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Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic midge, *Belgica antarctica*

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SUMMARY

During winter, larvae of the Antarctic midge, *Belgica antarctica* (Diptera, Chironomidae), must endure 7–8 months of continuous subzero temperatures, encasement in a matrix of soil and ice, and severely desiccating conditions. This environment, along with the fact that larvae possess a high rate of water loss and are extremely tolerant of desiccation, may promote the use of cryoprotective dehydration as a strategy for winter survival. This study investigates the capacity of larvae to resist inoculative freezing and undergo cryoprotective dehydration at subzero temperatures. Slow cooling to –3°C in an environment at equilibrium with the vapor pressure of ice reduced larval water content by ~40% and depressed the body fluid melting point more than threefold to –2.6°C. This melting point depression was the result of the concentration of existing solutes (i.e. loss of body water) and the *de novo* synthesis of osmolytes. By day 14 of the subzero exposure, larval survival was still >95%, suggesting larvae have the capacity to undergo cryoprotective dehydration. However, under natural conditions the use of cryoprotective dehydration may be constrained by inoculative freezing as result of the insect's intimate contact with environmental ice. During slow cooling within a substrate of frozen soil, the ability of larvae to resist inoculative freezing and undergo cryoprotective dehydration was dependent upon the moisture content of the soil. As detected by a reduction of larval water content, the percentage of larvae that resisted inoculative freezing increased with decreasing soil moisture. These results suggest that larvae of the Antarctic midge have the capacity to resist inoculative freezing at relatively low soil moisture contents and likely undergo cryoprotective dehydration when exposed to subzero temperatures during the polar winter.

Key words: Chironomidae, cryoprotective dehydration, freeze tolerance, supercooling.

INTRODUCTION

Cold-hardy invertebrates can be classified most simply as freeze tolerant or freeze intolerant. Freeze-tolerant species survive the freezing of their body fluids by promoting ice nucleation at high subzero temperatures and through the seasonal accumulation of cryoprotectants (Zachariassen, 1985; Duman et al., 1991; Lee, 1991). For freeze-intolerant species, by contrast, internal ice formation is ultimately lethal and survival depends upon prolonged, and often extensive, supercooling. Supercooling requires the absence or masking of potential ice nucleators within the body fluids as well as behavioral and/or physiological mechanisms that prevent inoculative freezing by environmental ice (Lee et al., 1995). Water also may be biologically unavailable in the form of ice, and, therefore, supercooled insects may be subjected to extended periods of desiccation as well (Lundheim and Zachariassen, 1993).

A third strategy of over-wintering, termed cryoprotective dehydration, has been described for several freeze-intolerant soil invertebrates (Holmstrup, 1992; Holmstrup and Westh, 1994; Holmstrup and Sømme, 1998; Holmstrup et al., 2002). In this strategy, supercooled invertebrates with high integumental permeability dehydrate when exposed to an environment at equilibrium with the vapor pressure of ice, owing to vapor pressure differences between supercooled water and ice at the same temperature (Holmstrup and Sømme, 1998). Such water loss continues until, at equilibrium, the vapor pressure of the body fluids equals that of the surrounding ice. At this time, the risk of freezing has been eliminated because the melting point (MP) of the animal's body fluids equals the ambient temperature (Holmstrup et al., 2002). Equilibration of the body fluid MP with that of the environment may also be facilitated by the accumulation of cryoprotectants (Holmstrup, 1995; Worland et al., 1998). More recently, cryoprotective dehydration has been reported for a freeze-tolerant nematode (Wharton et al., 2003) and an enchytraeid worm (Pedersen and Holmstrup, 2003), however, use of this strategy may be constrained by inoculative freezing of the body fluids as result of contact with environmental ice.

The terrestrial chironomid *Belgica antarctica* is the southern-most free-living holometabolous insect, being sporadically dispersed, but locally abundant, on the west coast of the Antarctic Peninsula. Detailed accounts of the life-history and ecology of *B. antarctica* are provided by Convey and Block (Convey and Block, 1996), Sugg et al. (Sugg et al., 1983), Usher and Edwards (Usher and Edwards, 1984), and references cited therein. Briefly, its 2-year life cycle includes four larval stages and over-wintering may occur in any instar. Larvae typically over-winter within the upper few centimeters of the substrate, with pupation and adult emergence occurring in spring and summer. The adults are wingless, like many

insects in wind-swept alpine and oceanic habitats, and live for fewer than 14 days.

