The Journal of Experimental Biology 209, 399-406
Published by The Company of Biologists 2006
doi:10.1242/jeb.02001

Rapid cold-hardening increases the freezing tolerance of the Antarctic midge *Belgica antarctica*

Richard E. Lee, Jr[1,*], Michael A. Elnitsky[1], Joseph P. Rinehart[2], Scott A. L. Hayward[2,3], Luke H. Sandro[1] and David L. Denlinger[2]

[1]*Department of Zoology, Miami University, Oxford, OH 45056, USA,* [2]*Department of Entomology, Ohio State University, 318 W. 12th Avenue, Columbus, OH 43210, USA and* [3]*School of Biological Sciences, Liverpool University, Crown Street, Liverpool, L69 7ZB, UK*

*Author for correspondence (e-mail: leere@muohio.edu)

Accepted 16 November 2005

Summary

Rapid cold-hardening (RCH) is well known to increase the tolerance of chilling or cold shock in a diverse array of invertebrate systems at both organismal and cellular levels. Here, we report a novel role for RCH by showing that RCH also increases freezing tolerance in an Antarctic midge, *Belgica antarctica* (Diptera, Chironomidae). The RCH response of *B. antarctica* was investigated under two distinct physiological states: summer acclimatized and cold acclimated. Summer-acclimatized larvae were less cold tolerant, as indicated by low survival following exposure to –10°C for 24 h; by contrast, nearly all cold-acclimated larvae survived –10°C, and a significant number could survive –15°C. Cold-acclimated larvae had higher supercooling points than summer larvae. To evaluate the RCH response in summer-acclimatized midges, larvae and adults, maintained at 4°C, were transferred to –5°C for 1 h prior to exposures to –10, –15 or –20°C. RCH significantly increased survival of summer-acclimatized larvae frozen at –10°C for 1 h compared with larvae receiving no cold-hardening treatment, but adults, which live for only a week or so in the austral summer, lacked the capacity for RCH. In cold-acclimated larvae, RCH significantly increased freeze tolerance to both –15 and –20°C. Similarly, RCH significantly increased cellular survival of fat body, Malpighian tubules and gut tissue from cold-acclimated larvae frozen at –20°C for 24 h. These results indicate that RCH not only protects against non-freezing injury but also increases freeze tolerance.

Key words: rapid cold-hardening, freezing tolerance, Chironomidae, *Belgica antarctica*.

Introduction

More than 15 years ago, we reported a rapid cold-hardening (RCH) response that protects various species of insects against cold shock (non-freezing) injury (Chen et al., 1987; Lee et al., 1987). The RCH response is manifested not only in measured survival but also in enhanced success in courtship, mating and fecundity (Rinehart et al., 2000; Shreve et al., 2000). Recently, we demonstrated that RCH also allows an organism's overall cold tolerance to track changes in environmental temperature, as would occur in natural diurnal thermal cycles (Kelty and Lee, 1999, 2001).

The distribution of the terrestrial chironomid *Belgica antarctica* extends further south than any other free-living holometabolous insect and, since Antarctic vertebrates are essentially marine, it is the largest terrestrial animal in Antarctica (Usher and Edwards, 1984; Sugg et al., 1983). This endemic species is sporadically dispersed, though locally abundant, on the west coast of the Antarctic Peninsula and its islands. Detailed accounts of the life history and ecology of *B. antarctica* are provided by Convey and Block (1996), Sugg et al. (1983), Usher and Edwards (1984) and references cited therein. Briefly, its two-year life cycle includes four larval stages, and overwintering may occur in any instar. Larvae feed on moss, terrestrial algae, particularly *Prasiola crispa*, plant and animal detritus and microorganisms. Pupation and adult emergence occur in spring and summer. Like many insects living in wind-swept alpine and oceanic habitats, the adults are wingless. Adults live for fewer than 10 days. Like the male swarms of winged midges in temperate and arctic regions, mating occurs in aggregations of flightless males. Females mate within one day of eclosion and lay several clusters of eggs within 1–2 days.

Although ambient air temperatures may reach winter lows of –40°C on Anvers Island, *B. antarctica* survives freezing to only –15°C (Baust and Lee, 1981), a relatively modest level of cold tolerance compared with many alpine and polar insects. Thermal buffering within its microhabitat explains this apparent anomaly; at 1 cm depth, substrate temperatures

remain between 0 and –2°C for more than 300 days of the year, rarely decreasing to –7°C (Baust and Lee, 1981). Buffering may be provided by more than 1 m of ice and snow that covers the hibernacula of overwintering larvae; thermal conditions on small islands and peninsulas are further ameliorated by surrounding seawater, where annual temperatures remain between 0 and –1.8°C (Baust, 1980; Baust and Lee, 1981). Unlike most temperate insects that markedly increase their cold tolerance in preparation for winter, *B. antarctica* retains its modest levels of freeze tolerance all year round. Larvae are sensitive to thermal stress and die within a week of exposure to 10°C (Baust and Lee, 1987).

Despite residing in a thermally buffered winter microenvironment, larvae experience highly variable and often unpredictable conditions during the summer. Accordingly, several recent studies have examined the potential for RCH in Antarctic arthropods (Worland et al., 2000; Worland and Convey, 2001; Sinclair et al., 2003b; Sinclair and Chown, 2003). In the present study, we test whether RCH can occur in larvae and adults of *B. antarctica* that were field collected in the summer or in larvae after cold acclimation in the laboratory. We report that RCH enhances freeze tolerance in this species, thus extending the role of RCH beyond chilling injury to include protection against freezing injury.

