distilled H_2O prior to the –5°C exposure to promote inoculative freezing (Lee et al., 2006). After exposure to –5°C, tubes were thawed and excess water was removed to prevent overhydration during recovery (Lopez-Martinez et al., 2009). Larvae in the 'supercooled' groups were gently blotted dry on an absorbent tissue prior to the experiment and placed in dry centrifuge tubes. To assure that larvae could remain supercooled for 12 h at –5°C, a subset of larvae were placed in direct contact with a thermocouple at –5°C and monitored for 12 h for the presence of a freezing exotherm. Out of 12 larvae tested, only two froze, indicating that nearly all larvae remain supercooled at –5°C for 12 h (data not shown).

Whole-animal survival

At the end of the 12 h recovery phase after each of five RCE cycles, larvae were assessed for survival. Concurrently, survival was measured each day for control larvae that were held continuously at 4°C. Groups of 15 larvae were placed in a drop of water under a microscope, and larvae that either moved spontaneously or in response to gentle prodding were considered to be alive. The mean survival for each treatment group is based on five replicates of 15 larvae each.

Cell survival of midgut tissue

To test for sublethal damage to tissues during RCE, the cell survival of midgut tissue was assessed. The midgut tissue of *B. antarctica* is particularly sensitive to cold (Lee et al., 2006; Teets et al., 2008), making it a suitable tissue for this experiment. After each of five cycles, midguts from control, frozen and supercooled larvae were dissected in Coast's solution (Coast and Krasnoff, 1988) and cell survival was assessed using the LIVE/DEAD sperm viability kit (Invitrogen, Carlsbad, CA, USA), as adapted by Yi and Lee (Yi and Lee, 2003). Only living, non-moribund larvae were dissected for cell survival determination. Tissues were stained and images were taken *via* fluorescent microscopy at the Campus Microscopy and Imagining Facility, Ohio State University. Living cells with intact membranes fluoresced green whereas dead cells with damaged membranes fluoresced red. Cell survival for each replicate was based on the mean count of three groups of 100 cells. For each treatment, four replicates were conducted.

Metabolite assays

To determine the effects of RCE on energy stores, colorimetric assays for lipid, glycogen, trehalose and glucose were conducted. Glycerol, a common cryoprotectant and carbon source in cold-acclimated insects, was also measured. After each of five RCE cycles, five replicates of 50 larvae from each treatment (i.e. control, frozen and supercooled) were quickly frozen at –80°C and shipped on dry ice to Ohio State University for biochemical analysis. Samples were thawed one at a time, and five larvae were set aside for lipid analysis. The remaining 45 larvae were blotted dry, weighed and homogenized in 7% perchloric acid. The homogenates were centrifuged at 14,000 × $\boldsymbol{g}$, and the supernatant was neutralized with 0.789 mol l^{-1} KOH. Neutralized extracts were stored at –70°C until analysis.

Lipid content was measured according to Sim and Denlinger (Sim and Denlinger, 2009), using vanillin reagent. Briefly, groups of five

larvae were homogenized in 1:1 chloroform:methanol, the solvent was evaporated and vanillin reagent was added to the sample. The absorbance of the solution was read at 490 nm in a NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) and compared with known standards. Glycogen content was measured according to Kepler and Decker (Kepler and Decker, 1984). Amyloglucosidase from *Aspergillus niger* (A1602, Sigma, St Louis, MO, USA) was used to liberate free glucose in the homogenate, and the glucose concentration was measured using a glucose assay kit (GAGO20, Sigma) by measuring the absorbance at 540 nm. The glycogen content was calculated by subtracting the free glucose content from the enzyme-digested glucose content and comparing this value with standards of known glycogen concentration. Trehalose content was also determined enzymatically, according to Chen et al. (Chen et al., 2002). Trehalose was hydrolyzed using trehalase from porcine kidney (T8778, Sigma), and the liberated glucose concentration was compared with trehalose standards, correcting for free glucose concentration. Finally, glycerol content was measured using Free Glycerol Reagent (F6428, Sigma). The suggested ratio of homogenate to reagent was increased by ~10× to get a signal, but otherwise the assay was conducted according to the manufacturer's protocol. For each metabolite measured, the content is expressed as μg metabolite mg^{-1} dry mass (DM).

Energy content calculations

After conducting assays for the major energy reserves, the total energy content (TEC) of the major energy reserves (lipid, glycogen, trehalose and glucose) was calculated according to Djawdan et al. (Djawdan et al., 1998). The formula for TEC is TEC=0.393(lipid content) + 0.176TCC, where TEC is in J mg^{-1} DM, lipid content is in μg lipid mg^{-1} DM and TCC is total carbohydrate content in μg carbohydrate mg^{-1} DM, and is the sum of glycogen, trehalose and glucose content. Additionally, as the TEC was dominated by lipids, the most abundant energy reserve, we also separately calculated the carbohydrate energy content (CEC), in J mg^{-1} DM, using the following formula: CEC=0.176TCC.