Although ambient air temperatures on the Antarctic Peninsula may reach winter lows of –30°C, larvae of *B. antarctica* survive freezing to only about –15 to –20°C (Baust and Lee, 1981; Lee et al., 2006). However, thermal buffering of the over-wintering hibernaculum, provided by the oceanic influence and up to a meter of ice and snow, apparently explains this anomaly; at 1 cm depth, substrate temperatures remain between 0 and –2°C for more than 300 days of the year, and rarely decrease below –5°C (Baust and Lee, 1981). As larvae maintain relatively constant supercooling points between –6 and –8°C throughout the year (Baust and Lee, 1987), freezing of body fluids during over-wintering likely occurs *via* inoculation from the external environment. Alternatively, upon freezing of the surrounding substrate larvae may dehydrate, equilibrating their body fluid MP with the ambient temperature, thereby remaining unfrozen during over-wintering. Such a strategy of cryoprotective dehydration necessitates a high rate of water loss and/or a resistance to avoid inoculation of the supercooled body fluids.

Anecdotal reports suggest that several Arctic chironomids dehydrate during the winter (Scholander et al., 1953; Danks, 1971), thus the capacity for dehydration may be present within this taxonomic group. In addition, *B. antarctica* is highly desiccation tolerant, as larvae tolerate dehydration to ~30% of their initial body mass (Baust and Lee, 1987; Hayward et al., 2007; Benoit et al., 2007), and possess a high rate of water loss even at high relative humidities. Therefore, the purpose of the present study was to assess the capacity of larval *B. antarctica* to resist inoculative freezing and undergo cryoprotective dehydration when exposed to subzero temperatures.

MATERIALS AND METHODS

Source of insects

Substrate containing larval *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. Samples were shipped frozen (approx. –5°C for 7 days) to Miami University and subsequently stored at 4°C (0 h:24 h L:D) prior to use. Larvae were handpicked from the substrate and held in water at 4°C for 12–24 h to ensure clearance of the gut (mean gut clearance ~6 h) (Baust and Edwards, 1979) and to standardize body water content prior to use. Only fourth instar larvae were used for experiments.

Microhabitat temperature

Ten miniature temperature loggers (HOBO Water Temp Pro, Onset Computer, Pocasset, MA, USA) were deployed in microhabitat sites containing larval and adult *B. antarctica* on Torgersen Island in January 2005. Loggers recorded temperature at 30-min intervals for the duration of the study period. The loggers were recovered in January 2006 and the resulting data analyzed using Boxcar Pro 4.3 software (Onset Computer, Pocasset, MA, USA).

Cryoprotective dehydration

The capacity of *B. antarctica* to undergo cryoprotective dehydration was assessed by exposing larvae to an environment at equilibrium with the vapor pressure of ice as described in Pedersen and Holmstrup (Pedersen and Holmstrup, 2003). Groups of five individuals were blotted dry and placed within 0.6 ml polyethylene microcentrifuge tubes. Larvae were confined by means of fine (~20 μm) nylon mesh that allowed free movement of water vapor. Microcentrifuge tubes were in turn placed within 15 ml glass vials containing ~5 g of crushed ice and closed with tightly fitting lids. Vials containing larvae were allowed to equilibrate in refrigerated baths at –0.6±0.1°C for 24 h. The temperature of the bath was then lowered incrementally (~0.5°C day^{-1}) to approximately –3.0±0.1°C and held there for an additional 10 days. A control group of larvae was held at –0.6±0.1°C until termination of the experiment (day 14).

Groups of larvae for body water content (WC) and body fluid melting point (MP) measurements were removed at 1- to 4-day intervals. The WC of individual larvae was assessed gravimetrically from measurements of fresh mass (to the nearest 0.01 mg) at the time of sampling, and dry mass (DM) after drying to constant mass at 65°C. Melting point determinations were made using a vapor pressure depression technique (Holmstrup and Sømme, 1998). Groups of five larvae were placed in a sample holder, crushed with a Teflon rod to expose the body fluids, and rapidly sealed within a C-52 sample chamber (Wescor Inc., Logan, UT, USA). Samples were then allowed to equilibrate for 30 min prior to measurement of the body fluid MP using a Wescor HR-33T Dew Point Microvoltmeter (Wescor Inc., Logan, UT, USA) operated in the dew point mode. Sample melting point was determined from standard curves produced from known salt solutions (Opti-Mole, Wescor Inc., Logan, UT, USA).

