Rapid cold hardening protects against sublethal freezing injury in an Antarctic insect

Running title: RCH and sublethal freezing injury

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SUMMARY STATEMENT

Rapid cold hardening has a well-established role in preventing death from cold, and here we show it also protects against nonlethal freezing injury in a freeze-tolerant Antarctic insect.

ABSTRACT

Rapid cold hardening (RCH) is a type of beneficial phenotypic plasticity that occurs on extremely short time scales (minutes to hours) to enhance insects' ability to cope with cold snaps and diurnal temperature fluctuations. RCH has a well-established role in extending lower lethal limits, but its ability to prevent sublethal cold injury has received less attention. The Antarctic midge, *Belgica antarctica* is Antarctica's only endemic insect and has a well-studied RCH response that extends freeze tolerance in laboratory conditions. However, the discriminating temperatures used in previous studies of RCH are far below those ever experienced in the field. Here, we tested the hypothesis that RCH protects against nonlethal freezing injury. Larvae of *B. antarctica* were exposed to either control (2°C), direct freezing (-9°C for 24 h), or RCH (-5°C for 2 h followed by -9°C for 24 h). All larvae survived both freezing treatments, but RCH larvae recovered more quickly from freezing stress and had significantly higher metabolic rates during recovery. RCH larvae also sustained less damage to fat body and midgut tissue and had lower expression of two heat shock protein transcripts (*hsp60* and *hsp90*), which is consistent with RCH protecting against protein denaturation. The protection afforded by RCH resulted in energy savings; directly frozen larvae experienced a significant depletion in glycogen energy stores that was not observed in RCH larvae. Together, these results provide strong evidence that RCH protects against a variety of sublethal freezing injuries and allows insects to rapidly fine-tune their performance in thermally variable environments.

INTRODUCTION

The ability to cope with thermal variability on seasonal and diurnal timescales is a critical adaptation for animals living in temperate and polar environments (Colinet et al., 2015), and climate change is increasing thermal variability across much of the planet (Dillon et al., 2016; Vasseur et al., 2014). To cope with low temperature stress, insects have evolved a suite of physiological and biochemical adaptations (reviewed by Lee, 2010; Overgaard and MacMillan, 2017; Teets and Denlinger, 2013). One such adaptation is rapid cold hardening (RCH), an adaptive plastic response in which brief chilling enhances tolerance to subsequent cold stress (Lee et al., 1987; Lee and Denlinger, 2010). One of the fastest known adaptive physiological responses to temperature, RCH has been observed across the arthropod phylogeny, and analogous responses are present in fish (Hazel and Landrey, 1988), amphibians (Layne and Claussen, 1987), and turtles (Muir et al., 2010). RCH can be induced by natural diurnal thermoperiods and ecologically relevant cooling rates (Kelty, 2007; Kelty and Lee, 1999; Kelty and Lee, 2001) and allows insects to cope with sudden cold snaps and optimize performance in thermally variable environments.

In terrestrial polar environments, insect diversity is severely suppressed, in large part due to short growing seasons and extreme low temperatures (Teets and Denlinger, 2014). Terrestrial Antarctica harbors only three insect species (Convey and Block, 1996), and of these the midge *Belgica antarctica* is the only endemic species and the world's southernmost insect (Lee and Denlinger, 2015). The physiological and molecular mechanisms by which this species tolerates environmental extremes (e.g., cold, desiccation, salinity, anoxia) are well-studied. While the long, cold winter is a conspicuous feature of Antarctic habitats, larvae of *B. antarctica* experience multiple freeze-thaw cycles throughout the year and thus maintain the ability to

66 survive internal ice formation (i.e., freeze-tolerance) year around (Baust and Lee, 1981; Elnitsky
67 et al., 2008; Kawarasaki et al., 2014a). Mechanistically, constitutive expression of heat shock
68 proteins (Rinehart et al., 2006) and antioxidants (Lopez-Martinez et al., 2008), aquaporins that
69 facilitate water movement during freezing (Goto et al., 2015; Yi et al., 2011), and plastic changes
70 in metabolic gene expression following freezing (Teets et al., 2013) likely contribute to the year-
71 round freeze tolerance of this species.

72 RCH was initially thought to be restricted to chill-susceptible and freeze-avoiding insects,
73 but the discovery of RCH in *B. antarctica* was the first case of RCH being described in a freeze-
74 tolerant insect (Lee et al., 2006b). In *B. antarctica,* RCH is elicited by temperatures between -3
75 and -12°C, occurs in as little as 30 min, and is activated more strongly when larvae are frozen
76 than supercooled (Kawarasaki et al., 2013). For example, a 2 h period of RCH at -5°C (the
77 conditions used in our experiments; see below) increases survival at -18°C for 24 h from ~10%
78 to >80%. Optimal RCH conditions can extend the lower limit of freeze tolerance below -20°C in
79 the laboratory (Kawarasaki et al., 2013), but microhabitat temperatures for larvae rarely drop
80 below -5°C, and -10°C is the lowest recorded microhabitat temperature in the field (Baust and
81 Lee, 1981; Elnitsky et al., 2008; Kawarasaki et al., 2014a). These temperatures are above the
82 supercooling point for most larvae (supercooling points are typically around -10°C for summer
83 acclimatized larvae; see Kawarasaki et al., 2014a), but larvae have a limited capacity to avoid
84 inoculative freezing at ecologically relevant soil moisture conditions (Kawarasaki et al., 2014b).
85 Thus, larvae have a high probability of freezing at sub-zero temperatures, but they can readily
86 survive freezing below -10°C, even in the summer (Kawarasaki et al., 2013; Lee et al., 2006b).
87 Therefore, most, if not all, freezing events in the field are nonlethal.

