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# Alternative overwintering strategies in an Antarctic midge: freezing vs. cryoprotective dehydration

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## Summary

**1.** Cryoprotective dehydration is a relatively new addition to our understanding of freeze avoidance strategies employed by polar invertebrates. Although the underlying cellular processes associated with this strategy are similar to those of freeze tolerance, little is known about potential trade-offs of overwintering in these physiological states.

**2.** This study compares the potential of larvae of the terrestrial midge *Belgica antarctica* (Diptera, Chironomidae) to overwinter in these two states. As the only insect with the capacity to tolerate freezing and to cryoprotectively dehydrate, it is an ideal model to compare the benefits and costs of these strategies.

**3.** Compared to summer-acclimated larvae, supercooling points of winter-acclimatized larvae were significantly depressed and were lower than observed minima for their microhabitat temperatures. Thus, if larvae avoid inoculative freezing from environmental ice, they could remain unfrozen *via* cryoprotective dehydration.

**4.** Both frozen and cryoprotectively dehydrated larvae readily survived a 32-day exposure to simulated overwintering temperatures.

**5.** Freezing had little effect on larval body water content and haemolymph osmolality. In contrast, cryoprotective dehydration at −5 °C resulted in a progressive loss of body water, ultimately reducing larval water content by 62%. This level of dehydration corresponded to an increase in haemolymph osmolality to *c.* 2750 mOsm $kg^{-1}$, depressing the haemolymph melting point to −4·9 °C.

**6.** Freezing and cryoprotective dehydration resulted in distinctly different patterns of glycogen breakdown. Whereas the glycogen content decreased only during the first 14 days in cryoprotectively dehydrated larvae, frozen larvae continued to break down glycogen throughout the 32-day subzero exposure. However, after recovery at 0 °C for 5 days, glycogen levels were similar in these two groups, as were the levels of total lipids.

**7.** Our results indicate that freezing and cryoprotective dehydration are both effective in promoting winter survival of larvae, with surprisingly few differences in energetic costs. Whether larvae freeze or become cryoprotectively dehydrated ultimately depends on the hydric condition of their microhabitat. The physiological flexibility of *B. antarctica* to overwinter in these alternative states likely contributed to its range distribution that extends further south than any other free-living insect.

**Key-words:** adaptations of polar arthropods, cellular dehydration, cold hardiness, cryoprotective dehydration, freeze concentration, freeze tolerance, supercooling

## Introduction

Traditionally, cold-hardy ectotherms are categorized into two groups based on their ability to survive internal ice formation (Costanzo & Lee 2013). Most of these species are freeze-intolerant, and winter acclimatization involves the removal or masking of endogenous ice nucleators and/or an increase in resistance to inoculative freezing to allow supercooling of their body fluids (i.e. the maintenance of liquid phase below the melting point). In contrast,

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although relatively uncommon, some species can survive freezing of their body fluids. Generally, freeze tolerance is accompanied by physiological changes that inhibit extensive supercooling by inducing ice nucleation at relatively high temperatures (e.g. Mugnano, Lee & Taylor 1996). Because of these physiological differences, freeze avoidance and freeze tolerance are generally considered to be mutually exclusive strategies for overwintering (Costanzo & Lee 2013). However, a few species of ectotherms can employ either, and whether these organisms overwinter in a frozen or an unfrozen state likely depends on the hydric and/or thermal condition within their hibernaculum (Ring 1982; Costanzo *et al.* 1998; Elnitsky *et al.* 2008).

Both overwintering in an unfrozen (i.e. supercooled) and a frozen state impose unique sets of limitations and advantages for ectotherms (see reviews by Block 1991; Costanzo & Lee 2013). The supercooled state is inherently precarious, and inoculative freezing may be unavoidable in certain environments. However, if an organism can remain unfrozen, additional stresses associated with mechanical injuries and homoeostatic disturbances caused by freezing and thawing of body fluids are avoided. Thus, freeze avoidance is advantageous in minimizing energetic expenditures associated with the repair of cell/tissue damages, especially when organisms are repeatedly threatened by acute exposure to subzero temperatures (e.g. Teets *et al.* 2011). Alternatively, if an environment is characterized by stable, low temperatures, freezing presents an energetic advantage since the metabolic rate is lower in a frozen compared to a supercooled animal at the same subzero temperature (Irwin & Lee 2002). Because many ectotherms do not feed during winter, conservation of energy reserves is critical for maximizing their post-winter reproductive fitness. In the case of *Eurosta solidaginis* larvae, freezing at −5 °C for 10 weeks promoted a higher rate of adult emergence than supercooling (Layne & Kuharsky 2001). Also, overwintering in a frozen state functions as a means to avoid organismal dehydration since freezing diminishes the gradient for water loss between supercooled body fluids and environmental ice (Zachariassen *et al.* 2008).

As an alternative to supercooling (and remaining fully hydrated), cryoprotective dehydration allows some polar invertebrates to remain unfrozen during winter (Holmstrup, Bayley & Ramløv 2002; Pedersen & Holmstrup 2003; Elnitsky *et al.* 2008). Because of their highly permeable integuments, the maintenance of supercooled state in the presence of environmental ice inevitably leads to loss of body water (Holmstrup *et al.* 2010). Driven by the vapour pressure difference between supercooled body fluids and the surrounding ice, this water loss continues until the melting point of body fluids is sufficiently depressed to match the environmental temperature. At this point, the organism is no longer supercooled, and the risk of internal ice formation is eliminated. Accompanied by the concomitant accumulation of cryoprotectants, this cryoprotectively dehydrated state promotes winter survival of permeable soil invertebrates inhabiting polar regions (Lundheim & Zachariassen 1993; Holmstrup & Sømme 1998; Pedersen & Holmstrup 2003; Wharton, Goodall & Marshall 2003; Worland & Block 2003; Elnitsky *et al.* 2008; Sørensen & Holmstrup 2011).

