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Slow dehydration promotes desiccation and freeze tolerance in the Antarctic midge *Belgica antarctica*

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Summary

Adaptations to low moisture availability are arguably as important as cold resistance for polar terrestrial invertebrates, especially because water, in the form of ice, is biologically inaccessible for much of the year. Desiccation responses under ecologically realistic soil humidity conditions – those close to the wilting points of plants [98.9% relative humidity (RH)] – have not previously been examined in polar insect species. In the current study we show that, when desiccated at 98.2% RH, larvae of the Antarctic midge *Belgica antarctica* are more tolerant of dehydration than larvae desiccated at lower humidities (75% RH), and develop an increased tolerance to freezing. The slow rate of desiccation at this high RH enabled more than 50% of larvae to survive the loss of >75% of their osmotically active water (OAW). Survival rates were further increased when rehydration was performed at 100% RH, rather than by direct contact with water. Two days at 98.2% RH resulted in a ~30% loss of OAW, and dramatically increased the freeze tolerance of larvae to –10 and –15°C. The supercooling point of animals was not significantly altered by this desiccation treatment, and all larvae were frozen at –10°C. This is the first evidence of desiccation increasing the freeze tolerance of a polar terrestrial arthropod. Maximum water loss and body fluid osmolality were recorded after 5 days at 98.2% RH, but osmolality values returned to predesiccated levels following just 1 h of rehydration in water, well before all the water lost through desiccation had been replenished. This suggests active removal of osmolytes from the extracellular fluids during the desiccation process, presumably to intracellular compartments. Heat-shock proteins appear not to contribute to the desiccation tolerance we observed in *B. antarctica*. Instead, we suggest that metabolite synthesis and membrane phospholipid adaptation are likely to be the underpinning physiological mechanisms enhancing desiccation and cold tolerance in this species.

Key words: desiccation, freezing tolerance, heat-shock proteins, Chironomidae, polar insects.

Introduction

The Antarctic continent is one of the earth's most challenging environments for terrestrial organisms. Temperatures conducive to growth occur for only a few weeks each year, and even during the favourable period, freeze–thaw cycles persist on a daily basis (Convey, 1996). Polar terrestrial environments are also effectively deserts (Campbell and Claridge, 1987), with adaptations to low moisture availability perhaps even more crucial than cold resistance (Kennedy, 1993). For the resident invertebrate fauna, this combination of conditions poses significant problems. As poikilotherms, their tissues, cells and molecules are exposed to the full effects of temperature variation, necessitating major adaptive responses to cold stress. Maintenance of water balance is also a particularly acute problem for invertebrates, owing to their high surface area to volume ratio (Hadley, 1994). Intriguingly, cold and desiccation response mechanisms demonstrate many similarities, including the use of low molecular mass solutes such as sugars and polyols (Young and Block, 1980; Womersley and Smith, 1981; Bayley and Holmstrup, 1999), membrane lipid alterations (Bennett et al., 1997; Holmstrup et al., 2002a) and the expression of molecular chaperones such as heat-shock proteins (Hsps) (Rinehart et al., 2000; Hayward et al., 2004). A connection between cold and desiccation tolerance has been identified in several invertebrate species, including nematodes (Forge and MacGuidwin, 1992), earthworms (Holmstrup and Zachariassen, 1996), tardigrades (Sømme, 1996), Collembola (Worland et al., 1998) and insects (Ring and Danks, 1994; Williams et al., 2004). This has led to the conclusion that many of the physiological and molecular

responses to cold may have originally been adaptations for desiccation stress (Ring and Danks, 1994; Block, 1996; Danks, 2000).