Survival was assessed in remaining larvae upon termination of the subzero exposure. Water (~80–100 μl) was added to the microcentrifuge tubes and larvae allowed to rehydrate/recover for 24 h at 4°C prior to survival assessment. Individuals displaying spontaneous movements were considered to have survived.

Cryoprotectant analysis

Cryoprotectant analysis was performed on larvae following slow cooling to –3°C in an environment at equilibrium with the vapor pressure of ice as described above. Control larvae were maintained at –0.6±0.1°C until termination of the experiment (day 14). Groups of ~25 larvae were weighed and immediately frozen at –80°C until whole body concentrations of cryoprotectants were determined. Prior to cryoprotectant analysis, larvae were homogenized in 7% perchloric acid and neutralized with equal volumes of 0.789 mol l^{-1} KOH. Glycerol content was determined enzymatically as described by Holmstrup et al. (Holmstrup et al., 1999). Sorbitol concentrations were measured on the same individuals using an enzymatic assay described (Bergmeyer et al., 1974). Trehalose content was determined following digestion with trehalase as described (Chen et al., 2002). Glucose concentration was determined using the glucose oxidase procedure (no. 510; Sigma, St Louis, MO, USA).

Exposure in frozen substrate

The ability to use the strategy of cryoprotective dehydration may be limited in *B. antarctica*, as over-wintering larvae are likely in direct contact with ice and may be susceptible to inoculative freezing. Therefore, to assess their ability to resist inoculative freezing and undergo protective dehydration larvae were slowly cooled in contact with frozen substrate. Groups of 10 larvae were placed in 35 mm diameter Petri dishes containing ~4 g of loosely packed substrate, predominantly sand and organic matter, collected from Torgersen Island. Larger stones were removed and the soil mixed to achieve a relatively homogeneous substrate. Substrate samples were dried at 65°C prior to rehydration to the desired soil moisture contents. The soil moisture content of field samples collected near Palmer Station was 1.10±0.08 g H_2O g^{-1} dry soil

(R.E.L. and L. Sandro, unpublished). However, owing to the sandiness of the soil, larvae in the field likely experience large fluctuations of soil moisture. Therefore, three soil moistures (0.80, 1.10 and 1.40 g H_2O g^{-1} dry soil) were tested. Petri dishes were subsequently sealed with Parafilm™ to prevent evaporation.

Larvae were allowed to equilibrate at –0.2±0.1°C for 24 h and an additional 24 h at –1.0±0.1°C within refrigerated baths. Freezing of the soil was then induced by lightly applying freezing spray to the exterior of the Petri dish; soils were allowed to freeze overnight at –1.0±0.1°C and visually confirmed to have frozen by the presence of ice crystals within the soil matrix. The temperature of the bath was then lowered incrementally (~0.5°C day^{-1}) to approximately –3.0±0.1°C and held there for an additional 7 days. Control groups of larvae were held in unfrozen substrate at –0.2±0.1°C until the termination of the experiment. The WC of individual larvae was assessed at approximately 7-day intervals. Frozen soils were rapidly thawed and larvae collected and gently blotted to remove surface water prior to determination of WC as described above. As it was not possible to monitor individual larvae for freezing exotherms, WC was used as an indication of whether larvae remained supercooled, and therefore lost body water, or were frozen inoculatively (i.e. maintained high WC) during the subzero exposure. Following termination of the exposure (day 16), remaining larvae were allowed to recover at 4°C for 24 h prior to survival assessment as described above.

Statistical analysis

Means were compared 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 mean ± 1 s.e.m. Statistical significance was set at $P<0.05$.

RESULTS

Microhabitat temperatures

Summer temperatures (i.e. January and February) in microhabitat sites of larval *B. antarctica* often exceeded 15°C (Fig. 1), and occasionally 20°C. Conversely, subzero temperatures also occurred during summer months. Microhabitat temperatures declined throughout the summer, finally stabilizing at subzero temperatures during mid to late-April, and remained below zero until mid-November (Fig. 1). Thanks to oceanic thermal buffering (Baust and Lee, 1981), as well as thermal buffering from snow and ice, winter temperatures in microhabitat sites generally remained between –1 and –3°C, and only rarely fell below –5°C.