Although frozen organisms do not lose body water to the environment, internal ice formation induces progressive dehydration at the cellular level (Mazur 2004). As ice forms extracellularly, only water molecules join the growing ice lattice, thereby concentrating extracellular solutes. Essentially, water is 'lost' as ice within the body, and freeze concentration of the remaining unfrozen fraction leads to a rapid efflux of water from cells. This cellular dehydration continues until the haemolymph melting point is colligatively depressed to match the environmental temperature, thus preventing lethal intracellular freezing (Lee 2010). Thus, at the cellular level, the underlying processes associated with freeze tolerance bear a striking resemblance to those of cryoprotective dehydration. However, the majority of organisms that overwinter *via* cryoprotective dehydration are freeze-intolerant, and therefore, freezing may impose additional cellular/physiological stresses beyond cellular dehydration. Although a few studies have compared these physiological states within the same species (Pedersen & Holmstrup 2003; Wharton, Goodall & Marshall 2003; Hayashi & Wharton 2011), little is known about potential trade-offs (e.g. sublethal energetic costs) of overwintering in a frozen vs. a cryoprotectively dehydrated state.

Endemic to the Antarctic Peninsula, the terrestrial midge, *Belgica antarctica* Jacobs (Diptera: Chironomidae) is the southernmost, free-living insect (Sugg, Edwards & Baust 1983; Usher & 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 −7 °C, owing to thermal buffering by the oceanic influence and accumulated snow (Baust & Lee 1981; Elnitsky *et al.* 2008). Larvae are freeze-tolerant year-round (Baust & Lee 1987) and survive internal ice formation at temperatures as low as −20 °C (Lee *et al.* 2006; Kawarasaki *et al.* 2013). However, in some relatively dry microhabitats, inoculative freezing may be avoided, thereby allowing larvae to become cryoprotectively dehydrated (Elnitsky *et al.* 2008). Although some species of insects appear capable of physiologically switching between freeze tolerance and freeze avoidance strategies (e.g. Brown, Bale & Walters 2004; Horwath & Duman 1984), whether larvae of *B. antarctica* overwinter in a frozen or a cryoprotectively dehydrated state is likely determined by the hydric condition of their microhabitat (Elnitsky *et al.* 2008).

The purpose of this study was to directly compare the alterative overwintering strategies of freezing and cryoprotective dehydration in larvae of *B. antarctica*. Since previous studies were limited to summer-collected larvae (e.g. Elnitsky *et al.* 2008), this study is the first to examine the

overwintering physiology of winter-acclimatized larvae of *B. antarctica*. First, we determined the supercooling capacity of winter-acclimatized larvae, because larvae must be able to remain supercooled at ecologically relevant temperatures for cryoprotective dehydration to occur. We then characterized larval responses to these physiological states by monitoring changes in water content, haemolymph osmolality and levels of cryoprotectants. Lastly, we compared larval cold tolerance and changes in energy reserves between frozen vs. cryoprotectively dehydrated larvae to elucidate potential trade-offs of overwintering in these alternative states.

## Materials and methods

### SOURCE OF INSECTS

For the experiment comparing the effects of freezing vs. cryoprotective dehydration, substrate containing larvae of *B. antarctica* was collected on Cormorant Island near Palmer Station on Antarctic Peninsula (64°46′S, 64°04′W) on 19–21 April 2011. After collection, larvae were handpicked from substrate in ice-cold water and placed on moist filter paper at 0 °C for 12–24 h to ensure clearance of the gut (mean gut clearance *c.* 6 h; Baust & Edwards 1979). Additionally, another sample was collected on 1 May 2011 to assess the supercooling capacity of winter-acclimatized larvae, and data were compared to those of larvae collected in February 2011 and maintained in the laboratory under summer conditions (2 °C).

### MICROHABITAT TEMPERATURE

Miniature temperature loggers (HOBO Water Temp Pro, Onset Computer, Pocasset, MA, USA) were deployed in microhabitat sites of *B. antarctica* in February 2011, and temperatures were recorded every 30 min. Loggers were recovered in February 2012, and the resulting data were extracted using BOXCAR PRO 4.3 software (Onset Computer, Pocasset, MA, USA).

### DETERMINATION OF LARVAL SUPERCOOLING POINT (SCP)

Supercooling points (SCPs) of larvae were determined immediately after rapid extraction from their natural substrates (i.e. without gut clearance). Larvae were gently blotted dry on an absorbent tissue and individually placed in direct contact with a thermocouple and cooled at *c.* 0·5 °C min$^{-1}$. The SCP was taken as the lowest temperature reached prior to the release of the latent heat of fusion when body water froze.

### PROTOCOL FOR LOW-TEMPERATURE EXPOSURE AND RECOVERY

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 (Pedersen & Holmstrup 2003; Elnitsky *et al.* 2008). To induce freezing, groups of 25 individuals were placed in 1·5-mL centrifuge tubes with *c.* 200 μL of ice-cold distilled water. A piece of crushed ice was added to each tube to ensure the water froze at or slightly below 0 °C. Consequently, these larvae froze inoculatively when bath temperatures reached the haemolymph melting point (*c.* −1·1 °C).

Both 15-mL vials and 1·5-mL centrifuge tubes containing larvae were initially equilibrated at −0·6 °C overnight. Temperatures of refrigerated baths were lowered incrementally (*c.* 0·5 °C day$^{-1}$) to −3 °C and held at this temperature until Day 14 (Fig. 1). Subsequently, bath temperatures were further lowered to −5 °C and held there until the termination of low-temperature exposure (Day 32).

Larvae were transferred to Petri dishes lined with moist filter paper for recovery. Following 24 h on moist filter paper at 0 °C, drops of water were added to each Petri dish so that larvae were submerged in ice-cold water for the subsequent 48 h. After this recovery period in water, larvae were allowed to recover on moist filter paper for an additional 48 h at 0 °C (total of 5-day recovery; Day 37).

### ASSESSMENT OF LARVAL SURVIVAL AND COLD TOLERANCE

Following the 32-day exposure, subsets of larvae from each group ($N$ = 50) were allowed to recover on moist filter paper for 24 h at 0 °C and assessed for survival. Larvae were judged to have survived if they moved spontaneously or in response to gentle prodding. Following this initial assessment, water was added to each Petri dish, and larvae were submerged for an additional 48 h before survival was assessed again. Each treatment group consisted of approximately 50 larvae.

To investigate the effects of freezing and cryoprotective dehydration on larval cold tolerance, subsets of larvae were removed from the experiment on Day 14 and abruptly exposed to −10 °C for 5 days or −20 °C for 24 h. Larval survival ($N$ = 50) was assessed as described above.