Northern blot hybridization

To further assess the level of cellular stress caused by RCE, we measured *hsp70* expression during RCE. Clones for *hsp70* and *28s* rRNA were obtained from previously reported sequences (Rinehart et al., 2006). For *hsp70,* the following primers were used: forward primer 5′-GATGCAGTCATCACAGTTCCAGC-3′ and reverse primer 5′-AACAGAGATCCCTCGTCGATGGT-3′. For *28s*, the following primers were used: forward primer 5′-ACTTGATT-GATGTTGGCCTGGTGG-3′ and reverse primer 5′-GCTAATT-GCTTCGGCAGGTGAGTT-3′.

After each RCE cycle, groups of 25 larvae were immediately frozen at –80°C. Larvae were homogenized in Trizol reagent, and RNA was extracted according to the manufacturer's protocol. For *hsp70* northern blots, 5 μg of RNA was separated on a 1.4% agarose, 0.41 mol l^{-1} formaldehyde gel. Because of the high signal strength, only 1 μg of RNA was used for the *28s* control northern blots. After electrophoresis, RNA was transferred to a positively charged nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, NJ, USA) using the Turboblotter rapid downward transfer system (Schleicher and Schuell, Inc., Keene, NH, USA). DNA clones for *hsp70* and *28s*, obtained by PCR, were labeled with the DIG-High Prime labeling kit (Roche, Basel, Switzerland). Hybridization was performed using the DNA Labeling and Starter Kit II (Roche) and the membranes were exposed on Blue Lite Autorad Film (ISC BioExpress, Kaysville, UT, USA) for 5 min to 24 h, depending on signal strength. All northern blots were performed in technical triplicate.

Statistical analysis

Means were compared using ANOVA with a Bonferroni correction for multiple comparisons, using Minitab v. 13 (Minitab, State College, PA, USA). The primary comparisons used the means at each time point for the control, frozen and supercooled treatments. Survival data were arcsin-square-root-transformed prior to analysis, and statistical significance was set at $P<0.05$. All data are reported as means ± s.e.m.

RESULTS

Whole-animal survival

Nearly all control larvae held at 4°C survived the duration of the experiment; 97.3±1.6% survived 1 day at 4°C, and at the end of 5 days 90.8±1.7% were still alive (Fig. 1A). Larvae in the RCE group (one to five cycles of 12 h at –5°C followed by 12 h at 4°C) that were supercooled at –5°C also had high survival; 96.0±2.2%

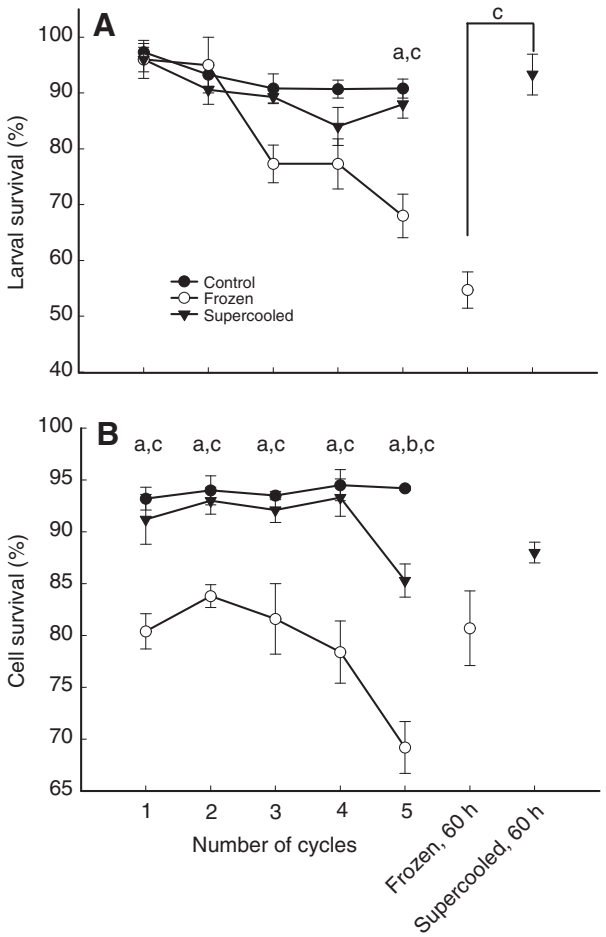

Fig. 1. Effect of repeated cold exposure (RCE) on (A) larval survival and (B) cell survival of *Belgica antarctica* larvae. Values are means ± s.e.m. Larval survival values are based on percentage survival of five replicates of 15 larvae each. Cell survival of each replicate is based on the mean count of three groups of 100 cells, and the mean for each treatment is based on four replicates. At each time point, 'a' indicates a significant difference between control and frozen larvae, 'b' a significant difference between control and supercooled larvae, and 'c' a significant difference between frozen and supercooled larvae (ANOVA, Bonferroni, $P<0.05$).

survived a single cycle and 88.0±2.5% survived five cycles (Fig. 1A). At no point did the survival of supercooled larvae differ significantly from the control, and the lowest survival observed was 84±3.4% after four cycles. However, survival of larvae frozen at –5°C steadily decreased over the course of five cycles (Fig. 1A), from 96.0±3.4% after a single cycle to 68.0±3.9% after five cycles. This survival level was significantly lower than that of both control and supercooled larvae after five cycles (P<0.05). For larvae continuously exposed to –5°C for 60 h, the survival of supercooled larvae was significantly higher than that of frozen larvae (93.3±3.3 *vs* 54.7±3.7%, respectively, P<0.05; Fig. 1A).