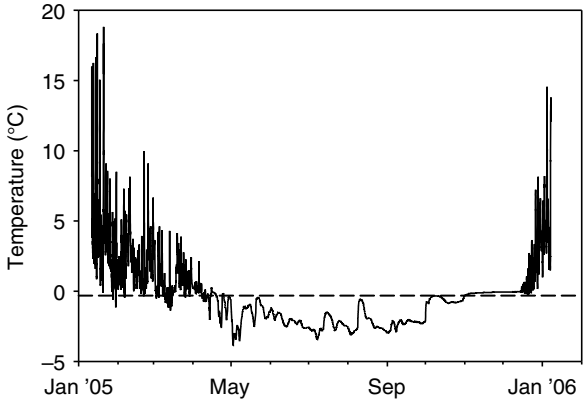

Fig. 1. Seasonal changes in temperature at a representative microhabitat of larval *Belgica antarctica* on Torgersen Island, near Palmer Station, Antarctica (64°46 S, 64°04 W). Microhabitat temperatures were measured in 2005–2006 using single-channel temperature loggers. The broken line indicates the equilibrium freezing point of the body fluids of fully hydrated, control larvae.

Cryoprotective dehydration

Larvae equilibrated to –0.6°C had a mean (±s.e.m.) WC of 2.67±0.05 g H_2O g^{-1} DM (N=15), with no significant change in the WC or DM of control animals during the experiment. By contrast, slow cooling in an environment at equilibrium with the vapor pressure of ice resulted in a significant ($P<0.001$) reduction of larval WC (Fig. 2A). Larval WC decreased rapidly until day 10, prior to leveling off for the remainder of the experiment. Water content was reduced by ~40%, to 1.63±0.03 g H_2O g^{-1} DM (N=15), by day 4; larval DM did not change significantly over the course of the experiment. These larvae appeared mildly dehydrated, however, survival at termination of the experiment was >96% (N=30). Similarly, all control larvae (N=30), maintained at –0.6°C, survived the 14-day exposure.

The MP of larvae equilibrated to –0.6°C was –0.74±0.02°C (N=6) and did not change in control animals during the course of the experiment. However, cooling while at equilibrium with the vapor pressure of ice resulted in a significant ($P<0.0001$) reduction of larval MP (Fig. 2B). In contrast to WC, the MP decreased throughout the 14-day experiment. Relative to the controls, the MP was reduced more than threefold, to –2.61±0.03°C (N=6), by day 14 in larvae exposed to the vapor pressure of ice. At the termination of the experiment, the body fluid MP had been

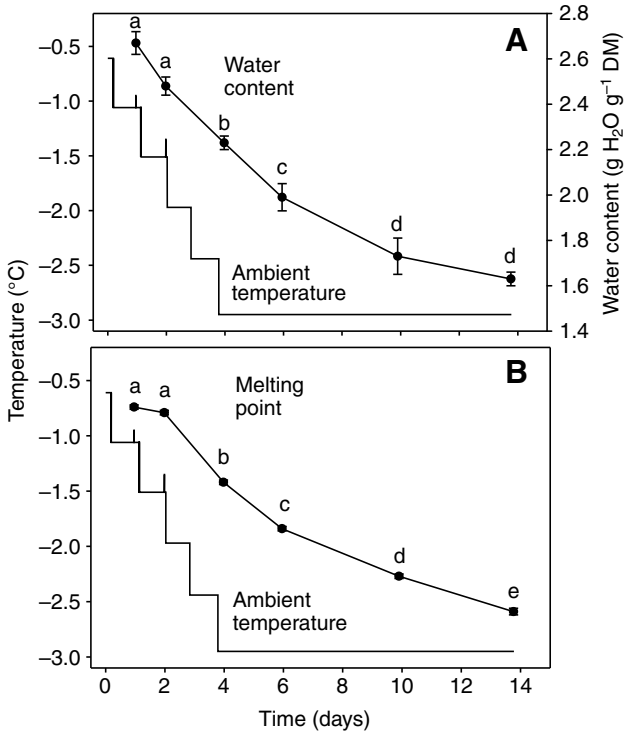

Fig. 2. Changes in (A) body water content (N=15) and (B) body fluid melting point (N=6) of larval *Belgica antarctica* during slow cooling to –3°C in an environment at equilibrium with the vapor pressure of ice. Different letters indicate significant differences between values (ANOVA, Bonferroni–Dunn test, $P<0.05$). Values are mean ± s.e.m.