For invertebrates with high integumental permeability, e.g. nematodes, enchytraeids, euedaphic Collembola and chironomid larvae, the risk of desiccation is particularly acute, yet these groups dominate the soil fauna in both the Arctic and Antarctic (Peterson and Luxton, 1982; Convey and Block, 1996; Wharton, 2003). It has been assumed that behavioural strategies of desiccation avoidance play a crucial role in survival (Hayward et al., 2000; Hayward et al., 2001), but considering the fact that relative humidities (RHs) as high as 98.2% RH can pose a significant desiccation risk (Bayley and Holmstrup, 1999), moist refuges with conditions near 100% RH may be scarce at high latitudes. The dehydration tolerance of polar invertebrates has typically been assessed under extreme conditions, e.g. 35% RH (Worland and Block, 1986), and generally these organisms are thought to have no physiological or metabolic means of regulating water loss (Harrison et al., 1991). For euedaphic invertebrates, however, Bayley and Holmstrup highlighted the importance of performing desiccation experiments at more ecologically relevant RH values (Bayley and Holmstrup, 1999), and in particular those close to the wilting point of plants (~98.9% RH). Under conditions of 98.2% RH, they identified a physiological response in the collembolan *Folsomia candida*, in which animals were able to actively combat water loss by regulating their internal osmotic pressure through sugar and polyol synthesis (Bayley and Holmstrup, 1999). Interestingly, this desiccation response also resulted in an increased tolerance to further desiccation (Sjursen et al., 2001) and cold (Bayley et al., 2001) stress. The capacity of polar terrestrial invertebrates to employ a similar strategy of cross-tolerance has not been addressed, but it could play an important role given that sugar and polyol synthesis represent perhaps the most important biochemical adaptation to both cold and desiccation (Storey, 1997).

The chironomid *Belgica antarctica* is the largest entirely terrestrial animal that lives in Antarctica, and it has the most southerly distribution of any free-living holometabolous insect (Sugg et al., 1983; Usher and Edwards, 1984). Endemic to the Antarctic Peninsula and its islands, *B. antarctica* has a two-year life cycle that includes four edaphic larval stages (Sugg et al., 1983). Overwintering can occur in any of the four larval instars, whereas the wingless adults live for fewer than 10 days during the brief Antarctic summer (Convey and Block, 1996). Larvae survive extracellular freezing, making *B. antarctica* the only freeze-tolerant insect on the continent, and can tolerate temperatures down to approximately –20°C (Baust and Lee, 1987; Lee et al., 2006). This is a somewhat modest level of cold tolerance in comparison with freeze-avoiding Antarctic species (see Cannon and Block, 1988), but it is more than sufficient for survival considering the thermal stability of *B. antarctica*'s snow-covered microhabitat, which typically remains between 0 and –2°C and rarely falls below –7°C (Baust and Lee, 1981). In common with many Chironomidae, *B. antarctica* larvae are not particularly resistant to water loss, but are highly desiccation tolerant (Baust and Lee, 1987). Indeed, *B. antarctica* appears highly resilient to a diverse range of environmental stressors (Baust and Lee, 1987), but few detailed physiological studies of this unique species have been undertaken.

This study is the first detailed assessment of the physiological response of an Antarctic terrestrial invertebrate to ecologically realistic desiccation stress. By investigating water loss at RH values specifically relevant to the soil environment, we have identified a level of desiccation tolerance in *B. antarctica* masked by more severe RH treatments. In addition, we tested the hypothesis that physiological adaptations to desiccation stress promote cross-tolerance to freezing in this species. We conclude that *B. antarctica* larvae can survive the loss of >75% of their osmotically active water (OAW) under gradual desiccation and rehydration, and the loss of as little as 30% of their OAW significantly increases their freeze tolerance. Heat-shock proteins (hsps) appear not to contribute to the desiccation response, and we predict that osmolyte and/or cryoprotectant synthesis and membrane phospholipid adaptation underpin the dramatic increase in freeze tolerance noted in desiccated larvae.

Materials and methods

Insects

Larvae of *Belgica antarctica* were collected from sites adjacent to penguin rookeries on Torgersen Island, near Palmer Station on the Antarctic Peninsula (64°46′ S, 64°04′ W) in January 2005 (the austral summer). Substrate temperatures ranged from 0 to 5°C during the collection period. Third- and fourth-instar larvae were hand picked from the substrate in ice-cold water and stored at 4°C in water for 2 days in order to synchronise their hydrated state prior to each desiccation treatment. Larvae of similar size were selected to limit effects of size differences on desiccation tolerance.

Desiccation treatments

Larvae were placed on nylon gauze netting (pore diameter 100 μm), which was then placed across the top of 50 ml centrifuge tubes containing 35 ml aqueous NaCl solutions. Saturated NaCl solutions provided 75% RH conditions, whereas 98.2% RH was achieved using 31.6 g of NaCl l^{-1} water (Bayley and Holmstrup, 1999). Controls were maintained under 100% RH conditions using demineralized water. The midge larvae (N=10 per tube) were thus suspended above the aqueous solution on the nylon gauze, which was then secured in position by a tightly fitting lid. The air in this small closed system rapidly equilibrated with the solution following Raoult's law. All desiccation treatments were performed in an environmental chamber that maintained a temperature of 4.0±0.2°C.