Previous work in *B. antarctica* documented sublethal costs of freezing stress. Multiple freeze-thaw cycles result in tissue damage, energy depletion, and upregulation of heat shock proteins before the onset of mortality (Teets et al., 2011), while simulated winter freezing depletes glycogen energy stores (Kawarasaki et al., 2014a). In other freeze-tolerant insects, similar sublethal costs have been observed. Multiple freeze-thaw cycles result in decreased body mass in a sub-Antarctic caterpillar (Sinclair and Chown, 2005), while repeated freezing as prepupae reduces adult fecundity in a temperate gall fly (Marshall and Sinclair, 2018). However, the extent to which RCH protects against sublethal freezing injury has not been assessed. Furthermore, most studies of RCH focus on extension of lower lethal limits, despite lethal cold events being rare in the field (Alvarado et al., 2015; but see Coello Alvarado et al., 2015; Findsen et al., 2013; Powell and Bale, 2006; Shreve et al., 2004).

Here, we investigated the extent to which RCH protects against sublethal freezing injury at ecologically relevant temperatures. We identified the lowest temperature that produced no significant mortality and tested the ability of a 2 h period of RCH to reduce freezing injury. The direct stepwise temperature shifts used to elicit RCH do not fully reflect natural conditions, but previous work has demonstrated that stepwise transfers provide the same protection as ecologically relevant cooling ramps, and that there is a narrow window of temperature that elicits RCH, including in *B. antarctica* (Chen et al., 1987; Coulson and Bale, 1990; Kawarasaki et al., 2012). Furthermore, in the field larvae are exposed to repeated freeze-thaw cycles that can have variable effects on physiology depending on the frequency and intensity of cold exposure (Marshall and Sinclair, 2012), and our experiments do not account for potential effects of multiple cold exposure. Nonetheless, our design allows us to rigorously test the hypothesis that RCH affords protection at temperatures likely to be encountered in the field.

To test our hypothesis that RCH protects against sublethal freezing injury in *B. antarctica,* summer-acclimatized larvae were exposed to nonlethal freezing for 24 h with and without a 2 h RCH pretreatment, and we measured a range of outcome variables that span levels of biological organization. In response to nonlethal freezing, we observed a reduction in locomotor activity, lowered metabolic rates, damage to midgut and fat body tissue, an increase in heat shock protein expression (consistent with damage to proteins), and a significant increase in glucose content coupled with a decrease in glycogen energy stores. All symptoms of freezing injury were reduced, at least partially, by RCH, indicating that RCH protects against multiple routes of sublethal freezing injury at ecologically relevant conditions.

MATERIALS AND METHODS

Insects

Larvae of *B. antarctica* were collected on various islands within a 3 km radius of Palmer Station (64°46'S, 64°04'W) in January 2018. Samples were returned to the laboratory and extracted from their substrate into ice water using a modified Berlese apparatus. After extraction, concentrated samples of larvae were immediately returned to natural substrate (containing rocks, moss, and the alga *Prasiola crispa*) and stored at 2°C for at least one week until used for experiments. Experiments were conducted within 2 weeks of collection. Prior to an experiment, larvae were sorted from their substrate in ice water and held on moist filter paper overnight. Only fourth instar larvae were used for experiments.

Cold treatments

Our goal was to assess the extent to which RCH prevents sublethal freezing injury. In a preliminary experiment, we assessed the freeze tolerance of summer-acclimatized fourth instar

larvae to establish conditions for later experiments. Groups of 20 larvae, N=3 per temperature, were exposed for 12 or 24 h to seven temperatures ranging from -3 to -21°C in 3°C increments. Immediately prior to cold exposure, larvae were submerged in ~50 µl water in a 1.5 ml microcentrifuge tube, and a small piece of ice was added to each tube to ensure that larvae froze via inoculative freezing. Larvae have a water-permeable cuticle and a limited capacity to avoid inoculative freezing (Elnitsky et al., 2008; Kawarasaki et al., 2014b), and thus submerged larvae in direct contact with ice will freeze at or near the body fluid melting point (~-0.6°C for summer acclimatized larvae). After cold exposure, larvae were placed in Petri dishes with moist filter paper, and survival was assessed 24 h later. Larvae that moved spontaneously or in response to gentle prodding were considered alive. Survival was identical for the 12 and 24 h exposure at each temperature, indicating that within these time frames temperature is the primary determinant of freezing injury, rather than exposure time. Thus, even though a 12 h exposure better reflects diurnal temperature fluctuations, we elected to use 24 h exposures for our experiment because of logistical constraints coordinating field work and laboratory work during our brief stay at Palmer Station. For 24 h exposures, survival was at or near 100% down to -9°C and dropped off rapidly at lower temperatures, and all larvae died at temperatures at or below -15°C (Fig. 1). Thus, we selected -9°C as our discriminating temperature for the RCH experiments.

For the remaining experiments, we used the following conditions: 1) Control (maintained at 2°C for the duration of the experiment), 2) Directly Frozen (DF, directly transferred from 2°C to -9°C, as described above, and held at -9°C for 24 h), 3) Rapid Cold Hardening (RCH; transferred from 2°C to -5°C for 2 h, then moved to -9°C for 24 h). For both freezing treatments, larvae were submerged in water containing a small piece of ice to ensure inoculative freezing at

high sub-zero temperatures. Previous work demonstrated that freezing at -5°C for 2 h elicits a maximal RCH response (Kawarasaki et al., 2013). After treatment, larvae were returned to Petri dishes with moist filter paper and kept at 2°C. All physiological experiments were conducted on station, and samples for gene expression and biochemical assays (see details below) were frozen at -80°C and shipped to the University of Kentucky on dry ice.

Recovery of locomotion

We first tested the hypothesis that RCH allows larvae to recover normal locomotion more quickly after freezing stress. Immediately after cold treatments, larvae were placed into individual wells of a 96-well plate containing 25 µl water. Plates were kept on a cooler of ice for observation. Larvae were observed under a stereo microscope until the first signs of spontaneous movement, which we recorded as the recovery time. In a separate experiment, we also measured locomotor activity after freezing stress. Midge larvae crawl through their substrate by simultaneously contracting their head capsule and an extension of the thorax called the anterior proleg. Thus, to measure locomotor activity, we placed individual larvae in a Petri dish with ice water and recorded the number of these contractions in a one-minute period. Cold-treated larvae were measured in separate groups of larvae 2 and 24 h after cold exposure, and untreated larvae were also included at both time points to control for any day-to-day variation in movement speed. For both recovery time and movement speed we measured 40 larvae per treatment group. For recovery time, 80 larvae were monitored simultaneously, while for movement speed a single larva was observed at one time. The same investigator (NMT) observed all samples.