Materials and methods

Source of insects

Substrate containing summer-acclimatized larvae, primarily fourth instars, of *Belgica antarctica* Jacobs was collected from sites near penguin rookeries on Torgersen Island, near Palmer Station on the Antarctic Peninsula (64°46′ S, 64°04′ W) in January 2005. Larvae were hand picked from the substrate in ice-cold water and stored at 4°C, 0 h:24 h L:D in water, to prevent desiccation and because larvae are commonly submerged in rain and/or melt water, for 2–4 days before the onset of experiments. Summer-acclimatized adults were collected with an aspirator from rocks and vegetation on Norsel Point, Humble Island, and Cormorant Island, also near Palmer Station, and were stored in the laboratory at 4°C for 1–3 days prior to use.

Samples of substrate containing larvae were shipped frozen (approximately –5°C for 7 days) to Miami University and subsequently stored at 4°C. These ‘cold-acclimated’ larvae were hand picked from the substrate in ice-cold water and stored at 4°C, 0 h:24 h L:D in water for 24 h to allow clearance of the gut (mean gut clearance ~6 h; Baust and Edwards, 1979) prior to experimental use.

Supercooling point, water content and osmolality determinations

For supercooling point determinations, larvae were blotted dry with absorbent tissue and single individuals were placed in direct contact with a thermocouple and cooled from 4 to –25°C at a rate of 1 deg. min^{-1}. The supercooling point was taken as the lowest temperature reached prior to the release of the latent heat of fusion as the result of freezing of the body water. Water content of individual larvae was assessed gravimetrically from measurement of fresh mass (to nearest 0.01 mg) at the time of sampling and dry mass after drying to constant mass at 65°C.

Hemolymph osmolality was determined using a vapor pressure depression technique (Holmstrup and Sømme, 1998). Groups of 5–10 larvae were placed in a sample holder and quickly crushed with a Teflon^{TM} rod to expose the body fluids. The sample was then allowed to equilibrate for 30 min following placement within a C-52 sample chamber (Wescor Inc., Logan, UT, USA). The osmolality of the sample was measured using a Wescor HR 33T Dew Point Microvoltometer operated in the dew point mode.

Whole-animal survival

Groups of 10 larvae were placed in 1.8 ml capped microcentrifuge tubes with ~50–80 μl ddH_2O. Adult temperature exposures were conducted in ‘dry’ tubes (i.e. containing no water). Microcentrifuge tubes containing larvae or adults were transferred from 4°C to refrigerated baths and directly exposed to –10, –15 or –20°C for 1 or 24 h. Individuals in the RCH group were held at –5°C for 1 h prior to direct transfer to the test temperature. During RCH, water in the tubes containing larvae was frozen at –5°C, suggesting that larvae, as result of their high susceptibility to inoculative freezing (M.A.E. and R.E.L., unpublished), were frozen inoculatively. Survival was assessed following a 24 h recovery at 4°C, as indicated by the larva’s ability to move either spontaneously or in response to gentle prodding. Three to six replicates were run per sample.

Low-temperature exposures were also conducted with larvae cooled in dry microcentrifuge tubes. Larvae were blotted with absorbent tissues before placement in the microcentrifuge tubes. Water (~50–80 μl) was added to dry tubes containing larvae immediately after removal from the temperature treatment to prevent desiccation during recovery. Survival was assessed as above. Data from these dry treatments did not differ significantly (P>0.05) from those containing water, thus only the results of ‘wet’ treatments are presented.

Cellular survival

Cold-acclimated larvae were divided into three groups: controls (maintained at 4°C), frozen directly (exposed to –20°C for 24 h) and rapidly cold-hardened (exposed to –5°C for 1 h prior to 24 h freezing at –20°C). Larvae were cooled in dry microcentrifuge tubes. Larvae in the RCH group were cooled individually in contact with thermocouples. Cellular survival of larvae frozen, as noted by the presence of an exotherm, during RCH did not differ (P>0.05) from larvae that remained supercooled at –5°C; therefore cellular survival is reported only for larvae frozen during the RCH treatment, to correspond to whole-animal RCH trials as described above.

Following a 24 h thaw at 4°C, groups of three larvae were dissected to assess cellular survival of several tissue types (fat body, gut, Malpighian tubules and salivary gland). Tissues were dissected in Coast’s solution (Coast, 1988) and cellular

survival was assessed using the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA) as modified by Yi and Lee (2003). Living cells with intact cell membranes fluoresced green or yellow-green, while red or orange-red fluorescence indicated dead cells. For fat body and gut, mean values of cellular survival are based on counts of three groups of 100 cells for each of the three replicates. Because the Malpighian tubules and salivary glands are composed of fewer cells, all visible cells were scored as either alive or dead in each of three replicates.

Statistical analysis

Means were compared, following tests for parametric assumptions, using Student's *t*-tests or analysis of variance (ANOVA) and Bonferroni–Dunn tests (Statview from SAS Institute, Cary, NC, USA). Survival data were arcsin-square root transformed prior to analysis. Data are presented as means ± s.e.m. Statistical significance was set at $P<0.05$.

Results

Intrinsic cold tolerance

Summer-acclimatized larvae were significantly ($P<0.05$) more tolerant of subzero temperatures than adults (Fig. 1). Nearly 90% of summer-acclimatized larvae survived a 24 h exposure at –5°C, while <50% of adults survived a similar exposure. No adults and <20% of summer-acclimatized larvae survived exposure at –10°C for 24 h. However, larval survival of subzero temperatures was significantly ($P<0.05$) higher following cold acclimation (Fig. 1). Nearly all cold-acclimated larvae survived a 24 h exposure at –10°C, and >40% survived at –15°C. No larvae survived exposure at –20°C for 24 h.