Survival and water loss

Samples were removed from each desiccation regime at set intervals over a 12-day period. Survival was assessed after 24

and 48 h of rehydration in water, and determined by the ability of larvae to move following gentle tactile stimulation.

Groups of 10 animals were weighed prior to desiccation (fresh mass), upon removal from each desiccation treatment (desiccated mass), and after 24 and 48 h of rehydration (rehydrated mass). Samples were then dried to constant mass at 60°C and their dry mass (DM) noted. From these values, mean initial water content and percentage water loss or gain could be calculated. At least three replicates of 10 animals were used for each time point under each treatment for both water content and survival assessments.

Osmotically active water (OAW) content and body fluid osmotic pressure

OAW content and body fluid osmotic pressure were calculated for larvae desiccated at 98.2% RH. Total water content was converted to osmotically inactive water (OIW) content according to the formula given in Worland et al. (Worland et al., 1998). In undesiccated controls the mean OIW content was 0.49±0.01 g water g^{-1} DM (N=5), equating to ~16% of the total water content. This value, generated for each sample, was then subtracted from total water content to give OAW content, expressed as g OAW g^{-1} DM. The osmolality of body fluids was determined using a vapour pressure depression technique (Holmstrup and Sømme, 1998). Groups of 10 larvae were placed in a sample holder and quickly crushed with a Teflon rod to expose the body fluids. The sample was then allowed to equilibrate for 30 min following placement within a C-52 sample chamber, which was connected to a Wescor HR 33T Dew Point Microvoltometer operated in the dew-point mode (Wescor Inc., Logan, UT, USA). This voltage reading was converted to osmotic pressure (bar) using van't Hoff's equation (1 bar=100 kPa). At least three replicates were performed for each time point during the desiccation and rehydration treatments.

Changes in osmolality and mass during rehydration

To assess the time frame of the rehydration process, and whether this changed depending on the extent of desiccation, the following experiment was performed. Samples were desiccated for either 24 h or 120 h (5 days) at 98.2% RH and then transferred to Eppendorf tubes containing water. The mass and osmolality of larvae was recorded before and after desiccation, and following either 1 or 24 h of rehydration, with surface water removed by blotting with filter paper. Three replicates (N=10 larvae) were performed for each data point.

Survival of freezing

To determine whether prior desiccation enhances the freezing tolerance of larvae, groups of 10 animals were transferred to 1.5 ml Eppendorf tubes and placed at either –10 or –15°C after 48 h at 98.2% RH. Larvae were removed at set intervals over a further 72 h period and transferred back to 4°C. At least three replicates were performed for each treatment and survival was assessed as previously described.

Supercooling point (SCP) measurements

Larvae desiccated for 48 h at 98.2% RH and controls (blotted dry with tissue paper) were placed in direct contact with a thermocouple cooled from 4 to –25°C at a rate of 1°C min^{-1}. The SCP 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.

Assessing hsp expression by northern blot hybridization

Clones of the genes encoding Hsp70 (GenBank accession number DQ459546), Hsp90 (DQAA459547), a small Hsp (DQ459548) and a 28s ribosomal RNA fragment (DQ459549) used as a control were described previously from *B. antarctica* (Rinehart et al., 2006).

Total RNA for northern blot hybridization was isolated using Trizol reagent from larvae desiccated under the following conditions: 0% RH for 20 h; 75% RH for 6, 24 and 48 h; and 98.2% RH for 6, 24, 96 and 120 h (N=25 per treatment). Twenty micrograms of each sample was heat denatured and separated by electrophoresis on a 1.5% agarose, 0.41 mol l^{-1} formaldehyde gel, transferred to a charged nylon membrane (Osmonics, Inc.) using a Turbo-Blotter (Schleicher and Schuell) and crosslinked by UV irradiation. Clones (hsp70, hsp90 and 28s) were digoxigenin (DIG)-labeled using DIG-high prime solution (Roche Applied Sciences, Inc.) for use as probes in northern hybridization using the Dig High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences, Inc.) following standard protocol. BioMax Chemiluminescence film (Kodak, Inc.) was then exposed to the blot for signal detection. Equal loading of samples was confirmed by alkaline stripping the membrane using 0.2 mol l^{-1} NaOH, 0.1% sodium dodecyl sulfate (SDS) followed by reprobing with the 28s rRNA probe. All northern analyses were run in triplicate.