Metabolic rate

Here we tested the hypothesis that RCH restores metabolic function after freezing stress. Larvae were exposed to control, directly frozen, and RCH treatments, as described above, and we measured oxygen consumption in separate groups of larvae after 2 and 24 h recovery. Oxygen consumption was measured by placing groups of 10 larvae into an Instech Fiber Optic Oxygen Monitor (Model FOL/C1T500P; Instech Laboratories, Plymouth Meeting, PA, USA) according to Elnitsky et al. (2009). In brief, larvae were equilibrated in the chamber containing 500 µl water at 4°C for 10 min prior to recording changes in dissolved oxygen consumption for at least 10 min. After measurement, each sample was weighed to the nearest 0.002 mg, and oxygen consumption was expressed as nmol O_2 min^{-1} mg^{-1} fresh mass (FM). The oxygen sensor was calibrated using solutions of 0% oxygen (produced by adding sodium dithionite crystals to the same water used for measurement) and a saturated oxygen solution at 4°C. For each treatment, we measured oxygen consumption in 5-6 groups of larvae.

Tissue damage

To measure tissue damage following freezing stress, we used a two-component dye exclusion assay modified from the LIVE/DEAD Sperm Viability Kit (ThermoFisher Scientific, Waltham, MA, USA) as described by Yi and Lee (2003). Larvae were exposed to control, directly frozen, and RCH treatments and allowed to recover for 24 h. After recovery, midgut and fat body tissue were dissected in ice-cold Coast's solution (Coast and Krasnoff, 1988) and transferred to a slide containing 25 µl SYBR-14 dye in Coast's solution. After 10 min, 25 µl propidium iodide in Coast's was added, and the tissues were stained for an additional 10 min. Samples were imaged on a fluorescent microscope, and live cells with intact membranes fluoresce green, while dead cells with damaged membranes fluoresce red. Cell survival was

199 determined by counting the proportion of live cells in a minimum of 300 cells per sample. For
200 each group, we imaged 4-5 tissue samples.

201 **Stress gene expression**

202 In this experiment, we compared the molecular stress response of larvae exposed to our
203 various cold treatments. We primarily focused on expression of transcripts encoding heat shock
204 proteins, a group of highly conserved stress genes (reviewed by Feder and Hofmann, 1999) with
205 an established role in stress responses in *B. antarctica* (Lopez-Martinez et al., 2009; Rinehart et
206 al., 2006; Teets et al., 2012b). Expression of heat shock protein transcripts is regulated by protein
207 denaturation, and in previous work we demonstrated that expression is correlated with other
208 measures of freezing damage (Teets et al., 2011). Thus, we used mRNA abundance of heat shock
209 proteins as a proxy for subcellular protein damage after freezing. We measured mRNA
210 expression of heat shock proteins from all five major families, small heat shock proteins (*sHsp*)
211 (GenBank: GAAK01009816), *hsp40* (GenBank: GAAK01004380), *hsp60* (GenBank:
212 GAAK01010161), *hsp70* (GenBank: GAAK01011953), and *hsp90* (GenBank:
213 GAAK01011429), as well as expression of *phosphoenolpyruvate carboxykinase* (*pepck*)
214 (GenBank: JX462659), a metabolic gene that is highly responsive to stress (Teets et al., 2013).
215 We measured gene expression in control larvae and after 2 and 24 h recovery from the directly
216 frozen and RCH treatments, N=5 per group.

217 RNA was extracted from groups of 20 larvae using Tri reagent (ThermoFisher) according
218 to the manufacturer's protocol. RNA was resuspended in Buffer RLT (Qiagen, Germantown,
219 MD, USA) and further purified using the RiboPure RNA Purification Kit (ThermoFisher).
220 Quantity and purity of RNA were assessed spectrophotometrically, and 500 ng RNA was used as
221 a template for first-strand cDNA synthesis using the qScript cDNA Synthesis Kit (Quanta Bio,

Beverly, MA, USA). cDNA was used as a template in qPCR reactions, with each 20 µl reaction containing 10 µl 2X PerfeCTa SYBR Green FastMix (Quanta Bio), 2 µl each primer at 2.5 µM concentration (250 nm final concentration), 2 µl cDNA, and 4 µl water. Primers for *ribosomal protein l19* (*rpl19*) (GenBank: JX462670) and *pepck* were obtained from Teets et al. (2013), while those for the heat shock proteins were designed against annotated genes in the *B. antarctica* genome (Kelley et al., 2014) (Table 1). Reactions were run for 40 cycles on a QuantStudio 6 Flex real-time PCR system (ThermoFisher) and cycle threshold (Ct) values were calculated. Gene expression was calculated using the $2^{-\Delta Ct}$ method as in previous studies (e.g., Teets et al., 2013). The Ct of each gene of interest was normalized to that of a reference gene, *rpl19*, and we calculated fold changes relative to the control group.

Metabolite assays

To assess potential energetic benefits of RCH, we measured levels of several energy stores. Metabolites were measured using colorimetric assays as described previously (Teets et al., 2011; Teets et al., 2012a). Carbohydrates were extracted in perchloric acid from groups of 20 larvae, and free glucose was measured using the Glucose Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). To measure trehalose, we treated samples with trehalase from porcine kidney (Sigma-Aldrich) to liberate glucose, and the resulting glucose was measured with the Glucose Assay Kit. Glycogen was measured similarly by treating samples with amyloglucosidase from *Aspergillus niger* (Sigma-Aldrich) prior to measuring glucose. Total lipids were measured in groups of five larvae by homogenizing larvae in 1:1 chloroform:methanol and using vanillin-phosphoric acid reagent to quantify lipids. Total proteins were measured by homogenizing groups of 20 larvae larvae in radioimmunoprecipitation (RIPA) buffer and quantifying proteins

with the Pierce BCA Protein Assay Kit (ThermoFisher). For all metabolites, sample absorbance values were compared to a standard curve and corrected for the dry mass of each sample.