Cold acclimation, in which larvae were exposed in frozen soil (approximately –5 to –10°C) for ~7 days, significantly ($P<0.05$) increased the larval supercooling point to –6.6°C (Table 1). However, cold acclimation was apparently not associated with increased cryoprotectant synthesis, as hemolymph osmolality was unchanged. Similarly, larval water content did not change significantly following cold acclimation.

Rapid cold-hardening: whole-animal freeze tolerance

Nearly all summer-acclimatized larvae (98.3±1.7%) survived freezing at –5°C for 1 h (Fig. 2A), but survival fell to <25% following freezing at –10°C for 1 h. RCH, in which larvae were frozen at –5°C for 1 h, significantly ($P<0.05$) increased the freezing tolerance of summer-acclimatized larvae following freezing at –10°C (Fig. 2A). Survival increased by 67.7% following RCH relative to larvae receiving no treatment prior to freezing. By contrast, summer-acclimatized adults lacked the capacity to undergo RCH (Fig. 2B): no individuals survived following RCH and exposure at –10°C for 1 h.

By contrast, cold-acclimated larvae were extremely tolerant of freezing at –10°C for 24 h (>97% survival; Fig. 3). However, only 42% of the cold-acclimated larvae survived freezing at –15°C for 24 h, and no larvae survived freezing at –20°C. Survival was significantly ($P<0.05$) greater following RCH: nearly 90% and 75% of the cold-acclimated larvae survived freezing at –15 and –20°C, respectively (Fig. 3). Among the individuals that survived freezing at –15°C, RCH larvae appeared much more active and mobile than those cold-acclimated larvae that survived but received no treatment prior

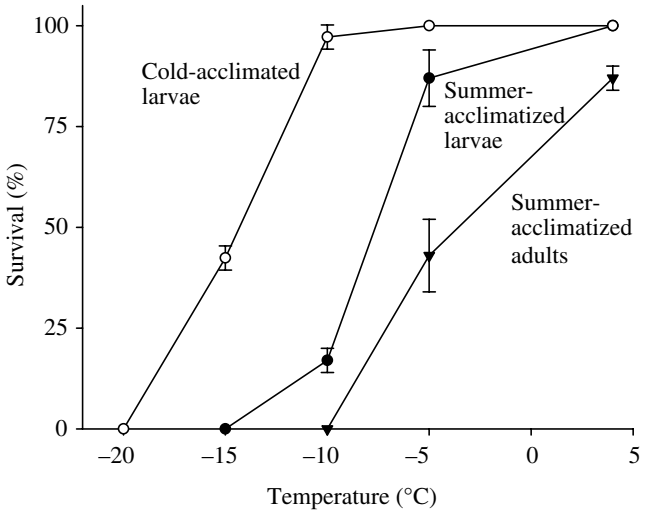

Fig. 1. Comparison of the cold tolerance of summer-acclimatized larvae and adults and cold-acclimated larvae of the Antarctic midge *Belgica antarctica*. Three replicates of 10 individuals were transferred from 4°C to the specified temperatures for 24 h. Survival was assessed following a 24 h recovery at 4°C, and all individuals that displayed at least some movement were deemed to have survived. Values are means ± s.e.m.

Table 1. *The effect of acclimation state and rapid cold-hardening (RCH) on the supercooling point, osmolality and water content of Belgica antarctica*

	Summer-acclimatized larvae		Cold-acclimated larvae
	Control (4°C)	RCH (–5°C/1 h)	Control (4°C)
Supercooling point (°C)	–8.6±0.9[a] (12)	–8.8±0.4[a] (12)	–6.6±0.3[b] (30)
Osmolality (mOsm kg^{-1})	373±29[a] (4)	390±4[a] (4)	417±18[a] (7)
Water content (g g^{-1} dry mass)	2.56±0.11 (10)	–	2.61±0.06 (12)

Different letters indicate significant differences between treatments (ANOVA, Bonferroni–Dunn test, $P<0.05$). Values are means ± s.e.m., with sample size in parentheses.

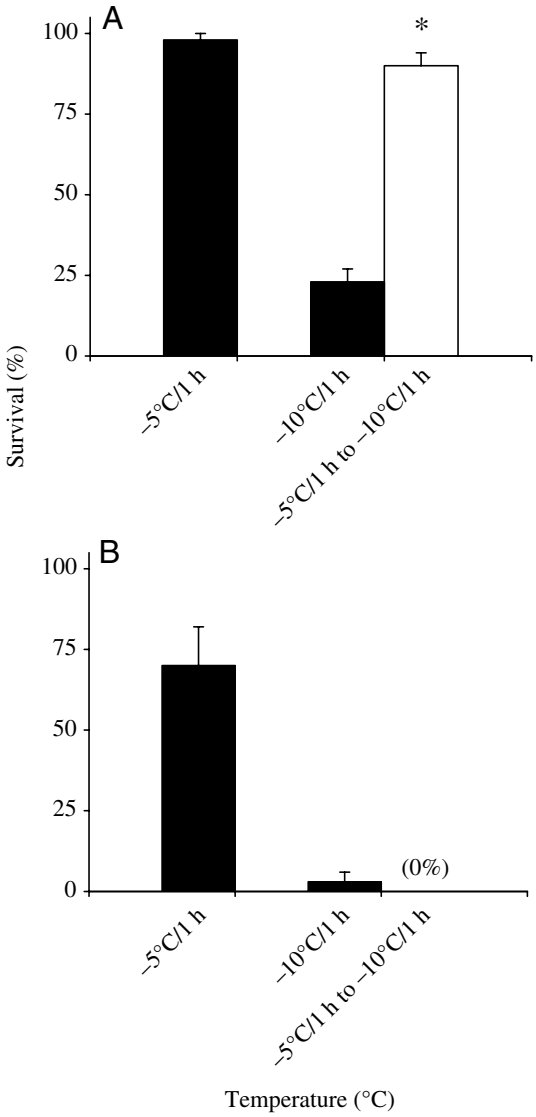

Fig. 2. Effect of rapid cold-hardening on the freeze tolerance of (A) summer-acclimatized larvae and (B) adults of the Antarctic midge, *Belgica antarctica.* Cultures were maintained at 4°C prior to exposure to the indicated temperatures. * denotes a significant (P<0.05) difference in survival between groups directly exposed to –10°C and those that were first held at –5°C for 1 h prior to the –10°C exposure. Values are means ± s.e.m. based on six replicates of 10 individuals.