Results

Desiccation survival and changes in water content

The mean fresh mass of *B. antarctica* larvae was 748±27 μg. The mean water content was 2.9±0.05 g g^{-1} DM, or 74.3±1.3% (N=100). The DM of samples did not change significantly throughout all desiccation treatments [one-way analysis of variance (ANOVA); P=0.20 and P=0.10 for 75% and 98.2% RH samples, respectively], indicating that all changes in mass were accounted for by water loss or gain.

When water loss exceeded ~50% larvae appeared shrivelled and motionless, although they rapidly rehydrated upon transfer to water, returning to their 'normal' shape within 24 h. Survival did not differ significantly between larvae rehydrated for 24 or 48 h, thus survival data are presented only for larvae rehydrated for 24 h.

Larvae maintained at 100% RH and 4°C experienced no significant changes in water content or mortality throughout the 12-day experimental period (Fig. 1A,B). Water was lost rapidly at 75% RH: within 2 days larvae lost >70% of their initial water content, retaining less than 1 g water g^{-1} DM (Fig. 1A).

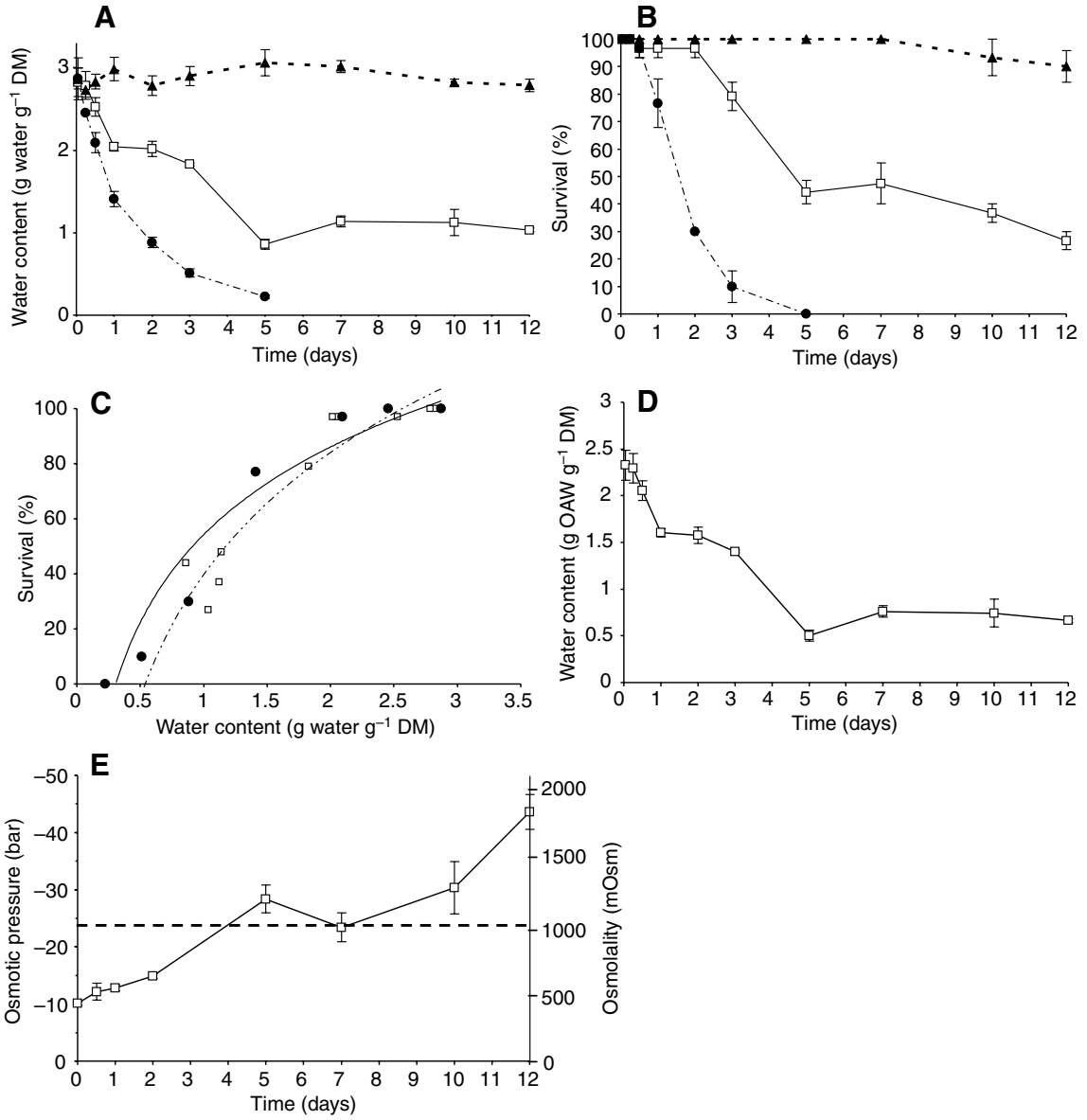

Fig. 1. (A) Change in water content (g) per gram of dry mass (DM). (B) Percentage survival of larvae maintained under 100, 98.2 and 75% relative humidity (RH) conditions for up to 12 days. (C) Comparison of survival at different water contents under desiccating conditions of 75 and 98.2% RH. Least-squares fit plotted using the following equation: y=cln x+b. (D) Change in osmotically active water content (OAW) during exposure to 98.2% RH. (E) Change in body fluid osmotic pressure (bar) and/or osmolality during exposure to 98.2% RH (1 bar=100 kPa). The horizontal line denotes the environmental water potential. 100% RH (closed triangles), 98.2% RH (open squares), 75% RH (closed circles). Values shown are means ± s.e.m. for three replicates of 10 individuals.

Survival also declined sharply at 75% RH, and no larvae survived more than 5 days at this relative humidity (Fig. 1B). The rate of water loss was considerably slower at 98.2% RH: within 2 days larvae still had ~2.0 g water g^{-1} DM, equating to a loss of less than 30% of their initial water content (Fig. 1A), and survival was close to 100% (Fig. 1B). Maximum water loss at 98.2% RH (~70%) occurred after 5 days, and was the only point