Cell survival of midgut tissue

As with whole-animal survival, RCE in the frozen state had a deleterious effect on cell survival of midgut tissue. After each of the five cycles, survival of midgut tissue from frozen larvae was significantly lower than that of either control or supercooled larvae (P<0.05; Fig. 1B). After one cycle, survival of midgut tissue was 80.4±1.7% for frozen larvae compared with 93.2±1.1% for control larvae and 91.8±2.4% for supercooled larvae. For cycles one to four, midgut cell survival of frozen larvae was ~80%, whereas that of control and supercooled larvae was >90% (Fig. 1B). After the fifth cycle, cell survival of supercooled larvae (85.3±1.6%) dipped significantly below that of control larvae (94.3±0.3%), but was still significantly higher than that of frozen larvae, which decreased to 69.2±2.5% (Fig. 1B). For larvae continuously exposed to –5°C for 60 h, cell survival after 12 h of recovery was 80.7±3.6% (frozen larvae) and 88.0±1.0% (supercooled larvae). However, this difference was not statistically significant (P>0.05).

Lipid content

For the first four cycles of RCE, lipid content did not change significantly, varying between 74.0±2.8 and 90.1±3.8 μg lipid mg^{-1} DM for the treatment groups (Fig. 2A). After the fifth cycle, the lipid content of frozen larvae was 25% less than that of supercooled larvae, a difference that was statistically significant (P<0.05). However, the difference in lipid content between control and frozen larvae after five cycles was not significant. As with the larvae exposed to five cycles of RCE, the lipid content of larvae continuously supercooled for 60 h (81.3±1.1 μg lipid mg^{-1} DM) was significantly higher (P<0.05) than that of larvae frozen for 60 h (60.6±2.3 μg lipid mg^{-1} DM).

Glycogen content

Similar to lipid content, glycogen content of larvae that were frozen during RCE was significantly lower than in controls and their supercooled counterparts (Fig. 2B). After one cycle of 12 h at –5°C and 12 h at 4°C, the glycogen content of frozen larvae was 19.7±1.1 μg glycogen mg^{-1} DM, but this value steadily decreased every cycle, and by the end of five cycles glycogen content was only 9.3±0.7 μg glycogen mg^{-1} DM (Fig. 2B). By the third cycle, glycogen content was significantly lower than that of control larvae, and by the fifth it was lower than that of both control and supercooled larvae (P<0.05). After five cycles, glycogen content of frozen larvae was ~43% less than that of both control and supercooled larvae. Similarly, larvae frozen at –5°C for 60 h had significantly less glycogen than those supercooled at –5°C for 60 h, with those being frozen having 26% less glycogen (Fig. 2B, P<0.05).

Trehalose content

The trehalose content of larvae exposed to RCE in the frozen state steadily decreased over the course of the experiment, starting at