Table 1. Estimated osmotic contribution of initial osmolytes in the hemolymph and osmolytes produced during slow cooling to –3°C in an environment at equilibrium with the vapor pressure of ice

		Cryoprotective dehydration	
	Control	Day 14	Day 6
Observed osmotic pressure of body fluids (mOsm) (*N*=6)	398±10	991±6	1392±16
Total body water content (g H_2O g^{-1} DM) (*N*=15)	2.67±0.05	1.99±0.06	1.63±0.03
OAW content (g H_2O g^{-1} DM)*	2.19	1.55	1.22
Loss of OAW (%)	–	29.2	44.3
Osmotic contribution of original solutes due to loss of OAW (mOsm)	–	562	715
Concentration of osmolytes (μg mg^{-1} DM) (*N*=5)			
Glycerol	~0	~0	~0
Sorbitol	~0	~0	~0
Glucose	2.15±0.24	12.41±0.12	18.46±0.24
Trehalose	3.32±0.52	19.48±0.67	37.42±0.31
Osmotic contribution of synthesized osmolytes† (mOsm)	–	67.2	155.9
Total explainable osmotic pressure (%)	–	63.5	62.6

OAW, osmotically active water; DM, dry mass.
Values are mean ± s.e.m.
*OAW was calculated from Worland et al. (Worland et al., 1998). [(OIW)=0.069(TBW) + 0.3, where OIW is osmotically inactive water content, TBW is total body water content and is the sum of OIW and OAW.]
†Assuming that osmolytes are dissolved in OAW.

depressed to within 0.4°C of the final ambient temperature (Fig. 2B). However, there remained a vapor pressure deficit of approximately –500 kPa between the body fluids of the larvae and the surrounding environment. Therefore, larvae would be expected to continue to lose water to their environment, further depressing the MP of the body fluids.

Cryoprotectant analysis

Of the cryoprotectants measured in larvae, only glucose and trehalose were found in significant concentrations (Table 1). Control larvae maintained relatively low concentrations of both glucose and trehalose throughout the experiment. Conversely, both glucose and trehalose were increased significantly (P<0.0001) in larvae that were cooled at equilibrium with the vapor pressure of ice. By day 14 of the subzero exposure, glucose and trehalose concentrations in larvae had increased by approximately nine- and 11-fold, respectively.

Exposure in frozen soil

The WC of larvae equilibrated at –0.2°C in contact with soils of different moisture content did not differ significantly (combined mean 2.48±0.04 g H_2O g^{-1} DM; N=30). Similarly, neither WC nor DM changed significantly in control animals during the 16-day exposure at any soil moisture tested. However, cooling larvae in contact with frozen substrate resulted in a clear separation, into high (>2.20 g H_2O g^{-1} DM) and low (<1.80 g H_2O g^{-1} DM) WC groups (Fig. 3). Larvae in the 'high' WC group, designated as frozen in Fig. 3, presumably froze inoculatively soon after the soil was frozen. These larvae remained in vapor pressure equilibrium with the surrounding environment, and, therefore, did not lose substantial amounts of water. For all levels of soil moisture tested, the mean WC of frozen larvae did not differ significantly from control larvae maintained at –0.2°C (Fig. 3). By contrast, larvae in the 'low' WC group, designated as dehydrated in Fig. 3, presumably remained unfrozen and dehydrated; larvae lost body water as a result of the vapor pressure gradient between the unfrozen body fluids and the surrounding environmental ice. By day 16, the WC of larvae that dehydrated was significantly (P<0.001) lower than that of control larvae for all three soil moistures tested (Fig. 3). At the termination of the experiment there was no significant difference in DM between 'high' and 'low' WC groups in any soil moisture tested.

Based upon reductions of larval WC during cooling in contact with frozen soil, the ability of larvae to resist inoculative freezing and, therefore, use a strategy of cryoprotective dehydration was significantly (P<0.001) affected by soil moisture content (Fig. 4); the percentage of larvae frozen inoculatively increased with increasing soil moisture. Less than 50% of larvae were frozen when cooled in contact with substrate at a soil moisture content of 0.80 g H_2O g^{-1} dry soil. However, at soil moisture contents of 1.10 and 1.40 g H_2O g^{-1} dry soil <30% of larvae were able to resist inoculative freezing and use a strategy of cryoprotective dehydration (Fig. 4). Regardless, larval survival of the 16-day exposure was >96% (N=30) for all soil moistures tested and did not differ significantly from control larvae maintained at –0.2°C.