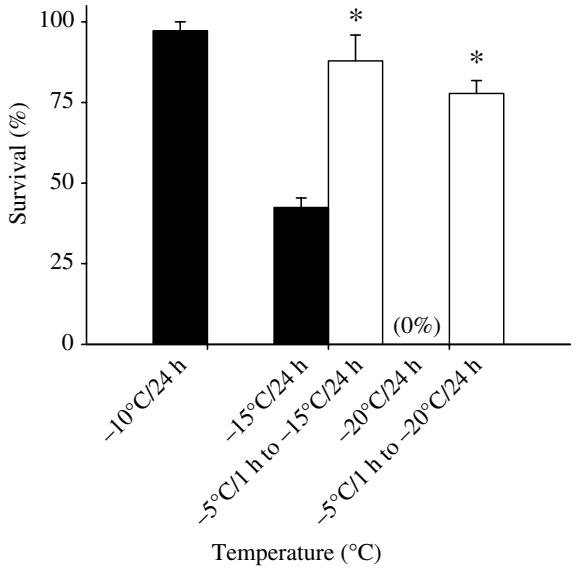

Fig. 3. Effect of rapid cold-hardening on the freeze tolerance of cold-acclimated larvae of the Antarctic midge *Belgica antarctica*. * denotes a significant (P<0.05) difference in survival between groups directly exposed to –15 or –20°C and those that were first held at –5°C for 1 h prior to the lower temperature exposure. Values are means ± s.e.m. based on six replicates of 10 individuals.

to freezing. Larvae that survived freezing at –20°C displayed only limited motility, suggesting some degree of freezing injury.

Cold-hardening is often accompanied by the synthesis of low-molecular-mass cryoprotectants, resulting in an increase in solute concentration that can be measured as an increase in hemolymph osmolality and depression of the supercooling point. No change was detected in either hemolymph osmolality or the supercooling point in response to RCH (Table 1). These observations suggest that additional cryoprotectants were not synthesized in response to RCH.

Rapid cold-hardening: cellular survival

Fat body, gut, Malpighian tubules and salivary gland sampled from control, cold-acclimated larvae maintained at 4°C had >97% cellular survival (Table 2). Cells of all tissue types from control larvae appeared healthy with intact cell membranes, as indicated by the observation that the vast majority of cells were stained by the membrane permeant SYBR-14 (green nuclei) while the dead-cell stain propidium iodide (red nuclei) was excluded (Fig. 4).

Cellular survival of fat body, gut and Malpighian tubules was significantly (P<0.05) lower than in the controls following larval freezing at –20°C for 24 h (Table 2; Fig. 4). Fat body cells were particularly susceptible to the freezing stress, as survival was reduced by 96% relative to controls; fat body cells incurred substantial damage to cell membranes, as nuclei were stained by propidium iodide (Fig. 4), and appeared more diffuse and loosely arranged compared with tissues from control larvae maintained at 4°C. Similarly, cellular survival of the gut and Malpighian tubules was reduced by 53 and 35%, respectively, following freezing. By contrast, survival of the salivary gland did not differ significantly relative to tissue from control larvae (Table 2; Fig. 4). Collectively, freezing of cold-acclimated larvae at –20°C with no prior treatment resulted in a decrease in cell survival of 47% relative to control larvae maintained at 4°C.

RCH larvae frozen at –5°C for 1 h prior to freezing at –20°C exhibited significantly (P<0.05) higher rates of cell survival for fat body, gut and Malpighian tubules relative to tissues from cold-acclimated larvae directly frozen at –20°C (Table 2). RCH increased cellular survival of fat body dramatically, by

48%, relative to larvae frozen with no prior treatment. While the majority of fat body cells maintained intact cell membranes, localized areas of higher cell mortality were present (Fig. 4). Similarly, RCH increased cell survival of gut and Malpighian tubules 31 and 18%, respectively. Survival of salivary gland from RCH larvae did not differ significantly from larvae frozen with no prior treatment or control larvae maintained at 4°C (Fig. 4). Combining survival for all tissues, RCH resulted in a 24% increase in cell survival relative to larvae frozen at –20°C with no prior treatment.

Table 2. *Cell viability of tissue from cold-acclimated larvae in three treatment groups*

	Cell viability (%)		
Tissue type	Control	Frozen, no prior treatment	Rapidly cold-hardened
Fat body	98.4±0.4[a]	2.8±0.3[b]	51.1±3.9[c]
Gut	98.1±0.6[a]	44.6±6.2[b]	75.6±4.3[c]
Malpighian tubules	97.8±0.7[a]	62.2±5.6[b]	80.4±2.1[c]
Salivary gland	98.5±0.7[a]	94.9±1.2[a]	95.3±1.3[a]

Values are means ± s.e.m. Treatment groups: control (4°C), frozen with no prior treatment (–20°C/24 h), and rapidly cold-hardened (–5°C/1 h to –20°C/24 h). Values for fat body and gut are based on three replicates of three counts of 100 cells.