Statistical analysis

All statistical analyses were conducted in JMP Pro 14 (SAS Institute Inc., Cary, NC, USA) and R statistical software. Recovery time data were analyzed using a log-rank test to compare recovery times between larvae that were directly frozen and those that experience RCH. Movement speed data were not normally distributed and were thus compared with a permutation ANOVA using the aovp function in the lmPerm package in R, followed by all pairwise permutation t-tests with the pairwise.perm.t.test function in the RVAideMemoire package in R . Cell viability data were analyzed with a generalized linear model using the glmer function in the lme4 package in R. The data were fit with a binomial error distribution, with treatment as a main effect and replicate nested within treatment as a random effect to prevent pseudoreplication. Respirometry data, gene expression data, and metabolite data (except for glucose) were compared with ANOVA followed by paired t-tests of all possible pairwise comparisons. Trehalose, glycogen, lipid, and protein contents were log transformed prior to statistical analysis. Glucose data were not normally distributed and were analyzed with permutation ANOVA and t-tests, as described above for the movement speed data. To correct for multiple comparisons for the entire study, p-values from all tests were combined and adjusted with the False Discovery Rate Correction method of Benjamini and Hochberg (1995) using the p.adjust function in R. All data used to generate the figures in this paper are available on Dryad (DOI: https://doi.org/10.5061/dryad.29p7ng2).

RESULTS

Recovery of locomotion

Following 24 h of freezing at -9°C, larvae treated with RCH had a median recovery time of 45 min while larvae that were directly frozen had a median recovery of 67.5 min, a difference that was highly significant (Figure 2a; Log-Rank test, FDR ,X^2 = 18.29, df = 1, p=3.34E-4). After 2 h recovery from freezing, larvae treated with RCH had significantly faster head capsule contractions than directly frozen larvae (Figure 2b; Permutation Test, FDR, p=0.9.72E-4), and most directly frozen larvae failed to regain normal contractile movements despite displaying irregular body contractions. After a 24 h recovery period, the median rate of movement of RCH and directly frozen larvae were statistically indistinguishable, and neither group returned to control levels.

Metabolic rate

After 2 h recovery from freezing at -9°C, there was a significant 22% decrease in oxygen consumption (Figure 2c; ANOVA, FDR, t_{22} = 3.76, p = 0.004), but this decrease in metabolic rate was prevented by RCH. The oxygen consumption rate of larvae treated with RCH (1.33 ± 0.02 nmol min^{-1} mg^{-1} FM) was significantly higher than that of directly frozen larvae (1.06 ± 0.05 nmol min^{-1} mg^{-1} FM) and statistically indistinguishable from that of control larvae (1.35 ± 0.04 nmol min^{-1} mg^{-1} FM). The same trend was apparent after 24 h recovery, although the difference between RCH and directly frozen larvae was not quite statistically significant (Figure 2c; ANOVA, FDR, t_{22} = 2.31, p = 0.061).

Tissue damage

RCH reduced damage to fat body and midgut tissue after freezing. Cell survival in control samples was high for both tissues (0.93 ± 0.03 for fat body; 0.96 ± 0.02 for midgut),

indicating minimal damage during dissection and processing (Figure 3). Cell viability was also measured after 24 recovery from direct freezing and RCH treatments. In both tissues, freezing significantly reduced cell viability; cell survival after freezing decreased to 0.47 ± 0.02 and 0.65 ± 0.05 in fat body and midgut, respectively. However, in both tissues, RCH significantly improved cell survival after freezing (Figure 3; GLM, FDR, $p<0.05$), although tissue damage was still higher than in untreated samples.

Stress gene expression

In this experiment, we measured the mRNA expression of five heat shock proteins and *pepck,* a stress-responsive metabolic gene (Figure 4). Expression of *sHsp* did not change after 2 h recovery from either cold treatment (directly frozen and RCH) but was strongly upregulated ~3-fold in both groups after 24 h recovery (Figure 4a). Transcripts for *hsp40*, *hsp70*, and *pepck* changed in some treatment groups but were never different between directly frozen and RCH larvae (Figure 4b, d, f). However, two of the transcripts, *hsp60* and *hsp90*, showed distinct expression patterns in directly frozen and RCH larvae. In both cases, expression was statistically indistinguishable after 2 h recovery, but after 24 h recovery expression was higher in the directly frozen group. For *hsp60* expression was 35% higher in directly frozen larvae (Figure 4c; ANOVA, FDR, $t_{19} = 2.68$, $p = 0.032$), and for *hsp90* expression was 56% higher in directly frozen larvae (Figure 4e; ANOVA, FDR, $t_{19} = 4.28$, $p = 0.002$).

Energy store analysis

In response to freezing, there was significant accumulation of glucose after 2 h recovery, with larvae that were directly frozen accumulating significantly more glucose than larvae treated with RCH (Figure 5a; Permutation Test, FDR, $p = 0.023$). However, after 24 h recovery, glucose

levels for both groups were indistinguishable from controls. Trehalose levels remained constant after 2 h recovery, but there was a significant and nearly identical 14% increase in both directly frozen and RCH larvae after 24 h recovery (Figure 5b; ANOVA, FDR, $p<0.05$). Glycogen content was significantly reduced by ~15% in directly frozen larvae after 24 h recovery relative to the other four groups (Figure 5c, ANOVA, FDR, $p<0.05$), such that RCH larvae had higher levels of glycogen after 24 h recovery. There was a slight elevation of lipid in directly frozen larvae after 24 h recovery relative to controls, but there were no significant differences between directly frozen and RCH larvae at either recovery time (Figure 5d). Protein content increased slightly in RCH larvae after 24 h recovery relative to controls but was otherwise invariant across all treatment groups (Figure 5e).