### MEASUREMENTS OF LARVAL WATER CONTENT AND HAEMOLYMPH OSMOLALITY

Water content and haemolymph osmolality were measured from subsets of larvae from each physiological state on Days 14, 32 and 37. Water content of individual larvae was assessed gravimetrically from measurements of fresh mass (to the nearest 0·002 mg) and dry mass (DM) after drying to constant mass at 65 °C. Haemolymph osmolality was determined using the vapour pressure depression technique (Holmstrup & Sømme 1998; Elnitsky *et al.* 2008). Groups of five larvae were placed in a sample holder and quickly crushed with a Pellet Pestle (Sigma-Aldrich, http://www.

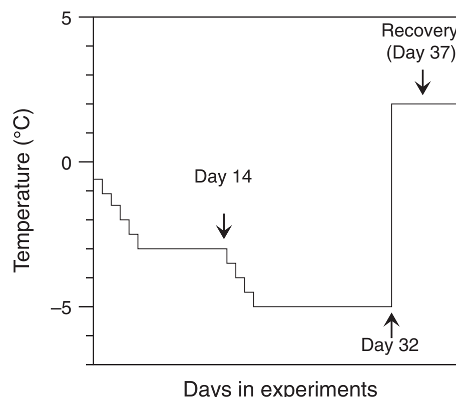


**Fig. 1.** Experimental protocol for the subzero exposure. Subsets of samples were removed from the experiment on Days 14, 32 and 37 to assess larval body water content, haemolymph osmolality, cryoprotectant and glycogen levels, and total lipid content.

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sigmaaldrich.com/catalog/product/sigma/z359947?lang = en®ion = US) to expose body fluids. Sample was then equilibrated for at least 30 min within a C-52 sample chamber, and osmolality was measured using a Wescor HR-33T Dew Point Microvoltmeter (Wescor Inc., Logan, UT, USA) operated in the dew point depression mode.

### DETERMINATION OF CRYOPROTECTANT ACCUMULATION

To determine whether larvae accumulated low molecular mass sugars during the low-temperature exposure, glucose and trehalose levels were measured from subsets of larvae from the experimental groups on Days 14, 32 and 37. These sugars were chosen because they are two of the major cryoprotectants for *B. antarctica* larvae (Baust & Lee 1983). Glycerol, a common cryoprotectant in many cold-hardy insects, was also measured. Immediately after sampling, five replicates of *c.* 25 larvae were quickly weighed and frozen at −80 °C for later analysis.

Prior to cryoprotectant determination, larvae were homogenized in 7% perchloric acid (PCA) and neutralized with KOH. Trehalose levels were determined following enzymatic digestion by trehalase from porcine kidney (T8778, Sigma, St. Louis, MO, USA). Glucose concentration was determined using the glucose oxidase reagent set (G7519, Pointe Scientific, Canton, MI, USA). Finally, glycerol concentration was determined using the free glycerol reagent (F6428, Sigma). The suggested ratio of homogenate to reagent was increased by *c.* 10X to get a signal, but otherwise the assay was conducted per manufacturer's protocol. For each cryoprotectant measured, content was expressed as μg mg$^{-1}$ DM.

### GLYCOGEN AND LIPID CONTENTS ANALYSIS

Glycogen levels were determined from aliquots of PCA homogenates for cryoprotectant analysis. Prior to neutralization with KOH, 20 μL of raw homogenate was incubated with amyloglucosidase from *Asperillus niger* (A1602, Sigma) at 40 °C to liberate glucose from glycogen (Keppler & Decker 1974). Following neutralization, the total glucose concentration was determined. Glycogen level was expressed as μg mg$^{-1}$ DM after subtraction of initial free glucose.

Total lipid content was measured from subsets of larvae sampled on Days 14, 32 and 37. Immediately after sampling, five replicates of five larvae each were quickly weighed and frozen at −80 °C until analysed. Total lipids were extracted using the modified Folch method (Folch, Lees & Sloane Stanley 1957) described by Christie (1982), and the solvent was evaporated in a vacuum. Residues were resuspended in $H_2SO_4$ and total lipid content was measured using vanillin–phosphoric acid reagent (Sim & Denlinger 2009). Values for total lipids are expressed as μg mg$^{-1}$ DM.

### STATISTICAL ANALYSIS

Larval survival data were analysed using a generalized linear model with the logistic link and binomial error distribution (Hosmer & Lemeshow 2000). All other data were analysed using the nonparametric, permutation test, since they did not meet the parametric assumption of normality (Edgington 1995). SCP data were compared between summer-acclimated and winter-acclimatized larvae. Temporal changes in body water content, haemolymph osmolality, each cryoprotectant, glycogen, total lipids and DM within each treatment group were analysed using the permutation test with a one-way ANOVA model; Bonferroni *post hoc* test distinguished means at different sampling points. The permutation test with a two-way ANOVA model was used to examine the mean of the variables as a function of two factors, treatment group (frozen and cryoprotectively dehydrated) and time (Days 14, 32 and 37), or the interaction of these two factors. The initial value was excluded from this analysis to make a 2 × 3 factorial design. When a significant interaction between the factors was found, the Bonferroni *post hoc* test was applied to distinguish between the two treatment means at each sampling point. All statistical analyses were performed using R (R Development Core Group 2013) with the LMPERM package (Wheeler 2010); statistical significance was set at $\alpha < 0{\cdot}05$.

## Results

### SEASONAL CHANGES IN MICROHABITAT TEMPERATURE

During the summer (from late December through February), temperatures in the microhabitat of *B. antarctica* fluctuated diurnally, acutely exposing larvae to both high (>10 °C) and subzero temperatures (Fig. 2). Microhabitat temperatures declined gradually until late March, when they decreased abruptly and remained below zero until