All cells of three replicates were counted for Malpighian tubules and salivary glands. Within a tissue type, different letters indicate significant differences between treatments (ANOVA, Bonferroni–Dunn test, $P<0.05$).

Discussion

Summers on the Antarctic Peninsula are characterized by highly variable thermal and hydric conditions. Within moss banks, we have measured microclimatic temperatures for *B. antarctica* larvae in excess of 20°C, while extreme values of >30°C are known from this region (Convey, 1997). At the other extreme, snow and freezing conditions can occur at any time during the summer. In the present study, we found that larvae could quickly increase their level of cold tolerance over timescales ranging from hours to days.

Cold acclimation of frozen larvae increases freeze tolerance and the supercooling point

Few summer-acclimatized larvae (<20%) survived 24 h at –10°C, while none survived –15°C (Fig. 1). By contrast, following 7 days of subzero cold acclimation, nearly all larvae survived –10°C and >40% survived –15°C. This increase is particularly remarkable because larvae were frozen at temperatures between –10 and –5°C during the 7-day acclimation period.

Previous reports have documented changes in frozen insects including mitochondrial structure, metabolite and cryoprotectant levels, and diapause status. Acclimation of frozen caterpillars of *Gynaephora groenlandica* at –15°C caused a reduction of the number of mitochondria and disruption of cristae in remaining mitochondria (Kukal et al., 1989). Changes in glycerol levels, energy charge and enzyme activity are reported from frozen larval and adult insects (Baust, 1972; Storey et al., 1981; Storey and Storey, 1981; Kukal et al., 1988). Irwin et al. (2001) demonstrated that diapause development occurs in fly larvae frozen at –20°C. In the present study, cold acclimation of frozen *B. antarctica* larvae markedly increased insect cold tolerance by decreasing the lower lethal temperature. This increase in freezing tolerance was apparently not caused by accumulation of cryoprotectants, since there was no increase in hemolymph osmolality (Table 1).

Subzero acclimation increased supercooling point values (–8.6°C) for summer-acclimatized

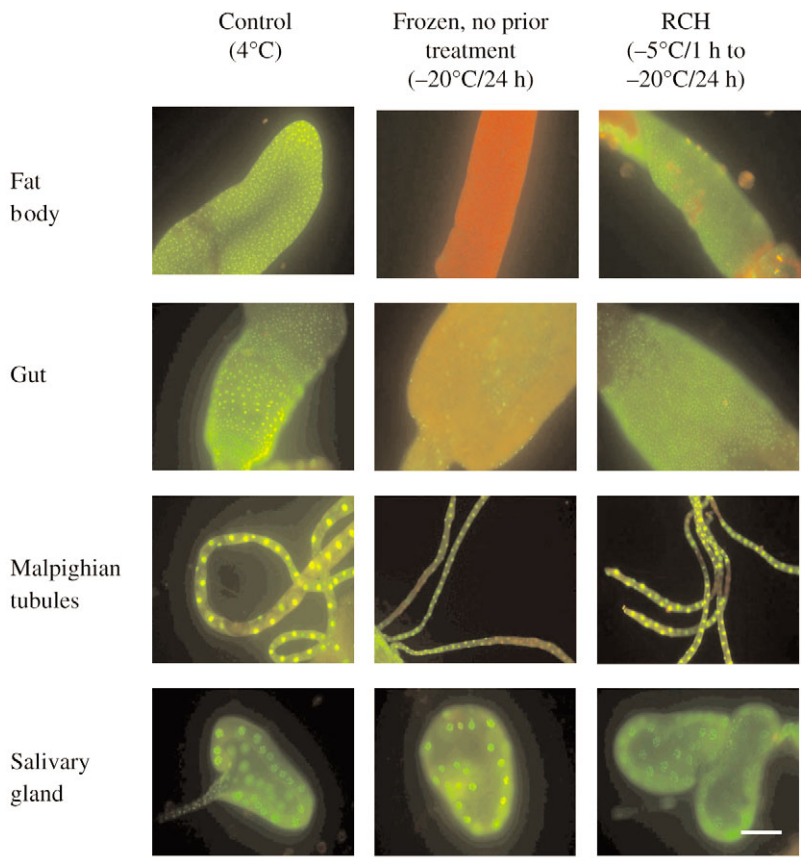

Fig. 4. The effect of rapid cold-hardening (RCH) on cellular survival of various tissues from cold-acclimated larvae of *Belgica antarctica*. Green indicates living cells, and red indicates dead cells. Scale bar, 300 μm for rapidly cold-hardened gut and Malpighian tubules, and Malpighian tubules frozen with no prior treatment; 150 μm for all other tissues.

larvae by 2° (Table 1). This elevation is consistent with previous reports for this species during the transition from summer to autumn. Between early February and the end of March, Lee and Baust (1981) reported an increase in the supercooling point from –10.2°C to –5.0°C; this change was closely correlated with decreasing microhabitat temperatures from 11.2°C to 0.8°C during this period. Similarly, a mean value of –6.2°C was reported for *B. antarctica* larvae collected in early April (Block, 1982). This trend suggests the seasonal production of endogenous ice nucleators, frequently found in freeze-tolerant insects, that function to promote protective freezing at high subzero temperatures (Zachariassen and Hammel, 1976; Baust and Edwards, 1979; Lee and Costanzo, 1998).

