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RESEARCH ARTICLE

The protective effect of rapid cold-hardening develops more quickly in frozen *versus* supercooled larvae of the Antarctic midge, *Belgica antarctica*

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SUMMARY

During the austral summer, larvae of the terrestrial midge *Belgica antarctica* (Diptera: Chironomidae) experience highly variable and often unpredictable thermal conditions. In addition to remaining freeze tolerant year-round, larvae are capable of swiftly increasing their cold tolerance through the rapid cold-hardening (RCH) response. The present study compared the induction of RCH in frozen *versus* supercooled larvae. At the same induction temperature, RCH occurred more rapidly and conferred a greater level of cryoprotection in frozen *versus* supercooled larvae. Furthermore, RCH in frozen larvae could be induced at temperatures as low as –12°C, which is the lowest temperature reported to induce RCH. Remarkably, as little as 15 min at –5°C significantly enhanced larval cold tolerance. Not only is protection from RCH acquired swiftly, but it is also quickly lost after thawing for 2 h at 2°C. Because the primary difference between frozen and supercooled larvae is cellular dehydration caused by freeze concentration of body fluids, we also compared the effects of acclimation in dehydrated *versus* frozen larvae. Because slow dehydration without chilling significantly increased larval survival to a subsequent cold exposure, we hypothesize that cellular dehydration caused by freeze concentration promotes the rapid acquisition of cold tolerance in frozen larvae.

Key words: Antarctic midge, rapid cold-hardening, freeze concentration, cellular dehydration.

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INTRODUCTION

Terrestrial habitats of polar regions represent some of the most challenging environments in the world, and the ecological success of arthropods in these regions depends primarily on their ability to cope with extreme abiotic conditions (Danks, 1999; Peck et al., 2006). For most of the year, temperatures are persistently below freezing, and water is present only as ice, a biologically unavailable form (Kennedy, 1993). Accordingly, polar arthropods exhibit a considerable degree of flexibility in their life-history strategies to exploit a short growing period during the summer (Convey, 2010). However, even during this favorable period, environmental conditions often change unpredictably, requiring adaptations that allow these organisms to swiftly adjust their physiological performance and tolerance to environmental stresses (Danks, 1999).

Endemic to the Antarctic Peninsula, the terrestrial midge *Belgica antarctica* Jacobs (Diptera: Chironomidae) is the southernmost free-living species of insect (Sugg et al., 1983; Usher and Edwards, 1984). During its 2-year life cycle, overwintering larvae are encased in a matrix of substrate and ice for up to 7–8 months each year. Although ambient air temperature reaches winter lows of –40°C, temperature within larval microhabitats rarely dips below –5°C, owing to thermal buffering by the oceanic influence and accumulated snow (Baust and Lee, 1981; Elnitsky et al., 2008). By contrast, late in the austral summer, larvae experience diurnal fluctuations and unpredictable weather conditions that result in sudden, acute exposure to subzero temperatures. In addition to remaining freeze tolerant year-round (Baust and Lee, 1987), these larvae are capable of quickly enhancing their cold tolerance by the process of rapid cold-hardening (RCH) (Lee et al., 2006b).

In contrast to cold acclimation, which takes place over the course of days to weeks, the RCH response describes the capacity of insects to dramatically enhance their cold tolerance within minutes to hours (Lee et al., 1987; Lee and Denlinger, 2010). Not only does this response allow insects to instantaneously acquire protection against deleterious effects of cold, but it also functions more generally to preserve or enhance their organismal performance within a thermally variable environment (Shreve et al., 2004). Documented in over 30 species from six different orders, it is now clear that the RCH response is widespread among freeze-intolerant insects (Elnitsky and Lee, 2009). However, only recently was the RCH response extended to freeze-tolerant insects when it was first reported in frozen larvae of *B. antarctica* (Lee et al., 2006b).

Despite the common perception, life in the frozen state is far from static, as significant biochemical and physiological changes occur in frozen ectotherms. During weeks of freezing, ATP levels decline and lactate accumulates in larvae of the goldenrod gall fly, *Eurosta solidaginis* (Storey and Storey, 1985a). In addition to accumulating glycerol, caterpillars of *Gynaephora groenlandica* reduce the number of mitochondria per cell during 3 months of freezing at –15°C, likely functioning to conserve energy (Kukal et al., 1988; Kukal et al., 1989). Even more complex processes associated with progression through the stages of diapause continue in frozen larvae of *E. solidaginis* (Irwin et al., 2001).

In addition to changes occurring during a long-term cold acclimation, some freeze-tolerant organisms can quickly enhance their cold tolerance in response to the onset of internal ice formation. In the wood frog, *Rana sylvatica*, glucose levels increase markedly within minutes of ice nucleation in body fluids, providing cryoprotection against freezing injury (Storey and Storey, 1985b; Costanzo et al., 1993). Similarly, the Siberian earthworm, *Eisenia nordenskioeldi*, accumulates significant amounts of glucose within 24 h after the onset of freezing (Holmstrup et al., 1999). However, little is known about insects' ability to undergo rapid acclimatory changes in response to the initiation of freezing or while frozen.

Whether larvae of *B. antarctica* freeze inoculatively by contact with environmental ice or remain supercooled during an acute exposure to cold likely depends on hydric conditions of their microhabitat (Elnitsky et al., 2008; Teets et al., 2011). The primary purpose of our study was to compare the RCH response in frozen *versus* supercooled larvae. Evidence for the induction of RCH was assessed based on organismal and cellular levels of freeze tolerance. Because our results indicated that RCH protection developed more quickly in frozen larvae, we further characterized the induction and the loss of RCH protection in the frozen state. Secondly, to determine whether cold acclimation following RCH confers additional cold hardening, we investigated the effect of increasing the duration of exposure to a mildly low temperature for up to 5 days. Lastly, because acclimation to mild dehydration confers cross-tolerance to cold (Hayward et al., 2007; Benoit et al., 2009; Elnitsky et al., 2009), we compared the effects of dehydration *versus* freezing in promoting cold tolerance.