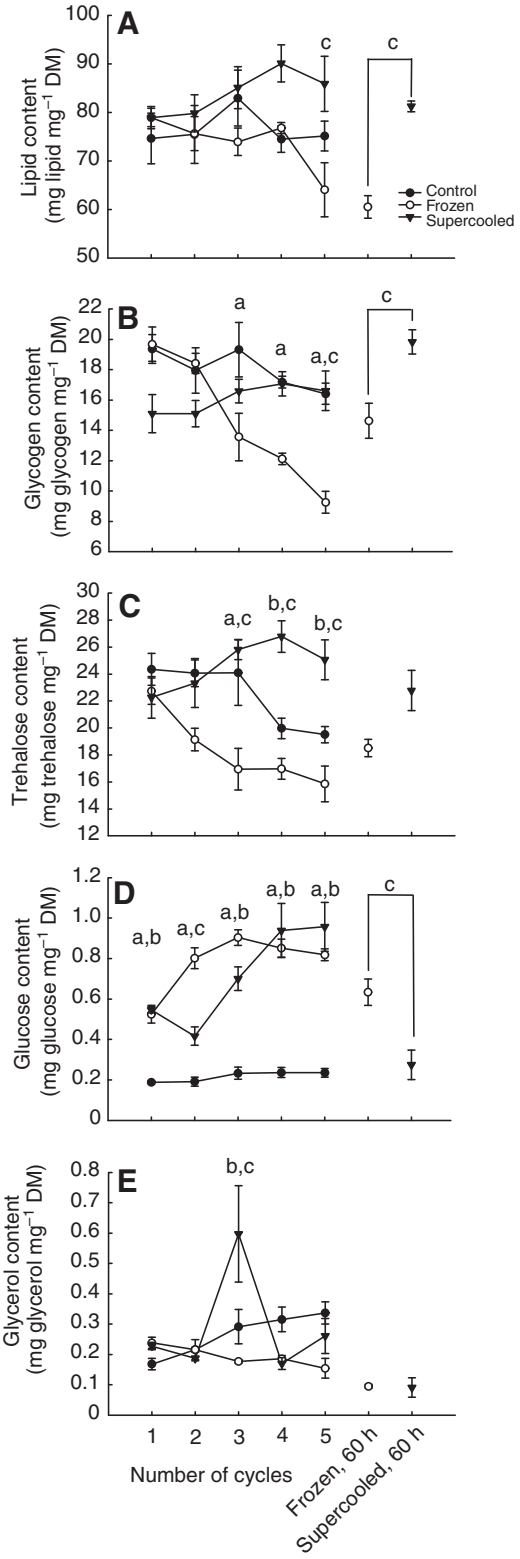

Fig. 2. (A) Lipid, (B) glycogen, (C) trehalose, (D) glucose and (E) glycerol content of *B. antarctica* larvae during RCE. Values are means ± s.e.m. The metabolite content is based on the mean of five replicates, five larvae per replicate for the lipid analysis, 45 larvae per replicate for the other metabolites. At each time point, 'a' indicates a significant difference between control and frozen larvae, 'b' represents a significant difference between control and supercooled larvae, and 'c' represents a significant difference between frozen and supercooled larvae (ANOVA, Bonferroni, P<0.05). DM, dry mass.

22.7±1.0 μg trehalose mg^{-1} DM after one cycle and ending at 15.9±1.3 μg trehalose mg^{-1} DM (Fig. 2C). However, trehalose content of supercooled larvae increased modestly over the course of the experiment, starting at 22.3±1.5 μg trehalose mg^{-1} DM after one cycle, peaking at 26.8±1.2 μg trehalose mg^{-1} DM and ending at 25.1±1.5 μg trehalose mg^{-1} DM (Fig. 2C). After three cycles of RCE, trehalose content of frozen larvae was significantly lower than that of both control and supercooled larvae, whereas after four and five cycles it was lower than that of supercooled larvae only (P<0.05). For larvae exposed to –5°C for 60 h, the trehalose content of frozen larvae was 18.5±0.6 μg trehalose mg^{-1} DM, which did not differ from that of supercooled larvae (22.8±1.5 μg).

Glucose content

The glucose content of control larvae did not change appreciably over the course of the experiment (Fig. 2D). However, larvae in both RCE groups experienced a dramatic increase in glucose content. After a single cycle of –5°C for 12 h and 4°C for 12 h, glucose content significantly increased nearly threefold in both frozen and supercooled larvae (P<0.05). For the duration of the experiment, the glucose content of RCE larvae was between two and four times that of their control counterparts, peaking at 0.90±0.04 μg glucose mg^{-1} DM for frozen larvae and 0.96±0.12 μg glucose mg^{-1} DM for supercooled larvae (Fig. 2D). Interestingly, for larvae continuously exposed to –5°C for 60 h, glucose content only increased in frozen larvae; the level for frozen larvae (0.63±0.06 μg glucose mg^{-1} DM) was significantly higher than that of supercooled larvae (0.27±0.07 μg glucose mg^{-1} DM).

Glycerol content

For most of the experiment, glycerol content did not significantly differ between control, frozen and supercooled larvae (Fig. 2E). There was a single peak of glycerol content after three cycles of RCE in supercooled larvae, in which glycerol content was more than double that of control larvae and more than triple that of frozen larvae (P<0.05). However, after the third cycle, the glycerol content of supercooled larvae returned to that of the other groups for the remainder of the experiment. Likewise, there was no difference in glycerol content between larvae frozen continuously for 60 h and supercooled larvae (Fig. 2E).

TEC and CEC

To summarize the results of the above experiments, we calculated the TEC of larvae based on the major energy reserves: lipid, glycogen, trehalose and glucose. TEC was largely unchanged for the first three cycles of RCE, ranging between ~3.5 and 4.0 J mg^{-1} DM (Fig. 3A) for all treatments. However, after four cycles, the TEC of both frozen and control larvae was almost 20% lower than that of supercooled larvae, a difference that was significant (P<0.05). The energy content of frozen larvae further decreased during the fifth cycle, so that after five cycles the energy content of frozen larvae (3.04±0.16 J mg^{-1} DM) was significantly lower than that of both control larvae (3.59±0.10 J mg^{-1} DM) and supercooled larvae (4.16±0.11 J mg^{-1} DM). For larvae continuously exposed to –5°C for 60 h, the energy content of frozen larvae (3.06±0.13 J mg^{-1} DM) was significantly less than that of supercooled larvae (3.89±0.07 J mg^{-1} DM).