DISCUSSION

Here, we provide several lines of evidence that RCH protects against sublethal freezing injury in larvae of *B. antarctica.* Previous work has demonstrated that RCH extends the limits of freeze tolerance (Kawarasaki et al., 2013; Lee et al., 2006a; Teets et al., 2008), yet the test temperatures in these studies (typically -15 to -20°C) are far colder than typical microclimate temperatures, which rarely approach -10°C (Kawarasaki et al., 2014a). Thus, lethal freezing events are rare or perhaps non-existent for *B. antarctica,* and the results presented here demonstrate that RCH can protect against nonlethal freezing injury at ecologically relevant temperatures. Other work in chill-susceptible insects has provided some evidence of the benefits of RCH at nonlethal conditions (Alvarado et al., 2015; Findsen et al., 2013; Kelty and Lee, 1999; Powell and Bale, 2006; Shreve et al., 2004), and by incorporating behavior, metabolic physiology, measurement of tissue damage, gene expression changes, and changes in

biochemical composition, our work demonstrates that RCH protects against multiple types of freezing injury that span levels of biological organization.

Preservation of locomotor function and metabolic rate

The current understanding of RCH suggests it is a plastic mechanism that allows ectotherms to "track" changes in environmental temperature in real-time (Lee and Denlinger, 2010). In our experiments, larvae treated with RCH regained motility 33% faster after a freezing event, and 2 h after freezing had already resumed normal locomotor behavior (albeit much slower than untreated larvae; Figure 2a, b). In chill susceptible insects, immobility from cold stress is a consequence of membrane depolarization at low temperatures (MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017), and recovery from chill coma requires restoration of ion balance to permit neuromuscular function (MacMillan et al., 2012). While these mechanisms are not as well-studied in freeze-tolerant insects, there is evidence of freezing-induced hyperkalemia in some species (Kristiansen and Zachariassen, 2001; Štětina et al., 2018), and current models of insect freeze tolerance propose that reversing these changes is essential for recovery during thawing (Toxopeus and Sinclair, 2018). Thus, it is likely that RCH either reduces the degree of ion dysregulation during freezing or allows larvae to restore ion gradients more quickly during recovery, thus permitting faster resumption of locomotor activity. Ecologically, quicker recovery from freezing would allow larvae to resume essential functions like feeding and microhabitat selection, which may be especially important during the brief austral summer.

Similar to locomotion, RCH also allowed larvae to maintain metabolic function after freezing. Larvae that were directly frozen had a 22% reduction in metabolic rate after 2 h recovery, and this reduction in metabolic rate was completely prevented by RCH (Figure 2c).

This pattern was also apparent after 24 h recovery, although the difference between directly frozen and RCH larvae was not quite statistically significant. This preservation of metabolic function by RCH likely allows larvae to kickstart the recovery process, which may explain the improvement of locomotor function immediately after freezing (Figure 2a,b). Post-freeze reduction in metabolic rate is also observed in the sub-Antarctic caterpillar *Pringleophaga marioni* (Sinclair et al., 2004), although RCH was not assessed in that study. Our results are consistent with the effects of freezing on mitochondrial function. In the freeze-tolerant goldenrod gall fly *Eurosta solidaginis,* freezing reduces mitochondrial cytochrome oxidase C activity (McMullen and Storey, 2008), and the activity of several mitochondrial enzymes is reduced in response to low temperature (Joanisse and Storey, 1994). Thus, our working model is that RCH protects against freezing-induced reductions in mitochondrial function, although we are unable to rule out other possibilities like changes in oxygen delivery. In chill-susceptible *Drosophila melanogaster,* cold acclimation prevents chilling-induced declines in mitochondrial coupling and ATP synthesis (Colinet et al., 2017).

Damage to tissues and proteins

While freezing at -9°C for 24 h was nonlethal, larvae experienced significant tissue damage. Nearly 55% of fat body cells and 35% of midgut cells died after freezing, and RCH reduced the amount of freezing damage by about half in each tissue (Figure 3). Thus, as we have observed previously for lethal freezing stress, RCH preserves cell viability and reduces damage to tissues (Kawarasaki et al., 2013; Lee et al., 2006b; Teets et al., 2008). Fat body and midgut tissues are critical for growth and development, so protection of these tissues by RCH is likely essential for resumption of normal activity.

We also measured mRNA expression of heat shock proteins as a proxy for protein damage at the cellular level. Heat shock proteins are well-studied in *B. antarctica* and are activated by a variety of stressors, including dehydration (Lopez-Martinez et al., 2009; Teets et al., 2012b), UV exposure (Lopez-Martinez et al., 2008), and repeated freeze-thaw cycles (Teets et al., 2011). Here, we show that beneficial hardening that reduces freezing injury also reduces expression of certain heat shock proteins. All heat shock proteins were elevated for at least some of the recovery times (Figure 4), which indicates that recovery from freezing activates the heat shock response. For *sHsp*, *hsp40,* and *hsp70,* expression patterns were indistinguishable between directly frozen and RCH at both time points. However, for *hsp60* and *hsp90,* expression was lower after 24 h recovery in RCH larvae. This result is consistent with RCH reducing cellular protein denaturation and thus reducing the signal for heat shock protein expression. *Hsp60* and *hsp90* are both regulated by heat shock factor, a transcription factor that is released from binding partners in response to protein denaturation (Feder and Hofmann, 1999). It is unclear at this point why *hsp60* and *hsp90* show this pattern, and not others, although it is worth noting that *hsp60* encodes a mitochondrial heat shock protein (Voos and Rottgers, 2002), which is consistent with the idea that RCH reduces damage to mitochondria (see above).

RCH provides energetic benefits

The ultimate measure of sublethal benefits of RCH would be a direct measure of fitness. However, *B. antarctica* has a two-year life cycle and is unamenable to laboratory rearing, which prevents measures of adult fitness after larval stress. Thus, we measured energy stores as a proxy for potential fitness benefits of RCH. Our earlier work showed that freezing is energetically costly for *B. antarctica* (Teets et al., 2011), and repeated freeze-thaw cycles in prepupae of the freeze-tolerant fly *E. solidaginis* reduce fecundity of adult females (Marshall and Sinclair, 2018).