RCH at subzero temperatures increases freezing tolerance

Cold tolerance in Antarctic and sub-Antarctic terrestrial arthropods, particularly Collembola and mites, has been studied extensively over the past 25 years (see reviews by Somme and Block, 1982; Convey, 1996, 1997; Sinclair et al., 2003a). Most insects in these regions are freezing intolerant, surviving subzero exposure by extensive supercooling of their body fluids; their lower lethal limit is usually defined by their supercooling point. Since these species are not susceptible to nonfreezing, chilling or cold shock injury, it is not surprising that rapid cold-hardening, *sensu stricto* as it was originally defined (Lee et al., 1987), has not been reported for Antarctic regions.

Traditionally, acclimation temperatures in the range of 0 to 6°C have been used to induce the RCH response (cf. Burks and Hagstrum, 1999; Coulson and Bale, 1990; Czajka and Lee, 1990; Koveos, 2001; Larson and Lee, 1994; Lee et al., 1987). This response can also be induced using slow rates of cooling or thermoperiods that mimic natural diurnal cycles (Kelty and Lee, 1999, 2001). Although acclimation at subzero temperatures has been reported rarely, Worland and Convey (2001) found a decrease in supercooling point values for a freeze-intolerant Antarctic springtail that was acclimated for 12 h at –2°C; however, acclimation at –5°C had no effect in this species. Similarly, Brown et al. (2004) reported a significant reduction of supercooling point values of larvae of the hoverfly *Syrphus ribesiii* following repeated subzero exposures. However, these larvae also displayed reduced freeze tolerance. To our knowledge, the –5°C that was used in the present study is the lowest temperature at which the RCH response has been induced.

In contrast to summer-acclimatized larvae, the freeze-intolerant adults did not undergo RCH (Fig. 2). Lack of the RCH response is, perhaps, not surprising for several reasons. Adults have a significant level of intrinsic cold tolerance as evidenced by the fact that a substantial number survived 24 h of exposure to –5°C (Fig. 1). Adults only live for a few days, emerging on warm, sunny days to mate and lay eggs (Sugg et al., 1983). Furthermore, the wingless adults are far more mobile than larvae and remain in or near thermally buffered microhabitats to which they could retreat during a cold spell.

Diversity in types of short-term cold-hardening responses

The original report on the RCH response described the swift acquisition of protection against chilling or cold shock injury, also called prefreeze mortality because it occurs at temperatures above the supercooling point and is not related to freezing of body fluids (Lee et al., 1987). Although this response is now well known in a range of insect orders and in the Acarina in temperate regions, the RCH response, according to the original definition, has not been found in Antarctic terrestrial arthropods (Worland and Convey, 2001; Sinclair et al., 2003b).

Nonetheless, other types of cold-hardening that occur over short time periods have been reported for Antarctic microarthropods. Supercooling points of the springtail *Cryptopygus antarcticus* track microhabitat temperatures that can result in significant changes in cold-hardiness within 12 h (Worland et al., 2000; Worland and Convey, 2001). For this species, and most other Antarctic mites and springtails, the supercooling point corresponds to the lower lethal temperature (Block and Somme, 1982; Somme and Block, 1982). Since no mortality occurs until the organism freezes at its supercooling point, this type of hardening differs from the RCH response, as originally defined, that protects against nonfreezing chilling injury. Similarly, diurnal variation in the supercooling points of other species of Antarctic Collembola indicates that cold tolerance can vary within hours (Sinclair et al., 2003b).

Only a minority of terrestrial arthropods are tolerant of freezing, and such species are especially rare in the sub-Antarctic region (Chown and Nicolson, 2004; Sinclair et al., 2003a). A notable exception to this pattern is caterpillars of *Pringleophaga marioni*, which survive freezing at high subzero temperatures (Klok and Chown, 1997; Sinclair and Chown, 2003). Both desiccation and high-temperature pre-treatments enhance freezing tolerance in this species; however, a variety of low temperature treatments, selected specifically to test for the presence of a RCH response, yielded no evidence for such a response (Sinclair and Chown, 2003). To our knowledge, the capacity for RCH in *B. antarctica* larvae described herein is the first for any freeze-tolerant insect.

RCH at the cellular level

Cold-hardening at the organismal level was paralleled at the cellular level, as RCH dramatically increased cellular survival of several tissue types in larvae of *B. antarctica* following freezing. When the RCH response was induced by exposing cold-acclimated larvae to –5°C for 1 h, cellular survival of Malpighian tubules and gut was ~18 and 31% higher, respectively, than tissues from larvae directly exposed to –20°C. An even greater increase was observed in survival of fat body cells: survival increased by 48% following RCH. A similar magnitude of increase in cellular survival of cold shock was seen following *in vivo* RCH of selected tissues from the flesh fly *Sarcophaga crassipalpis* (Yi and Lee, 2004). Yi and Lee (2004) also demonstrated that the RCH response was independent of the central nervous system and neuroendocrine control. It is now clear that the RCH response observed at the

organismal level is likewise operant at the cellular level and may afford protection against both non-freezing cold shock and freezing injury. In the case of *B. antarctica*, RCH may be especially important for cellular protection during unpredictable summer cold, when freezing may occur.

While gut, Malpighian tubules and salivary gland displayed substantially higher cellular survival, the fate of the fat body paralleled larval survival in being extremely susceptible to freezing, with <3% of cells surviving at –20°C. A similar pattern of cell survival was documented in the freeze-tolerant alpine cockroach *Celatoblatta quinquemaculata* (Worland et al., 2004). Following freezing to –12°C, no adult cockroaches survived, and cell survival of fat body declined to <10%. This contrasts the survival following freezing of fat body cells of the goldenrod gall fly, *Eurosta solidaginis* (Lee et al., 1993). While no larvae survived to adulthood, >60% of fat body cells survived following intracellular freezing at –80°C. Similarly, no larvae of the freeze-tolerant dipteran *Heliomyza borealis* survived freezing at –40°C, while >80% of fat body cells survived (Morton-Firth et al., 1996). This indicates, at least in *E. solidaginis* and *H. borealis*, that the freeze tolerance of fat body cells could not explain the lower limit of larval freeze tolerance, but this was not the case for *B. antarctica*. We assume that the most vulnerable tissue ultimately limits cold tolerance of the whole organism, and for *B. antarctica* the most vulnerable tissue may indeed be the fat body.