DISCUSSION

Cryoprotective dehydration in a polar insect

During winter *B. antarctica* larvae are likely to be encased in a matrix of soil and ice for 7–8 months. However, microhabitat temperatures remained between 0 and –3°C throughout much of winter, and only rarely fell below –5°C (Fig. 1) (Baust and Lee, 1981). Such conditions may result in the freezing of the body fluids through inoculation from environmental ice. Alternatively, if larvae can resist inoculative freezing, these environmental conditions would create a gradient for water loss from supercooled larvae due to the lower vapor pressure of the surrounding ice. Our results demonstrate that *B. antarctica* larvae do indeed dehydrate when exposed to an environment at equilibrium with the vapor pressure of ice (Fig. 2A). This water loss, in addition to the *de novo* synthesis of osmolytes, depressed the MP of the larvae's body fluids to nearly –3°C (Fig. 2B), suggesting *B. antarctica* larvae have the capacity to undergo cryoprotective dehydration at ecologically relevant subzero temperatures. Although protective dehydration has

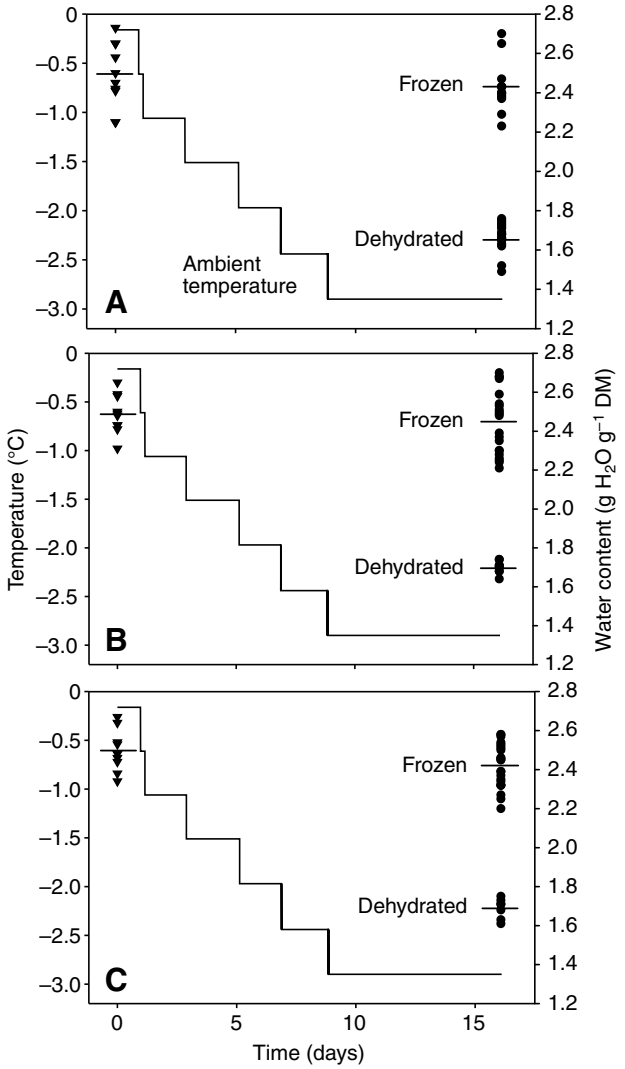

Fig. 3. Body water content (WC) of individual *Belgica antarctica* larvae (*N*=30) during slow cooling to –3°C in contact with substrates of varying moisture content (A) 0.80, (B) 1.10 and (C) 1.40 g H_2O g^{-1} dry soil. Triangles denote WC of individuals at day 0 and circles, the WC of frost-exposed individuals (day 16). Broken lines denote the mean WC of individuals at day 0 and 16, separated into 'high' (frozen) and 'low' (dehydrated) WC groups.