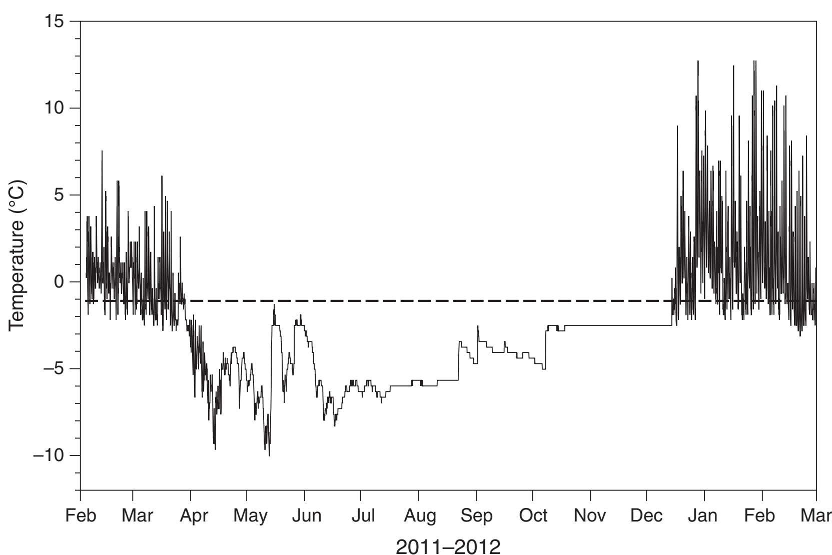


**Fig. 2.** Seasonal changes in temperature at a representative larval microhabitat of *B. antarctica* on Humble Island, near Palmer Station, Antarctica (64°46S, 64°04W). Microhabitat temperatures were measured in 2011−2012 using a single-channel temperature logger. The broken line indicates the equilibrium melting point of winter-acclimatized larvae (−1·1 °C; this study).

mid-December. Owing to thermal buffering by the ocean and accumulated snow cover (Baust & Lee 1981), the thermal environment of larvae during the winter was relatively stable compared to summer months; however, fluctuations in early winter could have acutely exposed larvae to temperatures as low as −10 °C.

#### SUPERCOOLING CAPACITY IN SUMMER-ACCLIMATED VS. WINTER-ACCLIMATIZED LARVAE

Summer-acclimated larvae exhibited relatively high SCPs (−9·8 ± 2·4 °C; $N = 17$; range from −22·3 to −5 °C). Although these larvae were collected in February 2011 and maintained under summer conditions in laboratory (2 °C) until 28 April 2011, SCP values were similar to those from previous studies of freshly collected summer larvae (Baust & Edwards 1979; Lee & Baust 1981; Hayward *et al.* 2007). By contrast, winter-acclimatized larvae, field-collected on 1 May 2011, exhibited significantly lower SCPs (mean; −15·1 ± 1·3°C; $N = 13$; range from −23·4 to −10·0 °C; $P = 0{\cdot}003$). Because the minimum temperature encountered by the larvae during the winter was only −10 °C (Fig. 2), initiation of freezing in these winter-acclimatized larvae would require inoculation by environmental ice.

#### PHYSIOLOGICAL EFFECTS OF CRYOPROTECTIVE DEHYDRATION VS. FREEZING

The physiological state of overwintering larvae of *B. antarctica* is likely to be strongly influenced by the hydric condition of their microhabitats (Elnitsky *et al.* 2008). In a wet microhabitat, larvae would freeze by inoculation from environmental ice at the melting point of their haemolymph (*c.* −1·1 °C). By contrast, in a relatively dry microhabitat, larvae may avoid inoculation and become cryoprotectively dehydrated as a result of water loss during prolonged supercooling. In the following sections, we compare the physiological effects of these alternative overwintering states.

### *Larval water content and haemolymph osmolality*

Prolonged supercooling in an environment at equilibrium with the vapour pressure of ice resulted in a progressive reduction in larval body water content ($F_{3,54} = 155{\cdot}6$, $P < 0{\cdot}0001$; Fig. 3a). After the first phase of subzero exposure (Day 14), in which larvae were equilibrated at −3 °C, larval body water content decreased by 41% compared to the initial value (from 2·57 ± 0·05 to 1·52 ± 0·07 g $H_2O$ $g^{-1}$ DM). Water loss continued during the second phase (from Day 14 to Day 32), as the environmental temperature was lowered to −5 °C. By Day 32, larval body water content had decreased by 62% to 0·98 ± 0·05 g $H_2O$ $g^{-1}$ DM. Correspondingly, haemolymph osmolality of larvae progressively increased from 620 ± 13 to 2745 ± 198 mOsm $kg^{-1}$ by Day 32 ($F_{3,20} = 95{\cdot}06$, $P < 0{\cdot}0001$; Fig. 3b). Based on the colligative properties of

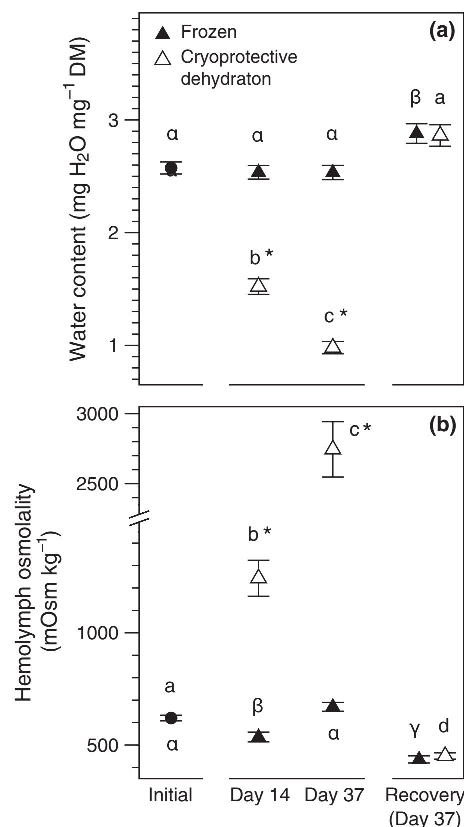


**Fig. 3.** Effects of freezing and cryoprotective dehydration on (a) larval body water content ($N = 15$) and (b) haemolymph osmolality ($N = 6$) of *B. antarctica*. Values are means ± SEM. Different Greek letters indicate significant differences among sampling points within the frozen group, while different English letters indicate significant differences within the cryoprotectively dehydrated group. Asterisk (*) indicates the value differs from the mean of the corresponding frozen group (Bonferroni, family-wise $P < 0{\cdot}05$).

solutions, the equilibrium melting point of these dehydrated larvae was calculated to be −4·9 °C. After recovery (Day 37), larvae fully rehydrated, and the haemolymph osmolality decreased to 451 ± 14 mOsm $kg^{-1}$, a value significantly lower than the initial value (Fig. 3a,b).