In our experiments, larvae that were directly frozen experienced a 16% decrease in glycogen content (Figure 5c), which is the major carbohydrate energy store in larvae. Lipids, the major energy store in terms of caloric content, did not differ between directly frozen and RCH larvae. However, it is worth noting that the vanillin assay we used is sensitive to the degree of saturation of fatty acids, so any changes in lipid saturation in response to freezing could obscure changes in bulk lipids (Williams et al., 2011).

The decrease in glycogen is perhaps explained by mobilization of glucose. Free glucose was mostly undetectable in untreated larvae, but directly frozen larvae had significantly higher levels of glucose after 2 h recovery, and this mobilization of glucose was muted in RCH larvae (Figure 5a). While we did not detect significant glycogen depletion at 2 h recovery, the amount of glucose liberated only represented ~1.5% of the available glycogen pool, so we were likely unable to detect any glycogen depletion at this time. Also, while glucose returned to baseline levels at 24 h recovery, it may have continued to increase during recovery in directly frozen larvae, which could explain the glycogen depletion in this group at 24 h recovery. Indeed, in our previous work on repeated freezing, elevated levels of glucose remained high after 12 h recovery (Teets et al., 2011), a time point our sampling scheme would have missed. Glucose likely serves as both a substrate for cryoprotectant synthesis and a fuel for metabolism (Calderon et al., 2009), and stress-induced mobilization of glucose in *B. antarctica* was previously observed in response to freezing and desiccation stress (Teets et al., 2011; Teets et al., 2012a). After 24 h recovery, both directly frozen and RCH larvae had a slight, but significant, increase in trehalose, the major blood sugar and a potent cryoprotectant (Crowe, 2007). While we are unable to account for the source of this extra trehalose, the lack of glycogen depletion in RCH larvae suggests the observed increase in trehalose could be the result of reduced breakdown rather than *de novo*

synthesis. However, without detailed analyses of metabolic flux, we are unable to reconcile the exact source of each metabolite change. Also, we note that our measurements were taken relatively soon after stress with a limited temporal resolution, so additional experiments are needed to conclude that the short-term energy deficits we are observing ultimately lead to fitness consequences.

The observed differences in glycogen content between directly frozen and RCH larvae are seemingly at odds with the metabolic rate data (Figure 2c). Metabolic rates were higher in RCH larvae, with a significant difference at 2 h recovery and a near significant difference at 24 h, yet RCH larvae had higher levels of glycogen after 24 recovery. We provide two possible explanations for this discrepancy: 1) While we held larvae on filter paper overnight to promote gut clearance, it is possible some food particles remained in the gut lumen. If that were the case, reduced gut damage in RCH larvae (Figure 3) may have promoted increased assimilation of any remaining gut content and allowed RCH larvae to maintain energy balance. The specific carbohydrate assays we use (glucose, trehalose, and glycogen) would be unable to detect residual plant sugars present in the gut lumen 2) Freezing stress may result in increased reliance on anaerobic metabolism, which use energy substrates less efficiently. Frozen insects (including *B. antarctica*) accumulate anerobic end products (Michaud et al., 2008; Storey et al., 1981), and an increased reliance on anaerobic metabolism could explain the increased glycogen depletion despite a lower oxygen consumption rate. Nonetheless, our results suggest a slight, but significant energetic benefit of RCH, which may ultimately provide a fitness advantage in the short growing seasons of terrestrial Antarctica.

Conclusions

We provide strong evidence that RCH protects against sublethal freezing injury at several levels of organization. Relative to directly frozen larvae, larvae treated with RCH regain locomotor activity more quickly, have higher metabolic rates, reduced damage to tissues, reduced damage to proteins, and higher levels of glycogen energy stores. Additional work is needed to address the extent to which these same types of injuries occur in the field or in response to ecologically relevant thermal regimes, to reconcile the apparent discrepancies between metabolic rate and energy stores, and to identify the exact sources for the observed shifts in carbohydrate metabolites. The observed symptoms of freezing injury are all consistent with current models of freeze tolerance (Toxopeus and Sinclair, 2018) and further highlight the multitude of challenges that must be overcome to cope with freezing. Our experiments add to this literature by demonstrating that beneficial acclimation through RCH can prevent or reduce organismal and suborganismal freezing injury at ecologically relevant temperatures.

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Competing interests

No competing interests declared

Author contributions

N.M.T. designed the experiment; N.M.T., Y.K., L.J.P., B.N.P., and J.D.G. collected samples and conducted physiological experiments; N.M.T. conducted molecular and biochemical analyses,

N.M.T. analyzed data, N.M.T., D.L.D., and R.E.L. wrote the paper; all authors contributed to drafts and approved the final version of the manuscript.

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Data availability

Data for this paper are provided in an excel spreadsheet (Table S1) and will be made available on Dryad (DOI: https://doi.org/10.5061/dryad.29p7ng2) at the time of acceptance.

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Figure Legends

Fig. 1. Freeze tolerance of summer acclimatized larvae. Larvae were exposed to the indicated temperatures for 24 h in groups of 20, n = 3 groups per temperature. Each sample is an independent replicate, and each sample was measured at the same time. Larvae were submerged in water with a small piece of ice to ensure inoculation, such that larvae were frozen at each of the indicated temperatures. Each point represents a single group of larvae, and the red dashed line is a logistic regression fit of the data. For each temperature, we measured three replicates; for some groups the replicates are obscured because all samples had the same survival.

Fig.2. RCH improves (A) recovery time and (B) movement speed immediately after freezing, and (C) it allows larvae to maintain a higher metabolic rate after freezing. In axis labels, C = control, DF = directly frozen, and RCH = rapid cold hardening. In (C), FM = fresh mass. In (A) and (B) jittered points represent individual larvae, and boxplots summarize distribution. In (C) bars mean ± SE, and overlaid jitter plots show individual data points. In (A), the p-value is the result of a Log-Rank test comparing recovery times of directly frozen and RCH larvae, followed by FDR correction, and n = 40 for each group. In (B-C) letters indicate statistically significant differences (Permutation test followed by FDR correction in B; ANOVA followed by pairwise t-tests with FDR correction for c; $p<0.05$). In (B), sample sizes for each

group, as they appear from left to right in the figure, are n = 40, 39, 39, 40, 40, and 35, while for (C) samples sizes are n = 6, 5, 5, 5, and 6. Each sample is an independent biological replicate, and the entire experiment was conducted one time.