MATERIALS AND METHODS

Source of insects

Substrate containing larvae of *B. antarctica* was collected on Cormorant Island, Humble Island and Christine Island near Palmer Station on the Antarctic Peninsula (64°46′S, 64°04′W) in February and March 2011. Prior to experiments, larvae were extracted from substrate into ice-cold water using a modified Berlese method and subsequently handpicked. Larvae were then placed on moist filter paper at 2°C for at least 12 h to ensure gut clearance (mean gut clearance ~6 h) (Baust and Edwards, 1979).

Microhabitat temperature

Miniature temperature loggers (HOBO Water Temp Pro, Onset Computer Corporation, Pocasset, MA, USA) were deployed on the Antarctic Peninsula in microhabitat sites containing larvae of *B. antarctica* in February 2011. Loggers recorded the temperature at 30 min intervals. The loggers were recovered in February 2012 and the resulting data were extracted using BoxCar Pro 4.3 software (Onset Computer Corporation).

Determination of larval supercooling point, water content and hemolymph osmolality

Larvae were gently blotted dry on an absorbent tissue and individually placed in direct contact with a thermocouple and cooled at ~0.5°C min^{-1}. The supercooling point was taken as the lowest temperature reached prior to the latent heat of fusion resulting from freezing of the body water. Water content of individual larvae was assessed gravimetrically from measurements of fresh mass (to the nearest 0.002 mg) and dry mass after drying to constant mass at 65°C.

Hemolymph osmolality was determined using the vapor pressure depression technique (Holmstrup and Sømme, 1998). Groups of five larvae were removed from experimental conditions, placed in a sample holder and quickly crushed with a Teflon rod to expose the body fluid. The sample was then equilibrated for 30 min within a C-52 sample chamber (Wescor, Logan, UT, USA). Osmolality was measured using a Wescor HR-33T Dew Point Microvoltmeter operated in the dew point depression mode.

Experimental conditions for assessing larval cold tolerance

The discriminating temperature for assessing the limit of larval cold tolerance was empirically determined by directly exposing groups of 25 larvae to various subzero temperatures for 24 h. Larvae were cooled in the presence of water (~200 µl), and a small piece of ice was added to ensure the water froze at or slightly below 0°C. Because larvae are highly susceptible to inoculative freezing (Lee et al., 2006b; Elnitsky et al., 2008), contact with surrounding ice would induce internal ice formation at the melting point of larval body fluids (~–0.7°C). Direct exposure to –18°C for 24 h caused >60% mortality in larvae and increased survival at this temperature was used as evidence for RCH induction.

To characterize the RCH response in frozen larvae, groups of 25 individuals were first exposed directly to various subzero temperatures ranging from –3 to –15°C for 2 h. Larvae were cooled in water (~200 µl) containing a small piece of ice to ensure larvae froze at their melting point (~–0.7°C). Immediately after this pre-treatment, tubes containing frozen larvae were directly transferred to a cold bath set at –18°C. Alternatively, induction of RCH in supercooled larvae was tested by exposing groups of 25 individuals to –3 or –5°C for 2 h in dry tubes (i.e. containing no water). A previous study demonstrated that nearly all larvae would remain supercooled at –5°C for up to 12 h when cooled in this manner (Teets et al., 2011). Ice-cold water and a small piece of ice were added to dry tubes containing supercooled larvae immediately prior the exposure to –18°C to assess changes in levels of freeze tolerance.

The rate of RCH induction was investigated by exposing larvae to –5°C for 15, 30, 60 or 120 min, and changes in cold tolerance were tested by a subsequent exposure to –18°C. RCH protection is transient and often lost upon return to milder temperatures (Chen et al., 1991). Therefore, the effect of warming on the loss of RCH protection was tested by allowing larvae to thaw at 2°C for 2, 6 or 12 h, following RCH at –5°C for 2 h, and cold tolerance at –18°C was subsequently examined.

We tested whether the protective effect of RCH could be enhanced by increasing the duration of cold acclimation from hours to days. Groups of larvae were held either frozen or supercooled at –3°C for various durations ranging from 2 h to 5 days and changes in cold tolerance were subsequently assessed as described above.

Assessment of organismal survival

Following exposure to the discriminating temperature, tubes containing larvae were quickly thawed and allowed to recover on moist filter paper for 24 h at 2°C. Larvae were judged to have survived if they moved either spontaneously or in response to gentle prodding. Each treatment group consisted of ~50 animals.

Assessment of cell survival of midgut tissue

Midgut tissue of *B. antarctica* is highly sensitive to sublethal damage from freezing (Lee et al., 2006b; Teets et al., 2008), making it a suitable tissue to assess the effect of RCH protection at the cellular level. Following a 24 h recovery from exposure to –18°C, midguts were dissected from living, non-moribund larvae in Coast's solution (Coast and Krasnoff, 1988). Tissue samples were stained using the

LIVE/DEAD sperm viability kit, as described previously (Yi and Lee, 2003), and images were documented using fluorescent microscopy. With this staining technique, living cells with intact membranes fluoresced green, whereas dead cells with damaged membranes fluoresced red. For each treatment, mean values of cell survival were determined from four individuals. Cell survival for each individual was based on the mean count of three groups of 100 cells from randomly selected regions. Nearly 90% survival in the untreated group indicated that damage inflicted during dissection of the midgut tissue was minimal.