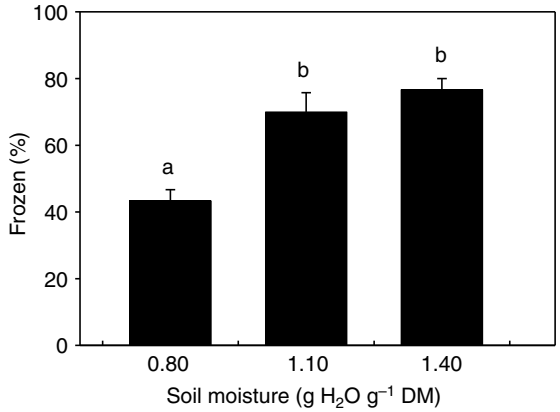

Fig. 4. Percentage of *Belgica antarctica* larvae frozen during cooling to –3°C in contact with substrates of varying moisture content, as detected by the maintenance of 'high' body water content, Different letters indicate significant differences between values (ANOVA, Bonferroni–Dunn test, $P<0.05$). Values are mean ± s.e.m. of three groups of ten individuals.

previously been documented in earthworm cocoons (Holmstrup, 1992; Holmstrup and Westh, 1994), Collembola (Worland et al., 1998; Holmstrup et al., 2002), a nematode (Wharton et al., 2003) and an enchytraeid worm (Pedersen and Holmstrup, 2003), our study is the first report of cryoprotective dehydration in a true insect.

The melting point depression observed in *B. antarctica* was a consequence of both water loss, and resultant increase in the concentration of the original solutes, as well as a concomitant synthesis of osmolytes (Table 1). As a result of the vapor pressure gradient between body fluids and the surrounding ice, larvae lost water throughout the subzero temperature exposure (Fig. 2A). At the termination of the experiment (day 14), larvae had lost ~40% of their total body water. However, estimates of the osmotically active water content, based upon measurements in Worland et al. (Worland et al., 1998), suggest that water loss, and the associated concentration effect, explained only approximately 57% and 51% of the observed change in osmotic pressure at days 6 and 14 during cryoprotective dehydration, respectively (Table 1). This is in contrast to the enchytraeid *Fridericia ratzeli*, in which MP depression to nearly –6°C during cryoprotective dehydration is accomplished largely (~83%) through water loss and the concentration of the original solutes (Pedersen and Holmstrup, 2003).

At the termination of the experiment, there still remained a vapor pressure gradient of approximately –500 kPa between the body fluids and the surrounding ice. Therefore, larvae would be expected to continue to lose water to their environment, further depressing the MP of their body fluids. Additionally, as the WC continues to decrease, even small reductions in WC would have a large effect on the resulting MP, as the effects of further water loss will increase the MP in a hyperbolic manner (Ring, 1982; Holmstrup and Westh, 1994). Such continued dehydration is unlikely to affect survival, at least in the short-term, as *B. antarctica* larvae tolerate the loss of ~70% of their total body water (Benoit et al., 2007).

During cryoprotective dehydration, larvae accumulated significant amounts of osmolytes that further depressed the MP of the body fluids (Table 1). Of the sugars and polyols assayed, only glucose and trehalose were detected in *B. antarctica* larvae; relative to control larvae, glucose and trehalose concentrations were approximately nine- and 11-fold higher, respectively, following cryoprotective dehydration. The Arctic collembolan, *Onychiurus arcticus*, likewise accumulates significant concentrations of both glucose and trehalose during cryoprotective dehydration (Worland et al., 1998). Furthermore, *F. ratzeli* accumulates similar concentrations of glucose during cryoprotective dehydration as we observed in *B. antarctica* larvae (Pedersen and Holmstrup, 2003). In addition to facilitating depression of the melting point, such sugars and polyols are well known to protect membranes and proteins against the deleterious effects of low temperature and desiccation (Crowe et al., 1992; Storey, 1997).

Although the observed increase in glucose and trehalose levels was substantial, these osmolytes accounted for a rather small fraction of the overall increase (<12%) in osmotic pressure during dehydration (Table 1). Together with the concentration of the original solutes, as the result of dehydration, the synthesis of the

measured sugars accounted for <65% of the observed change in osmotic pressure. The identity of the solutes making up the remainder of the observed osmotic pressure following dehydration is unknown. However, *B. antarctica* larvae are known to accumulate a variety of other sugars and polyols, including erythritol, sucrose and fructose (Baust and Edwards, 1979; Baust, 1980; Baust and Lee, 1983). Additionally, other cold-hardy insects accumulate significant concentrations of free amino acids during desiccation and acclimation to low temperature (Storey and Storey, 1988). Such increases in other cryoprotectants may account for a portion of the change in osmotic pressure during cryoprotective dehydration.