Body water content and haemolymph osmolality of frozen larvae also varied significantly throughout the experiment (body water content: $F_{3,53} = 6{\cdot}12$, $P = 0{\cdot}001$; haemolymph osmolality: $F_{3,20} = 33{\cdot}18$, $P < 0{\cdot}0001$; Fig. 3a,b). However, changes in these physiological parameters were relatively small in frozen larvae (water content: 2·53–2·57 g $H_2O$ $g^{-1}$ DM; haemolymph osmolality: 535–670 mOsm $kg^{-1}$) during the 32-day exposure period, compared to cryoprotectively dehydrated larvae. Accordingly, there was a significant interaction between treatment and time (body water content: $F_{2,83} = 56{\cdot}89$, $P < 0{\cdot}0001$; haemolymph osmolality: $F_{2,30} = 70{\cdot}39$, $P < 0{\cdot}0001$). After recovery (Day 37), both body water content and haemolymph osmolality of frozen larvae were statistically indistinguishable from the values observed from larvae that recovered from cryoprotective dehydration.

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### Larval cold tolerance

After 32 days of subzero exposure, larval survival was high in both treatment groups. Following 24 h of recovery on moist filter paper, 49 out of 50 frozen larvae exhibited spontaneous movements, and survival did not change during the subsequent 48-h recovery period in water. By contrast, cryoprotectively dehydrated larvae remained shrunken after 24 h on moist filter paper, and only 20 out of 50 larvae exhibited spontaneous movement. However, following a subsequent 48-h submergence in water, all larvae ($N = 50$) recovered.

To assess changes in cold tolerance associated with freezing and cryoprotective dehydration, subsets of larvae were removed from the experiment on Day 14 and abruptly exposed to −10 °C for 5 days or −20 °C for 24 h (Fig. 4). Both frozen and cryoprotectively dehydrated larvae tolerated −10 °C for 5 days with nearly 100% survival (50 out of 50 and 49 out of 50, respectively). By contrast, there was a statistically significant difference ($P < 0{\cdot}0001$) in survival at −20 °C for 24 h; whereas 47 out of 50 frozen larvae tolerated this exposure, only 21 out of 50 of cryoprotectively dehydrated larvae survived.

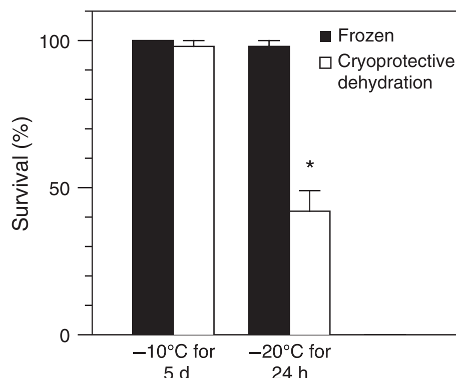


**Fig. 4.** Cold tolerance of frozen and cryoprotectively dehydrated larvae of *B. antarctica* were tested at −10 °C for 5 days or −20 °C for 24 h. Values were based on 50 larvae (±standard error of proportion). Asterisk (*) denotes the significant difference between frozen and cryoprotectively dehydrated groups.

### Cryoprotectant analysis

No significant changes in levels of glucose were induced by freezing or cryoprotective dehydration (freezing: $F_{3,16} = 3{\cdot}11$, $P = 0{\cdot}056$; cryoprotective dehydration: $F_{3,16} = 0{\cdot}853$, $P = 0{\cdot}485$; Table 1). Similarly, at the beginning of the study, trehalose levels in these winter-acclimatized larvae were already high compared to previous reports of summer-collected larvae (e.g. Elnitsky *et al.* 2008), and levels remained relatively constant throughout the experiment. Although ANOVA ($F_{3,16} = 11{\cdot}03$, $P = 0{\cdot}004$) and subsequent Bonferroni *post hoc* tests indicated significant changes in trehalose among sampling points in cryoprotectively dehydrated larvae, these small changes of *c.* 8 μg mg$^{-1}$ DM in trehalose level are unlikely to have biological significance. Glycerol remained barely detectable (*c.* 0·2 μg mg$^{-1}$ DM) throughout the experiment in both frozen and cryoprotectively dehydrated larvae.

### Changes in glycogen, total lipids and dry mass

Freezing and cryoprotective dehydration resulted in distinctly different patterns of glycogen breakdown (two-way ANOVA: treatment group × time interaction: $F_{2,24} = 37{\cdot}07$, $P < 0{\cdot}0001$; Fig. 5). Specifically, on Day 14, frozen larvae had significantly higher glycogen reserves than cryoprotectively dehydrated larvae (62·9 ± 1·7 vs. 41·2 ± 2·0 μg mg$^{-1}$ DM, respectively). However, during the second phase of subzero exposure (from Day 14 to 32), glycogen

**Table 1.** Effects of freezing and cryoprotective dehydration on cryoprotectant levels, lipid content and dry mass in larval *B. antarctica* during the 37-day experiment

| | | Day 14 | | Day 32 | | Recovery (Day 37) | |
|---|---|---|---|---|---|---|---|
| | Initial | Frozen | Cryoprotective dehydration | Frozen | Cryoprotective dehydration | Frozen | Cryoprotective dehydration |
| Glucose level (μg mg$^{-1}$ DM) ($N = 5$) | 0·26 ± 0·05$^{α/a}$ | 0·71 ± 0·19$^{α}$ | 0·50 ± 0·16$^{a}$ | 0·50 ± 0·07$^{α}$ | 0·55 ± 0·17$^{a}$ | 0·44 ± 0·06$^{α}$ | 0·50 ± 0·14$^{a}$ |
| Trehalose level (μg mg$^{-1}$ DM) ($N = 5$) | 52·94 ± 0·79$^{α/ab}$ | 53·25 ± 1·12$^{α}$ | 56·18 ± 1·09$^{b}$ | 51·28 ± 1·06$^{α}$ | 48·67 ± 1·20$^{a}$ | 54·03 ± 2·11$^{α}$ | 52·63 ± 0·43$^{a}$ |
| Lipid content (μg mg$^{-1}$ DM) ($N = 4–5$) | 151·8 ± 26·5$^{α/a}$ | 226·9 ± 25·2$^{α}$ | 168·8 ± 16·4$^{a}$ | 170·5 ± 24·6$^{α}$ | 157·9 ± 9·9$^{a}$ | 185·1 ± 22·5$^{α}$ | 154·7 ± 20·0$^{a}$ |
| Dry mass (mg individual$^{-1}$) ($N = 13–15$) | 0·31 ± 0·02$^{α/a}$ | 0·33 ± 0·02$^{α}$ | 0·31 ± 0·02$^{a}$ | 0·35 ± 0·02$^{α}$ | 0·30 ± 0·02$^{a}$ | 0·31 ± 0·02$^{α}$ | 0·32 ± 0·02$^{a}$ |

Values are means ± SEM. Different Greek letters indicate significant differences among sampling points within the frozen group, while different English letters indicate significant differences within the cryoprotectively dehydrated group. (Bonferroni, family-wise $P < 0{\cdot}05$).