Additionally, because TEC was dominated by lipid energy reserves, and because changes in carbohydrate content were much more dramatic, we also calculated the CEC in response to RCE. The CEC of larvae frozen at –5°C during RCE steadily declined over the course of the experiment, decreasing from 0.76±0.03 J mg^{-1} DM after one cycle to 0.46±0.03 J mg^{-1} DM after five cycles (Fig. 3B). Meanwhile, the CEC of control larvae decreased slightly, whereas that of supercooled larvae increased slightly, although these changes were not statistically significant (P>0.05). After five cycles of RCE, the CEC of frozen larvae was nearly 30% less than that of control larvae and nearly 40% less than that of supercooled larvae (Fig. 3B). The CEC of larvae frozen for 60 h (0.59±0.02 J mg^{-1} DM) was also significantly lower than that of larvae supercooled for 60 h (0.76±0.03 J mg^{-1} DM; Fig. 3B).

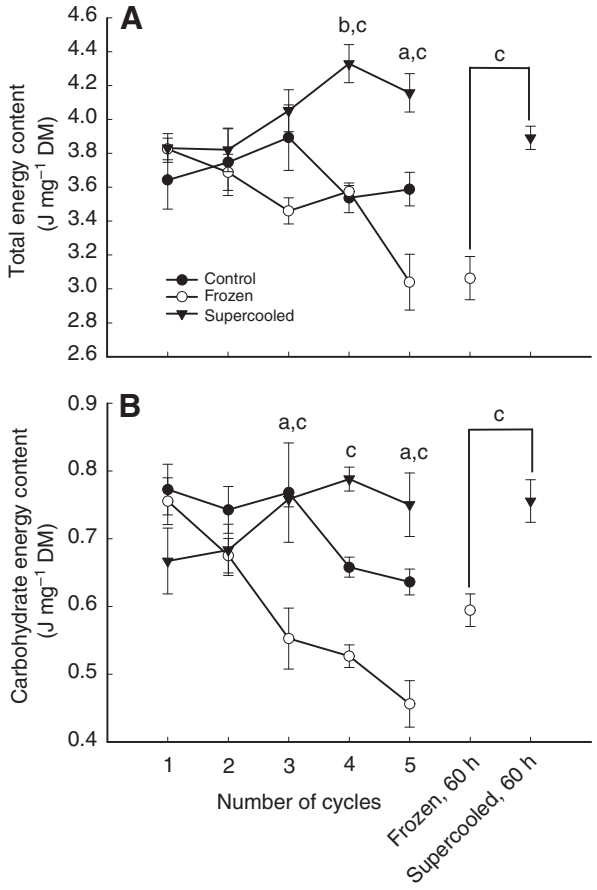

Fig. 3. (A) Total energy content (TEC) and (B) carbohydrate energy content (CEC) due to the major energy stores of *B. antarctica* larvae during RCE. Values are means ± s.e.m. TEC (J mg^{-1} DM) was calculated using the equation TEC=0.393(lipid content)+0.176(total carbohydrate content), where lipid content and carbohydrate content are in μg mg^{-1} DM. CEC (J mg^{-1} DM) was calculated using the equation CEC=0.176(total carbohydrate content) (Djawdan et al., 1998). Values are based on the mean of five replicates. At each time point, 'a' indicates a significant difference between control and frozen larvae, 'b' a significant difference between control and supercooled larvae, and 'c' a significant difference between frozen and supercooled larvae (ANOVA, Bonferroni, P<0.05).

Heat shock protein expression

Using northern blot hybridization, we measured the expression of *hsp70*, a common biomarker for environmental stress, in response to RCE. Over the 5 day experiment, expression of *hsp70* mRNA increased slightly in control and supercooled larvae (Fig. 4). However, this increase was not as dramatic as that observed in frozen larvae, where there was clear upregulation of *hsp70* over the course of the experiment. We measured expression after one, three and five cycles of RCE, and after each of these cycles the *hsp70* transcript

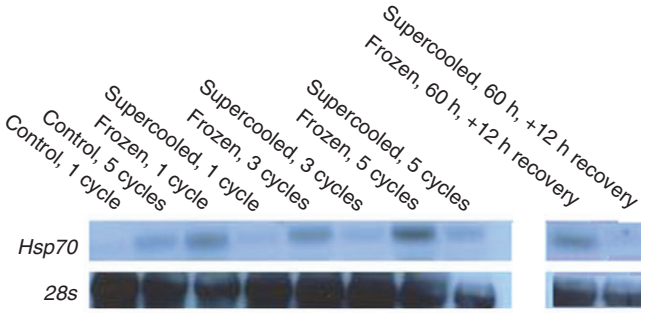

Fig. 4. mRNA expression of *hsp70* during RCE in *B. antarctica* larvae. The *28s* rRNA gene is used as a control.

level was clearly higher in frozen larvae than in supercooled larvae. Likewise, for larvae continuously exposed to –5°C, expression of *hsp70* was higher in the frozen group (Fig. 4). Expression of the control gene, *28s*, did not change appreciably over the course of the experiment.