Cryoprotectant analysis

To determine whether larvae accumulate low-molecular-mass sugars, we examined levels of glucose and trehalose because these are the major cryoprotectants for *B. antarctica* larvae (Baust and Lee, 1983). Immediately after acclimation, five replicates of ~25 larvae were quickly weighed and frozen at –80°C until whole-body concentration of sugars was determined. Prior to cryoprotectant analysis, larvae were homogenized in 7% perchloric acid and neutralized with KOH. Trehalose level was determined following enzymatic digestion by trehalase from porcine kidney (T8778, Sigma-Aldrich, St Louis, MO, USA) as per the manufacturer's instructions. Glucose concentration was determined using the glucose oxidase reagent set (G7519, Pointe Scientific, Canton, MI, USA).

Experimental conditions to compare effects of freezing *versus* dehydration

To achieve acclimation in the frozen state, larvae were exposed to –2°C in the presence of water (~200 μl) containing a small piece of ice. To induce cryoprotective dehydration, groups of five larvae were gently blotted dry and confined in 0.2 ml PCR tubes by means of fine nylon mesh. PCR tubes were then placed in 15 ml screw-cap vials containing ice at the bottom, and vials containing larvae were directly exposed to –2°C. Dehydration without chilling was induced at 2°C and 99% relative humidity (RH) generated in sealed desiccators using a saturated K_2SO_4 solution (Winston and Bates, 1960). Water potential values produced by both of these dehydrating conditions were calculated to be approximately –24 bar based on van't Hoff's equation. Groups of 10 larvae were confined to 0.6 ml microcentrifuge tubes by means of nylon mesh. Meanwhile, control animals were held at 2°C in a sealed Petri dish lined with moist filter paper. No significant mortality was observed during exposure to any of these experimental conditions.

Subsets of samples from treatments above were removed after 2 and 5 days to assess larval water content, hemolymph osmolality and freeze tolerance. Larval water content and hemolymph osmolality were determined as described above. Levels of larval freeze tolerance were evaluated by exposing larvae to –20°C for 24 h, and organismal survival following 24 h recovery was assessed as described above.

Statistical analysis

Organismal survival data were analyzed using a generalized linear model with the logistic link and binomial error distribution (Hosmer and Lemeshow, 2000). Cell survival data were analyzed using a generalized linear mixed model with the logistic link and binomial error distribution, in which regions within midgut tissue were considered as random effects. Differences in mean values for glucose and trehalose contents were tested for significance using one-way ANOVA. Differences in mean water content and hemolymph osmolality among treatment groups were compared using two-way ANOVA. As necessary, data were transformed to fulfill the ANOVA assumptions. Statistical significance was judged at $P<0.05$ and either Bonferroni or Tukey's *post hoc* test was applied where significant effects by factors were detected. Whereas binomial survival data were analyzed using SAS (SAS Institute, Cary, NC, USA),

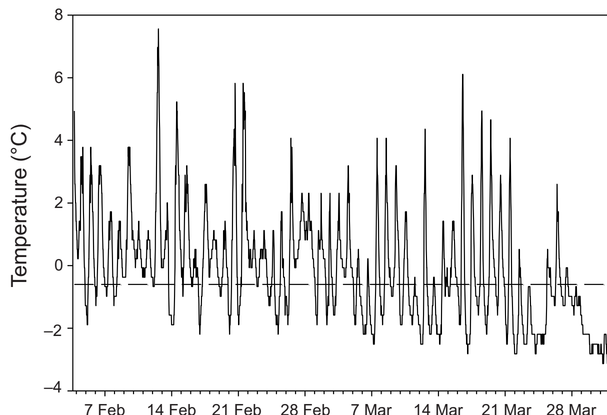

Fig. 1. Temperature fluctuations during the late austral summer (3 February–31 March 2011) at a representative microhabitat for larvae of *Belgica antarctica* on Humble Island, near Palmer Station, Antarctica (64°46 S, 64°04 W). The broken line indicates the equilibrium melting point of fully hydrated larvae (~–0.7°C).

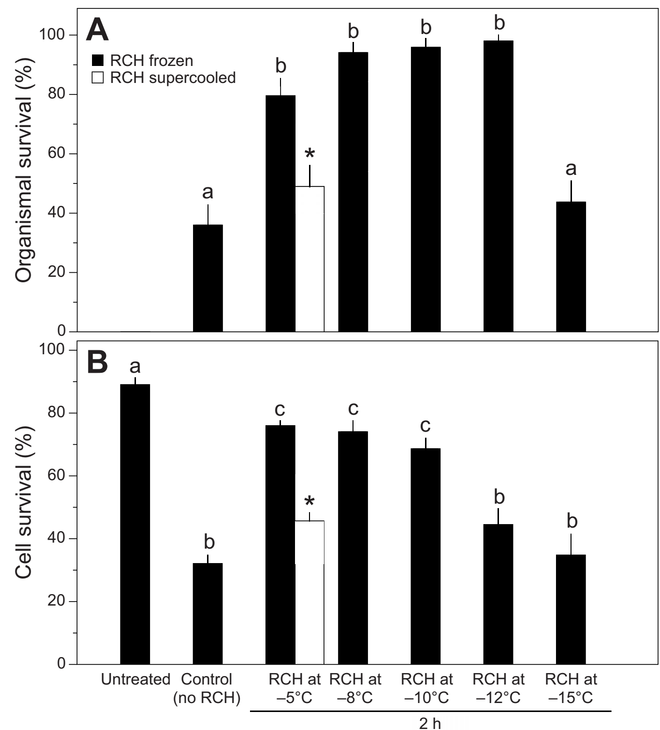

Fig. 2. Effects of rapid cold-hardening (RCH) at different temperatures on (A) organismal survival and (B) cell survival of larval midgut tissues in *B. antarctica*. RCH responses were induced by exposing larvae to various temperatures for 2 h, prior to testing their cold tolerance at –18°C for 24 h. The control group was directly exposed to –18°C for 24 h. Organismal survival was based on 47–51 larvae (±standard error of proportion). Cell survival was based on four or five replicates, with each replicate based on the mean of three groups of 100 cells (±s.e.m.). Values with different letters are significantly different within frozen groups. Asterisks (*) denote significant differences between frozen and supercooled groups (Bonferroni, family-wise $P<0.05$).

parametric analyses with ANOVA models were performed using R (R Development Core Team, 2013).