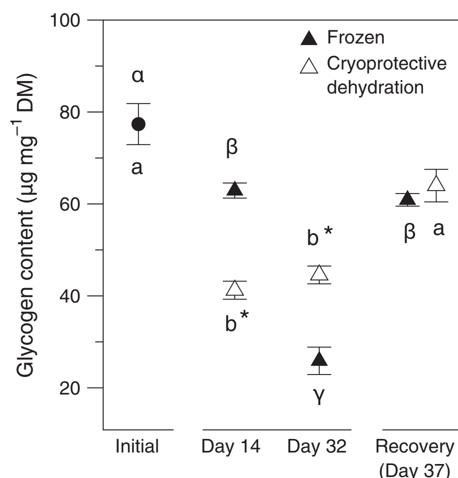


**Fig. 5.** Effects of freezing and cryoprotective dehydration on the glycogen level ($N = 5$) in *B. antarctica* larvae. Values are means ± SEM. Greek letters indicate significant differences among sampling points within the frozen group, while different English letters indicate significant differences within the cryoprotectively dehydrated group. Asterisks indicate the value differs from the mean of the corresponding frozen group (Bonferroni, family-wise $P < 0{\cdot}05$).

levels were further decreased to 25·9 ± 30 μg mg$^{-1}$ DM in frozen larvae, but remained unchanged in cryoprotectively dehydrated larvae. Despite these differences in the pattern of breakdown, glycogen returned to a similar level after recovery (Day 37) in both frozen and cryoprotectively dehydrated larvae (Fig. 5).

Levels of total lipids did not change during the experiment in either frozen or cryoprotectively dehydrated larvae (frozen: $F_{3,15} = 1{\cdot}709$, $P = 0{\cdot}208$; cryoprotectively dehydrated: $F_{3,16} = 0{\cdot}149$, $P = 0{\cdot}929$; Table 1). Similarly, no differences in larval dry mass (*c.* 0·31 mg individual$^{-1}$) were observed in these two groups (frozen, $F_{3,53} = 0{\cdot}796$, $P = 0{\cdot}502$; cryoprotectively dehydrated, $F_{3,54} = 0{\cdot}186$, $P = 0{\cdot}905$; Table 1).

## Discussion

During winter, larvae of *B. antarctica* are encased in a matrix of frozen substrate for 8–9 months (Fig. 2). Because of the high susceptibility of larvae to inoculative freezing (Lee *et al.* 2006; Elnitsky *et al.* 2008), direct contact with environmental ice would induce internal ice formation, forcing larvae to overwinter in the frozen state. Alternatively, if inoculative freezing is avoided, overwintering larvae may enter a cryoprotectively dehydrated state and remain unfrozen through the winter (Elnitsky *et al.* 2008).

### SEASONAL INCREASE IN SUPERCOOLING CAPACITY MAY PROMOTE CRYOPROTECTIVE DEHYDRATION

Because the vapour pressure gradient that drives the loss of body water is terminated once spontaneous ice formation occurs, the potential for larvae to overwinter in the cryoprotectively dehydrated state relies on their ability to initially remain supercooled at ecologically relevant temperatures (Holmstrup *et al.* 2010). Consistent with previous studies (Baust & Edwards 1979; Lee & Baust 1981; Hayward *et al.* 2007), summer-acclimated larvae exhibited relatively high SCPs, with some individuals spontaneously freezing at temperatures as high as −5 °C. Interestingly, SCPs of winter-acclimatized larvae were significantly lower than those of summer-acclimated larvae (range from −23·4 to −10·0 °C). This seasonal depression of the SCP would have allowed larvae to remain supercooled when the environmental temperature abruptly decreased in early April, exposing them to temperatures as low as −10 °C (Fig. 2). Whereas freeze-tolerant species typically increase their SCPs to initiate ice formation at relatively high subzero temperatures, the seasonal cold-hardening of freeze-intolerant species is accompanied by removing or masking of endogenous ice nucleators, increasing their capacity to remain supercooled (Lee & Costanzo 1998). The observed seasonal depression of the SCP would allow overwintering larvae to remain unfrozen *via* cryoprotective dehydration in 'dry' microhabitats.

### CRYOPROTECTIVE DEHYDRATION VS. FREEZING

In the present study, the alternative overwintering states of frozen vs. cryoprotectively dehydrated were compared using a two-phased low-temperature treatment (Fig. 1). During the first phase (Days 1–14), larvae were equilibrated at −3 °C following incremental decreases (0·5°C day$^{-1}$) in the environmental temperature. This initial treatment was comparable to that of a previous study investigating the effectiveness of cryoprotective dehydration in summer-acclimated larvae (Elnitsky *et al.* 2008). During the second phase (Days 14–32), the environmental temperature was further decreased to −5 °C, a more relevant overwintering temperature (Fig. 2).