DISCUSSION

Although previous studies on environmental stress tolerance in *B. antarctica* have consisted of a single stress exposure, in nature *B. antarctica* is exposed to repeated bouts of stress punctuated by periods of recovery. In the present study, we used a thermal regime of 12 h at –5°C followed by 12 h at 4°C to examine the physiological consequences of RCE in *B. antarctica*. Also, by holding larvae in the presence and absence of water at –5°C, we compared the effects of RCE in both the frozen and supercooled states. Overall, we observed deleterious effects of RCE in frozen but not supercooled larvae. Larvae exposed to multiple bouts of cold in the frozen state had higher mortality, more tissue damage, lower energy reserves and higher expression of heat shock proteins than supercooled larvae.

RCE in frozen larvae negatively impacts survival

At our test temperature, –5°C, over 95% of larvae survived a single 12 h exposure, whether in the frozen or supercooled state (Fig. 1A). However, as the number of RCE cycles increased, survival of frozen larvae steadily decreased below that of control and supercooled larvae; after three cycles survival decreased to 77%, and by the conclusion of five cycles survival was only 68%. Meanwhile, survival of supercooled larvae remained around 90% for the duration of the study. To our knowledge, only one other study has directly addressed the question of freezing *versus* supercooling in the same species. Layne and Kuharsky (Layne and Kuharsky, 2001) examined the effects of freezing and supercooling at –5°C in the goldenrod gall fly, *Eurosta solidaginis*, and found the opposite of our results; namely that larvae that were supercooled for 10 weeks at –5°C had much lower adult emergence rates than those frozen at –5°C for 10 weeks. Layne and Kuharsky (Layne and Kuharsky, 2001) postulated that the difference in survival could be attributed to higher water loss in the supercooled larvae, which was confirmed by Irwin and Lee (Irwin and Lee, 2002). In the present study, there was no significant change in water content (all larvae had ~73% water content; data not shown), so differences in survival must be due to some other parameter. Although it is likely that larvae would dehydrate when supercooled in nature, we maintained 100% RH in this study so that any observed changes could be attributed to freezing or supercooling and not to changes in water content.

For chill-susceptible insects, intermittent periods of recovery during a cold exposure often permit higher survival than a single prolonged exposure to cold (Chen and Denlinger, 1992; Colinet et al., 2006; Kostal et al., 2007; Leopold et al., 1998; Nedved et al., 1998; Renault et al., 2004). Periods of higher temperatures allow the insects to repair cold injury by restoring ion homeostasis (Kostal et al., 2007) and replenishing ATP levels (Dollo et al., 2010), for example. However, this benefit of RCE only appears to hold true for freeze-avoiding species. Although the literature on this topic is scarce, in both our study and in previous studies of the sub-Antarctic beetle *Hydromedion sparsutum* and the hoverfly *Syrphus ribesii*, RCE in the frozen state caused significant mortality (Bale et al., 2001; Brown et al., 2004). In our study, it appears that mortality due to freezing is simply a function of the total time spent in the frozen state; survival after five cycles of RCE (68.0%), in which 60 h of freezing are accumulated, was very similar to and not significantly different from the survival after 60 h of continuous freezing (54.7%). In the supercooled state, survival of larvae after five cycles of RCE (88.0%) was also very similar to survival of larvae exposed continuously to –5°C for 60 h (93.3%). What is not clear is whether a longer exposure to –5°C in the supercooled state would begin to produce mortality, and whether this mortality could be ameliorated by periodic recovery times, as is the case for previous studies of RCE.

Freezing, but not cold *per se*, causes significant tissue damage

As with whole-animal survival, RCE in the frozen state had a negative impact on cell survival of midgut tissue. Cell survival of midgut tissue was consistently 10–20% higher in supercooled larvae compared with their frozen counterparts at each time point (Fig. 1B). Similar results were obtained by Sinclair and Chown (Sinclair and Chown, 2005b), who showed that repeated freeze exposures in *P. marioni* cause considerable damage to gut tissue. In our study, although cell survival of frozen larvae was consistently lower in frozen larvae, the amount of mortality remained relatively constant until the fifth cycle. One possible explanation for this result is that insect midgut tissue has high cell turnover and rapid regeneration of dead cells (Okuda et al., 2007); thus the cumulative effects of multiple freeze cycles are not as dramatic because previously dead cells have already been removed. Also, to reduce variability, we only selected live, non-moribund larvae for dissection, so we likely missed the true range in cell survival.