Mechanism of rapid cold-hardening

The physiological mechanism of the RCH response remains poorly understood. Adults of *S. crassipalpis* increase hemolymph levels of the cryoprotectant glycerol threefold, to ~80 mmol l^{-1}, during RCH (Chen et al., 1987). While elevation of hemolymph glycerol may afford some protection against cold shock injury in *S. crassipalpis*, cryoprotectant synthesis clearly is not requisite for the RCH response, as adult *Drosophila melanogaster* do not synthesize carbohydrate cryoprotectants during RCH (Kelty and Lee, 2001). Similarly, no change in supercooling point or hemolymph solute concentration, suggestive of no increase in cryoprotectant synthesis, was observed for *B. antarctica* following RCH. Also, it is unlikely that the RCH response requires the synthesis of a new suite of proteins, as Misener et al. (2001) found that inhibition of protein synthesis did not inhibit RCH. Changes in the lipid composition of cell membranes may be involved in the RCH response (Overgaard et al., 2005), and such 'homeoviscous adaptations' could maintain cell membrane integrity when the cell is confronted with low temperatures and/or the stresses associated with ice formation within the body fluids (Hazel, 1995; Hazel and Williams, 1990), but, clearly, further experiments are needed to define the mechanisms responsible for the widely used RCH response.

This research was supported by NSF grants OPP-0337656 and OPP-0413786. We thank the support staff at Palmer Station for their excellent assistance with this project.

References

Baust, J. G. (1972). Insect freezing protection in *Pterostichus brevicornis* (Carabidae). *Nature* **236**, 219-220.

Baust, J. G. (1980). Low temperature tolerance in an Antarctic insect: a relict adaptation? *CryoLetters* **1**, 360-371.

Baust, J. G. and Edwards, J. S. (1979). Mechanisms of freezing tolerance in an Antarctic midge, *Belgica antarctica. Physiol. Entomol.* **4**, 1-5.

Baust, J. G. and Lee, R. E. (1981). Environmental "homeothermy" in an Antarctic insect. *Antarct. J. US* **15**, 170-172.

Baust, J. G. and Lee, R. E. (1987). Multiple stress tolerance in an Antarctic terrestrial arthropod: *Belgica antarctica*. *Cryobiology* **24**, 140-147.

Block, W. (1982). Supercooling points of insects and mites on the Antarctic Peninsula. *Ecol. Entomol.* **7**, 1-8.

Block, W. and Sømme, L. (1982). Cold hardiness of terrestrial mites at Signy Island, maritime Antarctic. *Oikos* **38**, 157-167.

Brown, C. L., Bale, J. S. and Walters, K. F. A. (2004). Freezing induces a loss of freeze tolerance in an overwintering insect. *Proc. R. Soc. Lond. B* **271**, 1507-1511.

Burks, C. S. and Hagstrum, D. W. (1999). Rapid cold-hardening capacity in five species of coleopteran pests of stored grain. *J. Stored Prod. Res.* **35**, 65-75.

Chen, C. P., Denlinger, D. L. and Lee, R. E. (1987). Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Physiol. Zool.* **60**, 297-304.

Chown, S. L. and Nicolson, S. W. (2004). *Insect Physiological Ecology: Mechanisms and Patterns*. New York: Oxford University Press.

Coast, G. M. (1988). Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domesticus*, and their response to diuretic hormone. *Physiol. Ent.* **13**, 381-391.

Convey, P. (1996). Overwintering strategies of terrestrial invertebrates in Antarctica – the significance of flexibility in extremely seasonal environments. *Eur. J. Entomol.* **93**, 489-505.

Convey, P. (1997). How are the life history strategies of Antarctic terrestrial invertebrates influenced by extreme environmental conditions? *J. Therm. Biol.* **22**, 429-440.

Convey, P. and Block, W. (1996). Antarctic Diptera: ecology, physiology and distribution. *Eur. J. Entomol.* **93**, 1-13.

Coulson, S. J. and Bale, J. S. (1990). Characterization and limitations of the rapid-cold hardening response in the housefly *Musca domestica* (Diptera: Muscidae). *J. Insect Physiol.* **36**, 207-211.

Czajka, M. C. and Lee, R. E. (1990). A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster*. *J. Exp. Biol.* **148**, 245-254.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu. Rev. Physiol.* **57**, 19-42.

Hazel, J. R. and Williams, E. E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog. Lipid Res.* **29**, 167-227.

Holmstrup, M. and Sømme, L. (1998). Dehydration and cold hardiness in the Arctic collembolan *Onychiurus arcticus* Tullberg 1876. *J. Comp. Physiol. B* **168**, 197-203.

Irwin, J. B., Bennett, V. A. and Lee, R. E. (2001). Diapause development in frozen larvae of the goldenrod gall fly, *Eurosta solidaginis* fitch (Diptera: Tephritidae). *J. Comp. Physiol. B* **171**, 181-188.

Kelty, J. D. and Lee, R. E. (1999). Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. *J. Insect Physiol.* **45**, 719-726.

Kelty, J. D. and Lee, R. E. (2001). Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophilidae) during ecologically based thermoperiodic cycles. *J. Exp. Biol.* **204**, 1659-1666.