#### *Body water content*

Prolonged supercooling in the presence of environmental ice resulted in a progressive loss of body water (Fig. 3a; Table 2). The amount of water lost during cryoprotective dehydration at −3 °C was similar to that observed in a previous study (Elnitsky *et al.* 2008). However, subsequent equilibration at −5 °C led to a further reduction in body water content; ultimately, after 32 days of low-temperature exposure, body water content was reduced by 62%, and the larvae appeared severely shrunken. The total body water content of an organism is divided into two fractions; whereas all solutes are dissolved in the osmotically active fraction, some water is bound to macromolecules and membranes and therefore is osmotically inactive (Zachariassen 1985). The body water loss promoting cryoprotective dehydration in the Arctic collembolan, *Onychiurus arcticus*, is primarily derived from the osmotically active fraction, while the osmotically inactive, bound water

**Table 2.** Estimated osmotic contribution of original solutes in the haemolymph due to loss of body water during cryoprotective dehydration

| | Initial | Cryoprotective dehydration | |
|---|---|---|---|
| | | Day 14 | Day 32 |
| Observed haemolymph osmolality (mOsm kg$^{-1}$) ($N$ = 6) | 620 ± 13 | 1243 ± 80 | 2745 ± 198 |
| Total body water content (mg $H_2O$ mg$^{-1}$ DM) ($N$ = 15) | 2·57 ± 0·05 | 1·52 ± 0·07 | 0·98 ± 0·05 |
| Loss of total body water (%) | – | 41 | 62 |
| OAW content (mg $H_2O$ mg$^{-1}$ DM)* | 2·09 | 1·12 | 0·61 |
| Loss of OAW (%) | – | 46 | 71 |
| Osmotic contribution of original solutes due to loss of OAW (mOsm kg$^{-1}$) | – | 1164 | 2119 |
| Osmolality not accounted for by loss of OAW (mOsm kg$^{-1}$) | – | 79 | 626 |
| Total explainable osmolality (%) | – | 93·6 | 77·2 |

OAW, osmotically active water; DM, dry mass. Values are mean ± SEM.
*OAW was calculated from Worland, Grubor-Lajsic and Montiel (1998) [(OIW) = 0·069(TBW) + 0·3, where OIW is the content of osmotically inactive water, TBW is total body water content and is the sum of OAW and OIW.]

fraction remains relatively unchanged (Worland, Grubor-Lajsic & Montiel 1998). Applying the regression between osmotically inactive water and total body water contents found in this collembolan, we calculated that cryoprotective dehydration at −5 °C in *B. antarctica* resulted in loss of *c.* 71% of the osmotically active fraction of body water (Table 2).

By contrast, the body water content of frozen larvae remained unchanged (Fig. 3a). However, freezing does induce dehydration at the cellular level. Using the haemolymph osmolality of frozen larvae (Day 32; 670 mOsm kg$^{-1}$; Fig. 3b) and the osmotically inactive, bound water fraction of 18·6% (estimated using the regression in Worland, Grubor-Lajsic & Montiel 1998), we calculated that 61·9% of the total body water was 'lost' as ice in these frozen larvae when equilibrated at −5 °C (Claussen & Costanzo 1990). This corresponds to a loss of *c.* 76% of the osmotically active fraction, resulting in a fourfold concentration of solutes. Therefore, during the 32-day low-temperature treatment, the level of cellular dehydration experienced by frozen larvae was similar to that of cryoprotectively dehydrated larvae, 76 vs. 71%, respectively.

### *Haemolymph osmolality, cryoprotectant levels and glycogen content*

As body water was lost to the environment, haemolymph osmolality of cryoprotectively dehydrated larvae progressively increased to *c.* 2750 mOsm kg$^{-1}$ after equilibration at −5 °C (Fig. 3b), depressing the melting point to *c.* −4·9 °C. Based on the regression presented by Worland, Grubor-Lajsic & Montiel (1998), we estimated that this substantial increase in haemolymph osmolality was largely (*c.* 77%) due to loss of the osmotically active fraction of body water (Table 2). The remaining 23%, corresponding to 626 mOsm kg$^{-1}$, appears to be a consequence of *de novo* synthesis of osmolytes.

In response to dehydration and cold acclimation, many insects accumulate large amounts of various low molecular mass sugars and polyols (Zachariassen 1985). Not only do these osmolytes function colligatively to decrease water loss and ice content, but they also have specific, non-colligative roles in protecting membranes and proteins from the deleterious effects of dehydration and cold. Previously, we found that cryoprotective dehydration in summer-collected larvae induced substantial accumulations of glucose and trehalose (nine- and 11-fold increases, respectively; Elnitsky *et al.* 2008). However, in the present study, levels of these low molecular mass sugars remained relatively unchanged throughout the 37-day experiment (Table 1). Because the initial level of trehalose in the untreated larvae (*c.* 53 μg mg$^{-1}$ DM) was even higher than that of summer-collected larvae cryoprotectively dehydrated at −3 °C (*c.* 37 μg mg$^{-1}$ DM; Elnitsky *et al.* 2008), this osmolyte likely had already increased under natural conditions before we collected them in late April.

The fact that trehalose and glucose levels did not increase during cryoprotective dehydration suggests the accumulation of other osmolytes are responsible for the 23% discrepancy (626 mOsm kg$^{-1}$) between the calculated vs. observed values of haemolymph osmolality (Table 2). Previous studies reported the presence of sucrose and erythritol in untreated larvae (Baust & Edwards 1979; Baust & Lee 1983). More recently, we used a high-throughput metabolomics approach to identify other osmolytes linked to dehydration tolerance in *B. antarctica* (Teets *et al.* 2012b). Most notably, the loss of body water triggers marked increases in levels of fructose, sorbitol and mannitol. *De novo* synthesis of these sugars and polyols may be correlated with the decrease in glycogen content observed during the first phase (Days 1–14) of our 37-day experiment (Fig. 5). Additionally, accumulation of other types of osmolytes, such as proline, an amino acid with a profound cryoprotective function (Koštál, Zahradníčková & Šimek 2011; Koštál *et al.* 2012), may contribute to cryoprotective dehydration in larvae of *B. antarctica* (Teets *et al.* 2012b).

Compared to cryoprotective dehydration, freezing did not induce substantial changes in haemolymph osmolality (Fig. 3b). Nevertheless, a significant reduction in glycogen

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content was observed during the 32-day low-temperature treatment (Fig. 5). Freezing triggers glycogenolysis in the wood frog, *Rana sylvatica*, promoting mobilization of their cryoprotectant, glucose (Storey & Storey 1985; Costanzo, Lee & Lortz 1993). Accumulation of glucose also occurs in response to freezing or cryoprotective dehydration in the freeze-tolerant earthworm, *Fridericia ratzeli* (Pedersen & Holmstrup 2003). Although levels of glucose and trehalose remained unchanged during the 32-day treatment (Table 1), accumulations of other low molecular mass sugars and polyols linked to dehydration tolerance may have also occurred in frozen larvae of *B. antarctica* (Fig. 5).