RESULTS

Microhabitat temperatures

During late summer, temperatures in the larval microhabitat of *B. antarctica* generally fluctuated between 5 and –3°C (Fig. 1). At this time, the melting point of larvae was ~–0.7°C, based on hemolymph osmolality (389±19 mOsm kg^{-1}; *N*=6). Within a 2-month period from 3 February to 31 March 2011, larvae occupying this microhabitat could have experienced as many as 40 cycles of freezing and thawing of their body fluids. However, because the supercooling point of larvae was –7.3±0.6°C (*N*=10), larvae may have remained supercooled if they avoided inoculative freezing by environmental ice.

Comparision of RCH in frozen *versus* supercooled larvae

When directly exposed to –18°C for 24 h, only 36% of larvae survived (Fig. 2A). However, following RCH at –5°C for 2 h in the frozen state, larval survival increased significantly to ~80%. By contrast, in supercooled larvae, exposure to –5°C for 2 h was insufficient to induce cryoprotection by RCH (*P*=0.19, compared with control with no RCH). Similarly, when larvae were acclimated at –3°C, RCH protection developed within 6 h in frozen larvae, whereas supercooled larvae required 24 h before the effect of RCH was evident (Fig. 4A). Furthermore, larvae frozen at –3°C for 24 h were better able to survive a more extreme freezing challenge of –21°C for 24 h than supercooled larvae (Fig. 4A). Overall, RCH occurred more rapidly and conferred a greater level of cryoprotection in frozen *versus* supercooled larvae.

Cell survival in midgut tissue closely matched survival at the organismal level. Following exposure to –5°C for 2 h, cell survival was significantly higher by ~30% in frozen larvae compared with supercooled larvae (Fig. 2B, Fig. 3). Similarly, greater RCH protection at the cellular level was observed in frozen larvae after acclimation at –3°C for 12 h (Fig. 4B).

Induction and the loss of RCH protection

Similar levels of cryoprotection were observed in frozen larvae held for 2 h at temperatures ranging from –5°C to as low as –12°C (Fig. 2A). Neither acclimation at –3°C nor acclimation at –15°C for 2 h elicited the RCH response at the organismal level (Fig. 2A, Fig. 4A). Cryoprotection in midgut tissue closely matched survival at the organismal level, with significant increases in cold tolerance observed after RCH at temperatures between –5 and –10°C (Fig. 2B, Fig. 4B).