Klok, C. J. and Chown, S. L. (1997). Critical thermal limits, temperature tolerance, and water balance of a sub-Antarctic caterpillar, *Pringleophaga marioni* (Lepidoptera: Tineidae). *J. Insect Physiol.* **43**, 685-694.

Koveos, D. S. (2001). Rapid cold hardening in the olive fruit fly *Bactrocera oleae* under laboratory and field conditions. *Entomol. Exp. Appl.* **101**, 257-263.

Kukal, O., Duman, J. G. and Serianni, A. S. (1988). Glycerol metabolism in a freeze-tolerant artic insect: an in vivo ^{13}C NMR study. *J. Comp. Physiol. B* **158**, 175-183.

Kukal, O., Duman, J. G. and Serianni, A. S. (1989). Cold-induced mitochondrial degradation and cryoprotectant synthesis in freeze-tolerant arctic caterpillars. *J. Comp. Physiol. B* **158**, 661-671.

Larsen, K. J. and Lee, R. E. (1994). Cold tolerance including rapid cold-hardening and inoculative freezing in migrant monarch butterflies in Ohio. *J. Insect Physiol.* **40**, 859-864.

Lee, R. E. and Baust, J. G. (1981). Seasonal patterns of cold-hardiness in Antarctic terrestrial arthropods. *Comp. Biochem. Physiol.* **70A**, 579-582.

Lee, R. E. and Costanzo, J. P. (1998). Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *Annu. Rev. Physiol.* **60**, 55-72.

Lee, R. E., Chen, C. P. and Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science* **238**, 1415-1417.

Lee, R. E., McGrath, J. J., Morason, R. T. and Taddeo, R. M. (1993). Survival of intracellular freezing, lipid coalescence and osmotic fragility in fat body cells of the freeze-tolerant gall fly *Eurosta solidaginis*. *J. Insect Physiol.* **39**, 445-450.

Misener, S., Chen, C. and Walker, V. K. (2001). Cold tolerance and proline metabolic gene expression in *Drosophila melanogaster*. *J. Insect Physiol.* **47**, 393-400.

Morton-Firth, C. J., Block, W. and Worland, M. R. (1996). Organism survival and cell integrity in a freeze-tolerant insect. *CryoLetters* **17**, 25-30.

Overgaard, J., Sorensen, J. G., Petersen, S. O., Loeschecke, V. and Holmstrup, M. (2005). Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J. Insect Physiol.* **51**, 1173-1182.

Rinehart, J. P., Yocum, G. D. and Denlinger, D. L. (2000). Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposure to high or low temperatures on fecundity in the flesh fly, *Sarcophaga crassipalpis*. *Physiol. Entomol.* **25**, 330-336.

Shreve, S. M., Kelty, J. D. and Lee, R. E. (2004). Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response. *J. Exp. Biol.* **207**, 1797-1802.

Sinclair, B. J. and Chown, S. L. (2003). Rapid responses to high temperature and desiccation but not low temperature in the freeze tolerant sub-Antarctic caterpillar *Pringleophaga marioni* (Lepidoptera, Teneidae). *J. Insect Physiol.* **49**, 45-52.

Sinclair, B. J., Addo-Bediako, A. and Chown, S. L. (2003a). Climatic variability and the evolution of insect freeze tolerance. *Biol. Rev.* **78**, 181-195.

Sinclair, B. J., Klok, C. J., Scott, M. B., Terblanche, J. S. and Chown, S. L. (2003b). Diurnal variation in supercooling points of three species of Collembola from Cape Hallett, Antarctica. *J. Insect Physiol.* **49**, 1049-1061.

Sømme, L. and Block, W. (1982). Cold hardiness of Collembola at Signy Island, maritime Antarctic. *Oikos* **38**, 168-176.

Storey, K. B. and Storey, J. M. (1981). Biochemical strategies of overwintering in the gall fly larva, *Eurosta solidaginis*: effect of low temperature acclimation on the activities of enzymes of intermediary metabolism. *J. Comp. Physiol.* **144**, 191-199.

Storey, K. B., Baust, J. G. and Storey, J. M. (1981). Intermediary metabolism during low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. *J. Comp. Physiol.* **144**, 183-190.

Sugg, P., Edwards, J. S. and Baust, J. (1983). Phenology and life history of *Belgica antarctica*, an Antarctic midge (Diptera: Chironomidae). *Ecol. Entomol.* **8**, 105-113.

Usher, M. B. and Edwards, M. (1984). A dipteran from south of the Antarctic Circle: *Belgica antarctica* (Chironomidae), with a description of its larva. *Biol. J. Linn. Soc.* **23**, 19-31.

Worland, M. R. and Convey, P. (2001). Rapid cold hardening in Antarctic microarthropods. *Funct. Ecol.* **15**, 515-524.

Worland, M. R., Convey, P. and Lukesova, A. (2000). Rapid cold hardening: a gut feeling. *CryoLetters* **21**, 315-324.

Worland, M. R., Wharton, D. A. and Byars, S. G. (2004). Intracellular freezing and survival in the freeze tolerant alpine cockroach *Celatoblatta quinquemaculata*. *J. Insect Physiol.* **50**, 225-232.

Yi, S.-X. and Lee, R. E. (2003). Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes. *J. Insect Physiol.* **33**, 523-528.

Yi, S.-X. and Lee, R. E. (2004). In vivo and in vitro rapid cold-hardening protects cells from cold-shock injury in the flesh fly. *J. Comp. Physiol. B* **174**, 611-615.

Zachariassen, K. E. and Hammel, H. T. (1976). Nucleating agents in the haemolymph of insects tolerant to freezing. *Nature* **262**, 285-287.