Fig. 3. RCH reduces tissue damage in (A) fat body and (B) midgut after freezing. Bars represent mean ± SE cell survival and overlaid jitter plots show individual data points. A representative microscopic image is placed above each bar; live cells fluoresce green while dead cells fluoresce red. Different letters indicate a significant difference between groups (ANOVA, Tukey, $p<0.05$). For (A) sample sizes are n = 4 for each group, and for (B) n = 5 for each group. Each sample is an independent biological replicate, and the entire experiment was conducted one time.

Fig. 4. RCH alters expression of stress protein transcripts. Whole-body expression of five heat shock protein genes, one from each of the major families, and *pepck,* a stress-inducible metabolic gene, were measured. Expression levels are normalized to a reference gene (*rpl19)* and converted to a fold-change scale. Bars represent mean ± SE, and overlaid jitter plots show individual data points. Different letters indicate significant differences between groups (ANOVA, FDR, $p<0.05$). In axis labels, DF = directly frozen, RCH = rapid cold hardening. For each group, n = 5 with the exception of RCH with 2 h recovery, for which n =4. Each sample is an independent biological replicate, and the entire experiment was conducted one time.

Fig. 5. RCH conserves glycogen energy stores relative to direct freezing. Levels of major energy stores were measured after direct freezing (DF) and rapid cold hardening (RCH) treatments. In (A) bars represent the median value while jittered points indicate individual samples. In (B-E) bars represent mean and overlaid jitter plots show individual data points. Letters represent significant differences between groups (Permutation test, FDR, $p<0.05$ for a;

ANOVA, FDR, $p<0.05$ for b-e). In (A-C) and (E), n = 5 for each group; for (D) n = 5 except for controls, for which n = 4. Each sample is an independent biological replicate, and the entire experiment was conducted one time.

Tables

Table 1. Primers used for qPCR.

Gene	GenBank Accession #	Forward Primer	Reverse Primer
rpl19	JX462670	ACATCCACAAGCGTAAGGCTGAGA	TTCTTGTTTCTTGGTGGCGATGCG
sHsp	GAAK01009816	GACACCCTTATCAGACGACTAC	CTTCTCGTTCTTCGTGCTTTG
Hsp40	GAAK01004380	ACTCTGACCGGAGAAGTGATA	CTCGCTTTGTTGGCTCTTTG
Hsp60	GAAK01010161	GTTGCAGGGAGTTGACATAC	GGCAACAGTTACACCATCTT
Hsp70	GAAK01011953	CTGCTTTGGCTTACGGTTTG	CCTTCGTCGATGGTCAAGATAG
Hsp90	GAAK01011429	CCGGTGGTAGCTTTATCATCTC	GGTAACGATGGCCTTGATCTTA
Pepck	JX462659	AAATGCCTGCACTCAGTTGGAACC	GCTCAGTGCTGGTTTGTGCAAGAT

Figures

Figure 1

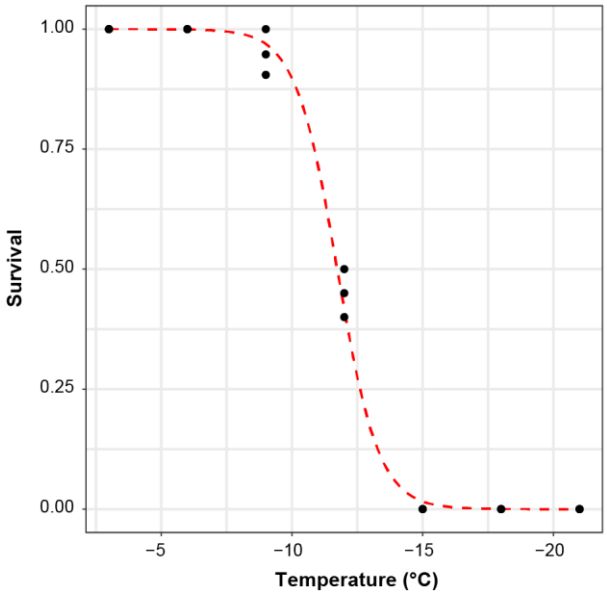
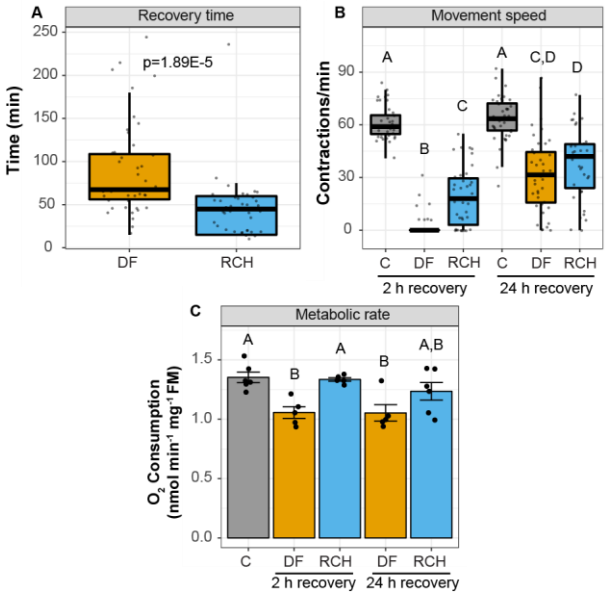


Figure 2


A
Recovery time
p=1.89E-5
Time (min)
250
200
150
100
50
0
DF
RCH
B
Movement speed
Contractions/min
90
60
30
0
A
B
C
A
C,D
D
C
DF
RCH
C
DF
RCH
2 h recovery
24 h recovery
C
Metabolic rate
O_2 Consumption (nmol min^{-1} mg^{-1} FM)
1.5
1.0
0.5
0.0
A
B
A
B
A,B
C
DF
RCH
DF
RCH
2 h recovery
24 h recovery