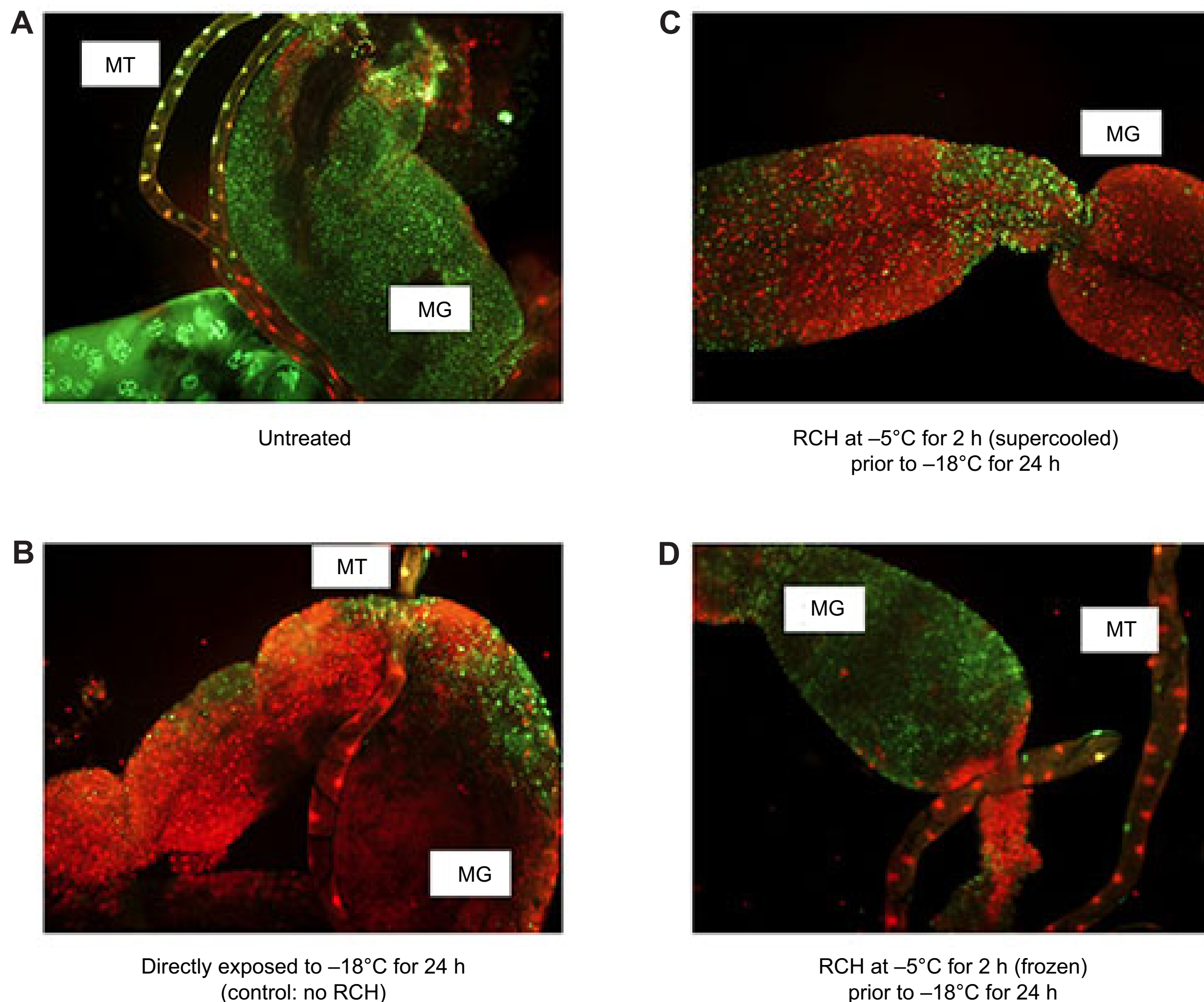

Fig. 3. Representative images of the effect of acclimation temperature on cell viability in midgut (MG) tissues of *B. antarctica* larvae. Tissues were dissected from larvae that survived exposure to –18°C for 24 h. Green indicates living cells, whereas dead cells are stained red. Malpighian tubules (MT) are also shown.

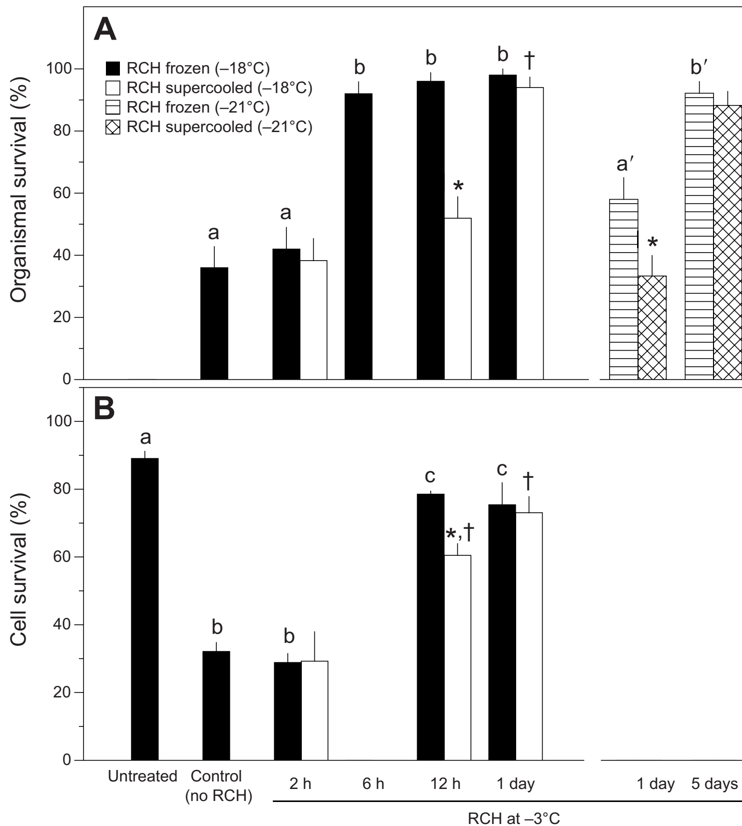

Fig. 4. Effect of varying the duration of acclimation at –3°C on (A) organismal and (B) cell survival of larval midgut tissues in *B. antarctica*. Prior to testing their cold tolerance at –18 or –21°C for 24 h, larvae were exposed to –3°C for various intervals. The control group was directly exposed to –18°C for 24 h. Organismal survival was based on 47–52 larvae (±standard error of proportion). Cell survival was based on four or five replicates, with each replicate based on the mean of three groups of 100 cells (±s.e.m.). Values with different letters are significantly different within frozen groups exposed to the same discriminating temperature. Asterisks (*) denote significant differences between frozen and supercooled groups. Crosses (†) denote significant differences between treatment and control groups (Bonferroni, family-wise $P<0.05$).

The protective effect of RCH was induced after as little as 15 min at –5°C in frozen larvae and continued to increase over 120 min of exposure (Fig. 5A). Similarly, cell survival in midgut tissue was enhanced significantly after 30 min at this temperature (Fig. 5B). The protection afforded by RCH was quickly lost upon thawing (Fig. 6). As few as 2 h at 2°C were sufficient to induce partial loss of the RCH protection acquired by frozen larvae held at –5°C for 2 h.

Effect of cold acclimation following RCH

Cold tolerance of *B. antarctica* larvae was progressively enhanced as the duration of exposure was increased for up to 5 days (Fig. 4). Because nearly all larvae survived exposure to –18°C after acclimation for 1 day at –3°C, a new discriminating temperature of –21°C for 24 h was used to distinguish the protective effects elicited by 1 and 5 days of exposure. Compared with larvae frozen at –3°C for 1 day, those acclimated for 5 days exhibited significantly higher survival, indicating that the cryoprotection by RCH was further enhanced by cold acclimation.

Cryoprotectant analysis

Mobilization of low-molecular-mass cryoprotectants often accompanies cold-hardening in ectothermic organisms. Although the result of ANOVA was significant ($F_{10,44}$=2.684, P=0.01), Tukey's *post hoc* test failed to detect significant differences in glucose levels (Table 1). By contrast, RCH and cold acclimation generally resulted in significant increases in trehalose levels by ~35% compared with the control group (Table 1). However, significant increases in trehalose levels were equally evident in supercooled larvae exposed to –5°C for 2 h, and in both frozen and supercooled larvae held at –3°C for 2 h, even though these treatments did not enhance organismal freeze tolerance (Fig. 2A, Fig. 4A).