RCE in the frozen state significantly depletes energy reserves

Adaptations to survive low temperatures, such as cryoprotectant synthesis and stress protein production, are energetically costly. As a result, we predicted that RCE would result in significant depletion of key energy reserves. Our results indicate that, although this is true when *B. antarctica* is frozen during RCE, this is not the case when *B. antarctica* is supercooled. By the end of five cycles of RCE, frozen larvae had lower levels of lipid, glycogen and trehalose compared with their supercooled counterparts (Fig. 2A–C). By pooling all the data, we observed that, by the fifth cycle of RCE, frozen larvae had 27% less TEC and 39% less CEC than supercooled larvae (Fig. 3). Also, as with survival, the energetic effects of RCE in the frozen state seem to be a function of the cumulative time frozen at –5°C; the metabolite and total energy profiles of larvae frozen continuously for 60 h are remarkably similar to those of larvae exposed to five cycles of RCE (Figs 2, 3).

Our results are in agreement with previous studies looking at energetic consequences of RCE. In the freeze-tolerant caterpillar *P. marioni*, although RCE does not result in changes of body composition, damage to the gut hinders feeding, resulting in

decreased body size (Sinclair and Chown, 2005b). In *Drosophila melanogaster*, RCE causes a decrease in glycogen and lipid stores, which reduces fecundity (Marshall and Sinclair, 2010). Although we were unable to rear larvae to adulthood and look at the fitness consequences of RCE, it is likely that the severe energy depletion caused by freezing ultimately would have fitness consequences, particularly given the extremely short growing season in Antarctica. Furthermore, Renault et al. demonstrated that body size positively correlates with the duration of cold survival in the beetle *Alphitobius diaperinus* (Renault et al., 2003), suggesting that higher energy reserves lead to prolonged cold survival. Thus, applying this to our results, it is possible that the reduced survival of frozen larvae was a direct consequence of the observed energy depletion.

Whereas there was clearly a decrease in energy reserves in the frozen larvae, the exact fate of these energy reserves is unclear. One possibility is that lipid, glycogen and/or trehalose serve as a carbon source for cryoprotectant production. In many cases, glycogen is the primary source of carbon for cryoprotectant production (Storey and Storey, 1991), and often glycogen content positively correlates with an organism's cold tolerance (Costanzo and Lee, 1993; Kostal et al., 2004; Overgaard et al., 2009). With this in mind, we measured the content of two known cryoprotectants in *B. antarctica*: glucose and glycerol (Baust and Edwards, 1979; Lee and Baust, 1981). Although glucose levels increased nearly fivefold (or ~0.54 μg larva^{-1}) in larvae exposed to RCE, this alone could only account for <10% of the reduction in glycogen in frozen larvae, which was ~8.3 μg larva^{-1}. At the same time, glycerol did not change during RCE, despite its known role as a protective compound during dehydration stress (Benoit et al., 2007). Also, to our surprise, trehalose content decreased in larvae frozen during RCE, despite its well-defined role as a cryoprotectant in *B. antarctica* (Elnitsky et al., 2008; Lee and Baust, 1981). However, *B. antarctica* accumulates several other cryoprotectants, including erythritol, sucrose and fructose, so it is possible that an increase in at least one of these compounds is at least partly responsible for the observed decrease in energy reserves during freezing.

A second, more likely explanation for the decreased energy reserves in frozen larvae is an elevated metabolic rate during recovery. Because freezing during RCE is clearly more damaging to the larvae (Figs 1, 4), the elevated repair cost could result in higher metabolism during recovery. Physiological responses to cold damage, including restoration of ion homeostasis (Kostal et al., 2007) and protein synthesis (Colinet et al., 2007), are energetically expensive, suggesting that frozen larvae may need to elevate their metabolic rate during recovery. In support of this, Block et al. found that the sub-Antarctic beetles *H. sparsutum* and *Perimylops antarcticus* both increase their metabolic rate during recovery from freezing, but not in response to chilling in a supercooled state (Block et al., 1998). Similarly, the freeze-tolerant wood frog *Rana sylvatica* elevates its metabolic rate several hours into recovery from freezing (Layne, 2000). Future work on the metabolic rate of *B. antarctica* in response to freezing could elucidate whether an elevated post-freeze metabolic rate is responsible for the observed energy depletion during RCE.

RCE elevates heat shock protein expression in frozen larvae

As a final measure of the relative costs of RCE in the frozen *versus* supercooled state, we measured expression of *hsp70*. This protein is perhaps the best studied of the heat shock proteins, a group of molecular chaperones that are elevated in response to numerous environmental stresses (Feder and Hofmann, 1999). Whereas most insects only express heat shock proteins transiently during stress, *B. antarctica* is unique in that heat shock proteins are expressed continuously (Rinehart et al., 2006). Despite the constitutive expression, heat shock protein transcript levels can still be elevated by a number of stresses, including UV exposure (Lopez-Martinez et al., 2008) and dehydration (Lopez-Martinez et al., 2009). In the present study, we observed that RCE in the frozen, but not supercooled, state enhances expression of *hsp70* (Fig. 4). The same is true of a continuous freezing exposure; larvae frozen at –5°C for 60 h had noticeably higher expression of *hsp70* compared with those supercooled at –5°C. Although Rinehart et al. (Rinehart et al., 2006) and Lopez-Martinez et al. (Lopez-Martinez et al., 2008) did not observe an increase in *hsp70* mRNA in response to freezing, their samples were only taken after a brief (i.e. 0–2 h) recovery period.