Glycogen binds three to five times its mass in water, and glycogenolysis results in the liberation of this large quantity of water (Gibbs, Chippindale & Rose 1997). Substantial reduction in glycogen content induced by internal freezing results in hyperhydration of the liver of Alaskan *R. sylvatica,* protecting this organ from dehydration (Costanzo *et al.* 2013). Such a release of previously unavailable water in *B. antarctica* may have counteracted the increase in haemolymph osmolality that was expected as a result of *de novo* synthesis of osmolytes in frozen larvae.

Although both treatments induced equivalent levels of cellular dehydration, freezing and cryoprotective dehydration resulted in distinctly different patterns of glycogen breakdown (Fig. 5). Whereas the glycogen content remained stable after the initial decrease during the first phase (Days 1–14) in cryoprotectively dehydrated larvae, frozen larvae continued to break down glycogen during the second phase (Days 14–32). This difference may be due to effects of these treatments on metabolic gene expression patterns (Teets *et al.* 2013). Cryoprotective dehydration at −3 °C (treatment equivalent to the first phase of our experiment) results in downregulation of transcripts for glycogen phosphorylase, a key enzyme responsible for glycogenolysis. In contrast, freezing at −5 °C for 24 h induces upregulation of the gene encoding this enzyme, a response that may explain the continued enhancement of larval cold tolerance in frozen larvae (Fig. 5).

### Larval cold tolerance

Our 32-day, low-temperature treatment ultimately exposed larvae to −5 °C for a total of 14 days (Fig. 1). Although larvae of *B. antarctica* are freeze-tolerant year-round, freezing at −5 °C for 60 h caused >60% mortality in summer-collected larvae (Teets *et al.* 2011). The nearly 100% survival (49 out of 50) by frozen larvae in the present study suggests that the level of larval freeze tolerance is enhanced by seasonal cold-hardening. Additionally, the slow cooling regime we used may have initially promoted rapid cold-hardening that augmented increased cold tolerance caused by the 32-day period of acclimation at subzero temperatures (Kawarasaki *et al.* 2013).

Cryoprotective dehydration was equally effective for winter-acclimatized larvae to survive the 32-day low-temperature treatment. Consistent with a previous report (Benoit *et al.* 2007), cryoprotectively dehydrated larvae were unable to absorb water from the saturated air, and submergence in water was required for these severely shrunken larvae to fully rehydrate. Therefore, cryoprotective dehydration in *B. antarctica* differs from that in Collembola and earthworm cocoons, in which increased environmental temperatures within the subzero range promote rehydration from saturated air, allowing melting points of these organisms to 'track' environmental temperature (Holmstrup, Bayley & Ramløv 2002). Once larvae of *B. antarctica* become cryoprotectively dehydrated, they would be expected to remain in this state until spring when meltwater from the accumulated snow inundates their microhabitats.

### Post-recovery energy reserve contents

One of our main objectives was to compare potential trade-offs of overwintering in a frozen vs. a cryoprotectively dehydrated state. Because adults have non-functional mouthparts and feeding opportunities in spring before pupation may be limited (Peckham 1971; Sugg, Edwards & Baust 1983), depletion of energy reserves during winter would be expected to negatively affect reproductive fitness. In summer-collected larvae, freezing at −5 °C for 60 h substantially reduces levels of total lipids and glycogen (Teets *et al.* 2011). Similarly, loss of as little as 26% of body water at 2 °C significantly depletes energy reserves (Teets *et al.* 2012a). In contrast, winter-acclimatized larvae maintained the initial level of total lipids throughout the 37-day experiment (Table 1). Although a substantial depletion of glycogen reserves occurred during the 32-day, low-temperature treatment, most of this energy reserve was replenished after recovery in both frozen and cryoprotectively dehydrated larvae by Day 37 (Fig. 5). In *R. sylvatica*, glucose mobilized in response to tissue freezing is usually reconverted to glycogen within 24–48 h of thawing (Costanzo & Lee 2013). Similar reconversions of mobilized osmolytes likely occurred in larvae of *B. antarctica* during recovery (Fig. 5).

The fact that post-recovery levels of glycogen and total lipids did not differ between two treatment groups suggests energetic costs of overwintering are similar. Compared to supercooling, freezing induces a metabolic depression at the same subzero temperature (Irwin & Lee 2002). Likewise, acclimation of *B. antarctica* larvae to a desiccating environment (Benoit *et al.* 2007) or hyperosmotic seawater (Elnitsky *et al.* 2009) results in a reduced oxygen consumption compared to fully hydrated larvae at the same temperature. Therefore, cryoprotective dehydration may also function to suppress larval metabolic activity. This hypothesis is supported by genome-wide expression data, as cryoprotective dehydration in *B. antarctica* is accompanied by across-the-board downregulation of genes involved in central metabolism and energetically expensive cellular processes such as membrane transport (Teets *et al.* 2012b). Future study is needed to directly compare the metabolic

rates of frozen vs. cryoprotectively dehydrated larvae, as well as the fitness consequences (e.g. adult emergence and fecundity) of overwintering in these alternative physiological states.

## Conclusion

Winter-acclimatized larvae of *B. antarctica* readily tolerated extensive loss of body water associated with cryoprotective dehydration at −5 °C. Although depression of the SCP in winter-acclimatized larvae suggests they enhance their supercooling capacity, overwintering *via* cryoprotective dehydration also requires avoidance of inoculative freezing from contact with environmental ice (Elnitsky *et al.* 2008). Due to global climate change, the Antarctic Peninsula is experiencing radical changes, including localized reduction in snow cover, glacial melting and increased precipitation as rain (Fox & Cooper 1998; Peck, Convey & Barnes 2006), factors that are likely to increase the chance of inoculative freezing in larval hibernacula. However, throughout the year hydric conditions within the diverse range of microhabitats used by these larvae may vary depending on substrate type, sun exposure, slope, vegetation and drainage. Furthermore, even at the same moisture content, variations in substrate composition (e.g. soil types and organic contents) influence the likelihood of inoculative freezing by contact with environmental ice (Costanzo *et al.* 1997, 1998). Therefore, in hibernacula, where the potential for inoculative freezing is low, larvae may be forced to overwinter in the cryoprotectively dehydrated state. Our results indicate that freezing and cryoprotective dehydration are equally effective in promoting winter survival of larvae, with surprisingly few differences in energetic costs. The physiological flexibility of *B. antarctica* to overwinter in these alternative states likely contributed to its range distribution that extends further south than any other free-living insect.

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