It should also be noted that estimates of the contribution of dehydration and the synthesis of osmolytes to account for the observed increase in osmotic pressure during cryoprotective dehydration are highly dependent upon the fraction of the total water content of larvae that is osmotically active (Table 1). This is especially true when total body water is low, as even a slight change in the osmotically active water (OAW) content would significantly affect estimates of the contribution to the measured osmotic pressure. Our estimates in accounting for the observed osmotic pressure necessarily rely on measures of osmotically active and inactive water from the collebolan *O. arcticus* (Worland et al., 1998), as, unfortunately, similar data do not exist for the midge larvae. These values may or may not be representative for *B. antarctica* larvae, and, therefore, may also account for a portion of the unexplained increase in osmotic pressure during dehydration.

Resistance to inoculative freezing

The ability to use the strategy of cryoprotective dehydration for subzero temperature survival may be constrained by inoculative freezing, as over-wintering larvae are likely to be in intimate contact with environmental ice. During the period in which the larvae are in a supercooled state (i.e. during cooling), they are at risk of inoculation from the surrounding ice. If the insect remains unfrozen during cooling, body water is lost, as a result of the vapor pressure gradient, the melting point of the body fluids equilibrates with the ambient temperature, and the risk of freezing is eliminated. Therefore, a high rate of water loss, which *B. antarctica* larvae certainly possess (this study) (Benoit et al., 2007), allows the organism to 'track' environmental changes and rapidly equilibrate the MP of the body fluids to that of the ambient temperature, thereby eliminating the risk of freezing.

Based upon reductions of larval WC, the resistance to inoculative freezing of *B. antarctica* depended on the moisture content of the soil in which they were cooled. As the water content of the soil increased, and therefore the amount of ice surrounding the larvae, the percentage of larvae that froze likewise increased (Fig. 4). At soil moistures of >1.10 g H_2O g^{-1} dry soil, fewer than 30% of larvae resisted inoculative freezing. These larvae almost certainly froze as a result of inoculation during cooling to –3°C, since the supercooling point of larvae remains between approximately –6 and –8°C year-round (Baust and Lee, 1987). Additionally, larvae were probably frozen soon after freezing of the substrate, as indicated from the maintenance of the high body water content in frozen larvae (Fig. 3). By contrast, at 0.80 g H_2O g^{-1} dry soil, nearly 60% of larvae remained unfrozen and dehydrated. This suggests that under relatively dry conditions larvae can resist inoculative freezing and use a strategy of cryoprotective dehydration to survive subzero temperatures.

The rate of cooling is also likely to determine the resistance to inoculative freezing and the use of cryoprotective dehydration. Wharton et al. (Wharton et al., 2003) demonstrated that in the Antarctic nematode *Panagrolaimus davidi*, slower cooling rates significantly increase the percentage of nematodes that resist freezing and undergo cryoprotective dehydration. The cooling rate of 0.5°C day^{-1} used in our study corresponded to a realistic, but relatively rapid rate of cooling compared to natural conditions (Fig. 1). As winter in the Antarctic begins and soils freeze, the cooling of larvae would likely be moderated by the thermal inertia of the soil and buffering from snow and ice. At slower cooling rates, water loss from the larvae, and the corresponding MP depression, could probably keep pace and 'track' changes of ambient temperature, therefore, increasing the likelihood that larvae would undergo cryoprotective dehydration.

Cryoprotective dehydration *versus* freezing for winter survival

Our results suggest that *B. antarctica* larvae can survive ecologically relevant subzero temperatures using either freeze tolerance or cryoprotective dehydration. The strategy used for winter survival probably depends upon the ambient environmental conditions upon entrance into winter. At relatively low soil moistures, the high subzero temperatures and slow rates of cooling within the larval microhabitat, in addition to the extreme tolerance of desiccation and high rate of water loss of *B. antarctica*, increase the likelihood that larvae can resist inoculative freezing and undergo cryoprotective dehydration during the polar winter. However, even if larvae are frozen after a period of dehydration this may only enhances survival, as mild dehydration increases the freeze tolerance of *B. antarctica* larvae (Hayward et al., 2007). Finally, although there did not appear to be differences in survival of low temperature between freeze tolerance and cryoprotective dehydration, future studies should address the long-term fitness consequences (e.g. Irwin and Lee, 2002) of the use of these strategies.

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