Once again, our results for *hsp70* point towards a higher cost of RCE in the frozen state. Because *hsp70* is primarily responsive to protein damage, our results indicate that freezing during RCE likely leads to protein denaturation. Thus, *hsp70* expression during RCE is dependent on freezing and not simply the drop in temperature. To our knowledge, this is the first study showing differential expression of a heat shock protein depending on whether the animal is frozen or supercooled.

Ecological relevance of this study

One caveat of this study is that our temperature fluctuations were likely of greater magnitude and periodicity than typical microhabitat conditions. Nonetheless, in the late summer and early winter, larvae are exposed to periodic freeze–thaw cycles in their natural habitat [see microclimate data from Elnitsky et al. (Elnitsky et al., 2008)]. Also, depending on the buffering capacity of its particular microhabitat, larvae could conceivably experience greater fluctuations in temperature that more closely resemble the rapidly changing ambient temperatures. A second caveat is whether larvae can use supercooling for subzero exposures longer than 12 h. Out of 12 larvae tested, only two froze during a 12 h exposure to –5°C, and did so during the initial rapid cooling phase. This suggests that the supercooled state is stable once the animal reaches –5°C. Finally, our results lead to the question if larvae can regulate whether they freeze or remain supercooled. The variation in moisture conditions within their environment (Elnitsky et al., 2008) suggests that larvae may avoid freezing by moving to a dry area where the risk of inoculative freezing is reduced. Thus, we feel that our experiments reasonably reflect natural conditions and can be used to make inferences about the physiological responses of larvae to acute fluctuating temperature exposures in their natural environment.

Although this study and our previous ones have focused on acute exposures to cold, overwintering conditions – which feature several months of continuous subzero temperature – offer different challenges for larvae. Here, larvae can either remain frozen for the duration of the winter or avoid inoculative freezing by supercooling. Even though supercooling is a possibility for overwintering larvae, it is more likely that overwintering larvae that avoid freezing would undergo cryoprotective dehydration because of the influence of surrounding ice and snow. Based on the results of the present study, we hypothesize that a freeze-avoiding overwintering strategy such as cryoprotective dehydration would be favorable compared with long-term freezing. Future studies by our group will address the overwintering physiological ecology of *B. antarctica*.

Finally, our results may have implications for global climate change. Although increases in air temperature would seemingly benefit polar insects by extending the growing season, this is likely not the case (Bale and Hayward, 2010). As the Earth warms, climate models predict more extreme variation in temperature (Easterling

et al., 2000). Also, although snowfall in the Antarctic Peninsula has increased over the past 150 years (Thomas et al., 2008), there are localized decreases in snow cover on the peninsula (Fox and Cooper, 1998) due to increased melting. Consequently, the microhabitat of *B. antarctica*, which is normally thermally buffered by snow and ice cover, could potentially experience more extreme changes in temperature and a greater number of freeze–thaw cycles. Additionally, the increased precipitation and snowmelt could enhance soil moisture, thereby increasing the likelihood of inoculative freezing. For example, a 40% increase in soil moisture doubles the likelihood of inoculative freezing during gradual chilling in *B. antarctica* (Elnitsky et al., 2008). Given the results of the present study, both of these potential consequences of climate warming (increased number of freeze–thaw cycles and increased risk of inoculative freezing) would be detrimental to the survival of *B. antarctica*.

Conclusions

We found that RCE in the frozen state, but not the supercooled state, was deleterious for larvae of *B. antarctica*. Larvae exposed to multiple bouts of cold in the frozen state had lower survival, greater tissue damage, lower energy reserves and increased protein damage compared with supercooled larvae. Also, the accumulated damage from RCE appears to be a function of the total time spent in the frozen state; larvae frozen continuously for 60 h experienced similar levels of damage and energy depletion as those that accumulated 60 h of freezing over five cycles of RCE.

Thus, it appears that although *B. antarctica* is freeze tolerant, it is preferable to remain supercooled during brief, repeated cold exposure. This begs the question as to why *B. antarctica* isn't strictly freeze avoiding. However, given the rapid changes in moisture content in its environment and the frequent changes in temperature, larvae are at continual risk of inoculative freezing. For this reason, freeze tolerance is common among insects in the Southern Hemisphere, where oceanic influences can cause summer cold snaps that put insects at higher risk of inoculative freezing (Sinclair et al., 2003; Sinclair and Chown, 2005a). Therefore, we assert that *B. antarctica* is obligately freeze tolerant, but likely prefers dry microclimates so that it can remain unfrozen at subzero temperatures. Over the short term, this can be accomplished by supercooling (present study), whereas cryoprotective dehydration provides a means to avoid freezing during long-term chilling (Elnitsky et al., 2008). In subsequent studies, we will track *B. antarctica* larvae in the field to determine which cold-tolerance strategy (i.e. freeze tolerance or freeze avoidance) is used by *B. antarctica* larvae in their natural environment.

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