Effects of low temperature and dehydration on larval cold tolerance

In this experiment, we investigated the role of cellular dehydration in promoting larval cold tolerance. Both dehydration at –2°C in the presence of environmental ice (i.e. cryoprotective dehydration) and exposure to 99% RH at 2°C (i.e. slow dehydration without chilling) substantially reduced larval water content by ~45% after 5 days of exposure (Table 2). In response to these reductions in water content, hemolymph osmolality of dehydrated larvae increased to ~1000 mOsm kg^{-1}. Compared with dehydrated larvae, frozen individuals maintained relatively constant water content and hemolymph osmolality.

Slow dehydration (99% RH, 2°C) elicited the most rapid acquisition of cryoprotection, significantly enhancing larval freeze tolerance within 2 days (Table 2). By contrast, significant increases in larval survival at –20°C were evident only after 5 days in frozen and cryoprotectively dehydrated treatments. After 5 days of exposure, levels of cold tolerance were indistinguishable among experimental groups.

DISCUSSION

RCH at subzero temperatures

In temperate and tropical insects, RCH is induced at temperatures between 0 and 10°C (Elnitsky and Lee, 2009). However, larvae of *B. antarctica* are capable of undergoing RCH at subzero temperatures below the melting point of their body fluids (present study; Lee et al., 2006b; Teets et al., 2008). Similarly, in the subantarctic midge, *Eretmoptera murphyi*, the RCH response in supercooled larvae extends the lower limit of freeze tolerance (Everatt et al., 2012). In addition, other freeze-intolerant species of Antarctic arthropods, namely collembolans and mites, can swiftly increase their cold tolerance by decreasing their supercooling point

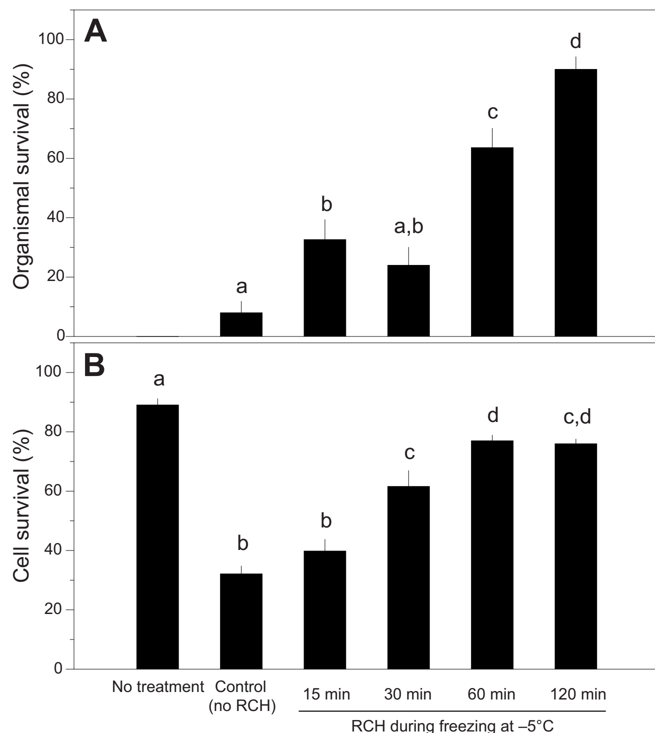

Fig. 5. Effect of varying the duration of exposure to –5°C on (A) organismal survival and (B) cell survival of larval midgut tissues in *B. antarctica*. Protective effects of RCH were tested by subsequently assessing larval cold tolerance at –18°C for 24 h. The control group was directly exposed to –18°C for 24 h. Organismal survival was based on 49–55 larvae (±standard error of proportion). Cell survival was based on four or five replicates, with each replicate based on the mean of three groups of 100 cells (±s.e.m.). Values with different letters are significantly different (Bonferroni, family-wise $P<0.05$).

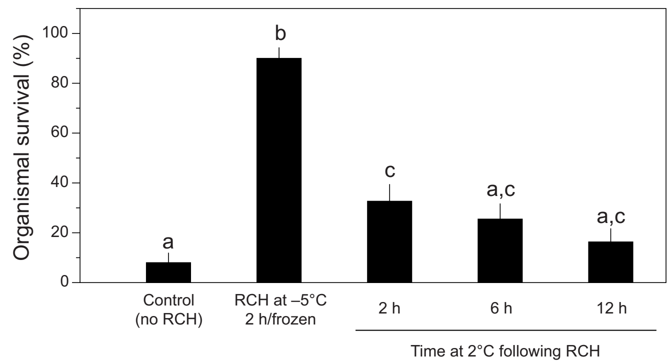

Fig. 6. Time course for the loss of protective effects generated by RCH. Following RCH at –5°C for 2 h in the frozen state, larvae were allowed to thaw at 2°C for various intervals and survival was assessed after subsequent exposure to –18°C for 24 h. The control group was directly exposed to –18°C for 24 h. Organismal survival was based on 49–51 larvae (±standard error of proportion). Values with different letters are significantly different (Bonferroni, family-wise $P<0.05$).

(Worland and Convey, 2001; Sinclair et al., 2003; Hawes et al., 2007). Although this swift increase in supercooling capacity is distinct from the RCH response (Lee et al., 2006b), these general capacities for rapid physiological changes at subzero temperatures reflect the importance of these adaptations for life in perpetually cold, but unpredictable and highly variable, conditions of the maritime Antarctic (Fig. 1) (Convey, 1996; Convey, 2010; Danks, 1999; Peck et al., 2006).