757 Figure 3

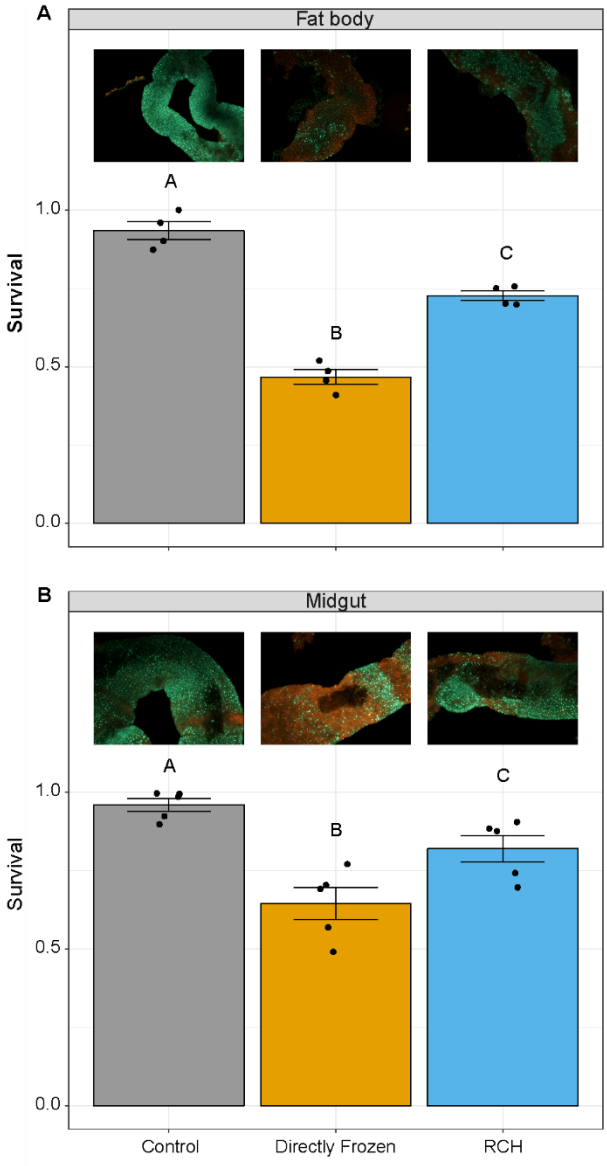


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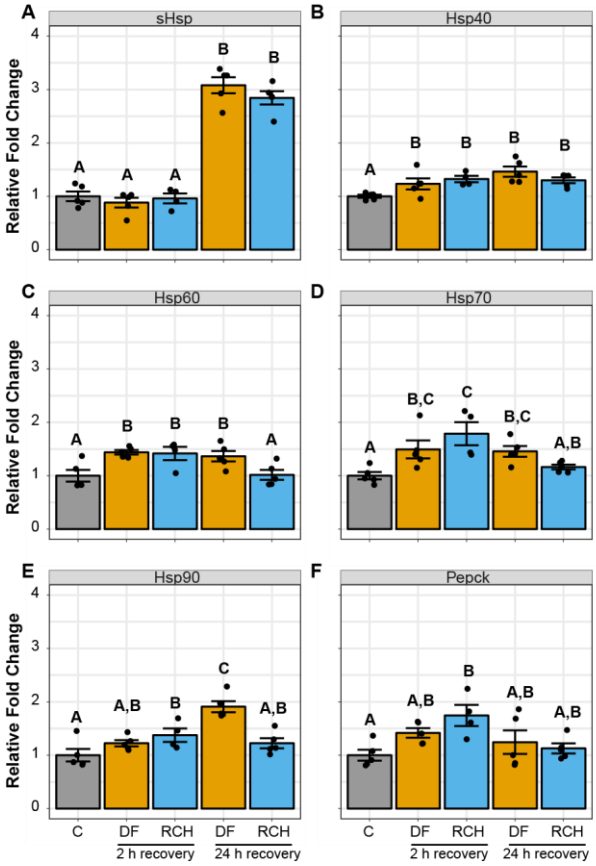
Figure 4


A
sHsp
B
Hsp40
C
Hsp60
D
Hsp70
E
Hsp90
F
Pepck
Relative Fold Change
0
1
2
3
4
C
DF
RCH
DF
RCH
2 h recovery
24 h recovery

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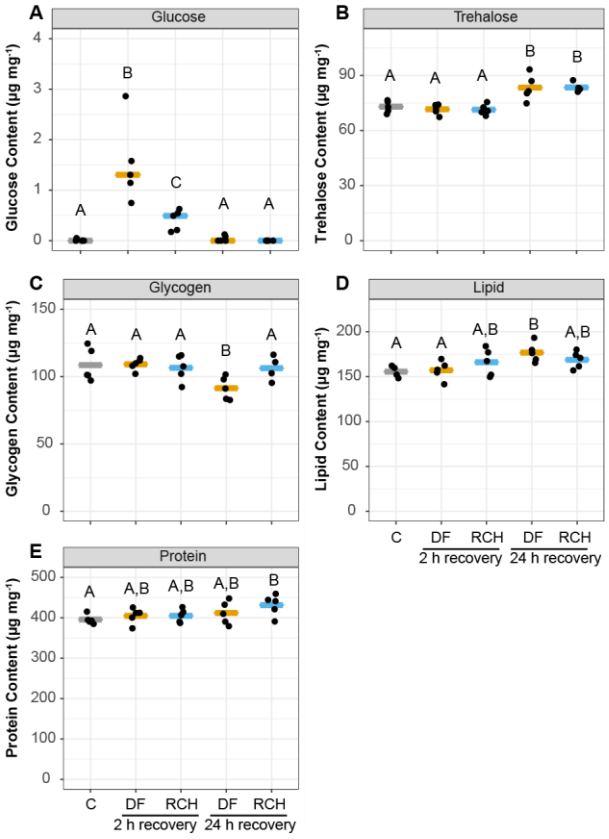

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