A notable characteristic of *B. antarctica* larvae is their capacity to rapidly cold-harden even while frozen (Lee et al., 2006b; Teets et al., 2008). In the present study, we demonstrated that RCH in frozen larvae could be induced at temperatures as low as –12°C, which is the lowest temperature known to induce the RCH response (Fig. 2A). This ability of *B. antarctica* to undergo RCH while frozen differs from that of *E. murphyi*, in which only supercooled larvae exhibit the RCH response (Everatt et al., 2012). The lack of an RCH response in frozen larvae of *E. murphyi* suggests that internal ice formation inhibits the RCH response in this species (Everatt et al., 2012).

Comparison of RCH in frozen *versus* supercooled larvae

During an episode of subzero exposure, whether larvae freeze inoculatively by contact with environmental ice or remain supercooled depends on hydric conditions within the microhabitat (Elnitsky et al., 2008). Therefore, we compared the efficacy of the RCH response in frozen *versus* supercooled larvae. Surprisingly, when acclimated for 2 h at –5°C, survival following a subsequent cold exposure at –18°C was significantly higher in frozen larvae compared with supercooled ones (Fig. 2A,B). Similarly, when larvae were acclimated at –3°C, RCH protection developed more rapidly in frozen larvae (Fig. 4A,B). Although accumulation of trehalose enhances freeze tolerance in larvae of *B. antarctica* (Benoit et al., 2009), trehalose levels were equally elevated in both frozen and supercooled larvae, and thus could not explain the difference in RCH protection (Table 1).

Why was the RCH response induced more rapidly in frozen larvae compared with supercooled ones? The primary difference between these treatments is that as ice forms extracellularly, only water molecules join the growing ice lattice, resulting in the freeze concentration of solutes in the remaining unfrozen body fluids (Mazur, 2004). Freeze concentration leads to a rapid efflux of water from cells, causing progressive cellular dehydration that continues until the freezing point of the intracellular fluid is colligatively depressed to match the environmental temperature. Based on the equation by Clausen and Costanzo (Clausen and Costanzo, 1990) and the hemolymph osmolality of larvae (389 mOsm kg^{-1}), we calculated the amount of ice that would form in larvae equilibrated at –3°C. Assuming a bound water fraction of 18.5% as per Worland et al. (Worland et al., 1998), 62.5% of body water froze, thereby concentrating the original solutes threefold. Although excessive levels of freeze concentration and cell shrinkage may result in lethal freezing injury and define the lower limit of freeze tolerance, we hypothesize that moderate cellular dehydration functions to enhance induction of the RCH response.

Cellular dehydration is critical for successful cryopreservation of isolated cells (Mazur, 2004). In the two-step freezing method for cryopreservation, cells are first slowly cooled to a moderately low temperature to allow them to dehydrate as ice forms in the surrounding solution, and are then rapidly transferred to an ultralow, storage temperature (Farrant et al., 1977). The protocol used in our study to test for RCH resembles this two-step freezing method. Exposure to mild freezing during RCH is equivalent to the initial phase of slow cooling in the two-step freezing method because it allows cells to effectively exchange water and cryoprotectants between extracellular and intracellular compartments, thereby decreasing the chance of lethal intracellular ice formation.

Table 1. The effect of rapid cold-hardening and cold acclimation on glucose and trehalose levels in larvae of *Belgica antarctica*

Treatment	Glucose (μg mg^{-1} dry mass)	Trehalose (μg mg^{-1} dry mass)
Control	0.86±0.12[a]	32.01±1.53[a]
−5°C for 2 h (frozen)	1.00±0.09[a]	42.97±4.22[b]
−5°C for 2 h (supercooled)	0.99±0.26[a]	48.06±1.50[b]
−8°C for 2 h (frozen)	0.94±0.14[a]	51.71±1.05[b]
−3°C for 2 h (frozen)	1.44±0.27[a]	43.14±2.32[b]
−3°C for 2 h (supercooled)	0.93±0.17[a]	48.86±1.90[b]
−3°C for 12 h (frozen)	1.53±0.17[a]	38.69±2.25[a,b]
−3°C for 12 h (supercooled)	1.17±0.15[a]	42.21±0.98[b]
−3°C for 1 day (frozen)	1.43±0.07[a]	43.99±3.53[b]
−3°C for 1 day (supercooled)	1.63±0.22[a]	41.05±0.61[a,b]
−3°C for 5 days (frozen)	1.45±0.12[a]	44.89±1.30[b]

Values are means ± s.e.m. (*N*=5). Different letters indicate significant difference between treatments (Tukey, family-wise *P*<0.05).

Dehydration at the cellular level is caused by various forms of environmental stress, including freezing, desiccation and salinity. Furthermore, adaptive mechanisms against these forms of osmotic stresses are commonly shared, and acclimation to one often promotes cross-tolerance to others (Holmstrup et al., 2010). For example, prior desiccation without chilling significantly improves freeze tolerance of the Antarctic nematode *Plectus murrayi* (Adhikari et al., 2010). Similarly, in *B. antarctica*, acclimation of larvae to either a desiccating environment (Hayward et al., 2007; Benoit et al., 2009) or hyperosmotic seawater (Elnitsky et al., 2009) in the absence of chilling increases freeze tolerance.

In the present study, we investigated the role of cellular dehydration in enhancing larval cold tolerance by comparing effects of acclimation for 2 or 5 days in frozen *versus* dehydrated larvae (Table 2). Based on the hemolymph osmolality of frozen larvae (555 mOsm kg^{-1}) and assuming 18.5% bound water, we calculated the amount of ice formed in larvae after equilibration at –2°C to be 41% of the total body water (Claussen and Costanzo, 1990). We then selected two conditions, with (–2°C) and without chilling (2°C), to induce an equivalent level of cellular dehydration. After 5 days of exposure, slow dehydration (99% RH, 2°C) and cryoprotective dehydration at –2°C caused a similar reduction in larval water content of 45 and 48%, respectively. Although larval freeze tolerance increased to a similar level after 5 days of exposure to these comparable osmotic stresses, slow dehydration without chilling was the most effective, enhancing larval survival to –20°C within 2 days (Table 2). Considering the relatively long duration of the exposure (i.e. days), this dehydration-induced increase in freeze tolerance was likely accompanied by changes in gene expression (Teets et al., 2012a; Teets et al., 2013a). Because the RCH response occurs too quickly to allow changes in mRNA levels (Teets et al., 2012b), future study is needed to examine whether cellular dehydration without chilling could elicit larval cold-hardening within minutes to hours.

In addition to a brief chilling, mild desiccation is now known to quickly increase cold tolerance of other freeze-tolerant insects. In larvae of *E. solidaginis*, as little as 10% reduction in body water content over a few hours significantly increases larval survival of freezing at –15°C by 30–40% (Levis et al., 2012). Similarly, modest reduction in body water content (12.1%) during exposure at 10°C for 6 h enhances freeze tolerance of a subantarctic caterpillar (Sinclair and Chown, 2003). The rapidity with which mild desiccation induces cold-hardening closely resembles the RCH response. The signal transduction mechanism of RCH involves cold-sensing by calcium signaling (Teets et al., 2008; Teets et al., 2013b) and phosphorylation of the p38 mitogen-activated protein kinase (MAPK) (Fujiwara and Denlinger, 2007). As these pathways are also linked to defense against osmotic and cell-volume disturbances in a variety of organisms (e.g. Xiong et al., 2002; Cowan and Storey, 2003), cellular dehydration in the absence of chilling may elicit similar responses to those of RCH, including an increase in membrane fluidity (Overgaard et al., 2005; Overgaard et al., 2006; Michaud and Denlinger, 2006; Lee et al., 2006a) and blocking of apoptotic pathways (Yi et al., 2007; Yi and Lee, 2011). Consequently, we speculate that internal ice formation enhances signal intensity for RCH induction in *B. antarctica* by causing cellular dehydration (and also by creating a steeper gradient for

Table 2. Effects of freezing, cryoprotective dehydration and slow dehydration on larval water content, hemolymph osmolality and freeze tolerance in *B. antarctica*

Condition	Duration (days)	Water content (mg H_2O mg^{-1} dry mass)	Hemolymph osmolality (mOsm kg^{-1})	Survival to −20°C for 24 h (%)
Control	2	2.93±0.08[a]	488±45[a]	0 (0/49)[a]
	5	2.92±0.07[a]	497±26[a]	0 (0/49)[a]
Frozen at –2°C	2	2.78±0.07[a,b]	528±34[a,*]	8 (4/50)[a]
	5	2.44±0.05[b]	555±30[a,*]	45 (24/53)[b]
Cryoprotective dehydration at –2°C	2	2.12±0.05[c]	887±84[b]	21 (9/44)[a,b]
	5	1.50±0.06[d]	983±60[b]	51 (25/49)[b]
Slow dehydration (99% relative humidity, 2°C)	2	2.00±0.05[c]	1147±60[b]	51 (26/51)[b]
	5	1.64±0.07[d]	1191±137[b]	61 (26/43)[b]

Values for water content (*N*=15) and hemolymph osmolality (*N*=5) are means ± s.e.m. Different letters indicate significant differences between treatments (Tukey, family-wise *P*<0.05).

*Measured after larvae were quickly thawed.

Survival data are presented with sample size in parentheses. Different letters indicate significant differences between treatments (Bonferroni, family-wise *P*<0.05).

calcium influx), thus conferring enhanced cryoprotection in frozen larvae compared with larvae that are supercooled.

Conclusions

Although our study focused on its effects in enhancing cold tolerance, RCH is now recognized as a general adaptation for swift acclimation within a thermally variable environment (Elnitsky and Lee, 2009). Rather than enhancing low-temperature survival, the primary role of RCH in nature is to fine-tune cellular and physiological states to ameliorate subtle, sublethal effects of chilling on organismal performance and reproductive fitness (Rinehart et al., 2000; Shreve et al., 2004). In the present study, we not only confirmed that *B. antarctica* undergoes RCH at temperatures below the melting point of their body fluids, but that RCH protection develops more quickly and effectively in frozen larvae.

As a result of global climate change, the Antarctic Peninsula has experienced localized reduction in snow cover (Fox and Cooper, 1998). With the loss of protective snowpack, microenvironments of *B. antarctica* may be exposed to more severe cold in winter as well as to greater and more frequent temperature fluctuations throughout the year (Bale and Hayward, 2010; Bokhorst et al., 2012). Additionally, greater amounts of meltwater will increase moisture content within microhabitats, thereby increasing the likelihood of larvae freezing by inoculation (Elnitsky et al., 2008). Therefore, larval ability to undergo RCH in the frozen state may become even more critical for protecting *B. antarctica* as their environment continues to change.

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AUTHOR CONTRIBUTIONS

Y.K., R.E.L and D.L.D. conceived the study; Y.K., N.M.T., and R.E.L. designed the experiments; Y.K. and N.M.T. collected data; Y.K. and R.E.L. analyzed the data and wrote the paper; Y.K., N.M.T., D.L.D. and R.E.L. contributed substantially to interpreting the data and developing the manuscript and take full responsibility for the content of the paper.

COMPETING INTERESTS

No competing interests declared.

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