at which the water content of larvae dropped below 1 g water g^{-1} DM at this humidity (Fig. 1A). The relationship between water content and survival differed between 75 and 98.2% RH (Fig. 1C), with survival after 5 days at 98.2% RH (~50% survival at 0.86 g water g^{-1} DM) significantly higher than for the equivalent water content at 75% RH (30% survival of 0.88 g water g^{-1} DM, two-tailed t-test; P<0.05). Between 5 and 12 days at 98.2% RH, the water content remained relatively stable (Fig. 1A), although survival declined slightly during this period (Fig. 1B).

Effect of desiccation on OAW and osmotic pressure

The mean OAW content of larvae declined more than fourfold, from 2.3 g g^{-1} DM to 0.5 g g^{-1} DM during the first 5 days of desiccation at 98.2% (Fig. 1D). Thus, ~50% of

larvae survived the loss of more than 75% of their OAW. The OAW content of larvae then remained stable between 5 and 12 days.

In accordance with the loss of OAW, a consistent and significant increase in the osmotic pressure and/or osmolality of body fluids was observed during the first 5 days of desiccation at 98.2% RH (Fig. 1E, two-tailed *t*-test; $P<0.01$). In fact, after 5 days at 98.2% RH, larvae reached an osmotic pressure exceeding the water potential of the environment, approximately –25 bar. Although a slight decrease in the osmolality of body fluids was noted between 5 and 7 days, the osmotic pressure did not decline significantly below that of ambient conditions. After 7 days at 98.2% RH the osmotic pressure of body fluids again increased and remained hyperosmotic in relation to the environment for the remainder of the experiment.

Influence of rehydration rate on survival

To assess the impact of rehydration rates following desiccation in *B. antarctica* we compared survival of larvae that were rehydrated either by submergence in water or by being transferred to 100% RH (Fig. 2). After 3 days of desiccation, corresponding to the loss of ~40% of initial water content, the difference in survivorship between the two rehydration regimes was not significant (two-tailed *t*-test; $P=0.24$). The survivorship of larvae rehydrated at 100% RH after 7 or 12 days of desiccation at 98.2% RH, however, was significantly higher than that for larvae rehydrated in water (two-tailed *t*-test; $P<0.05$ for both time points). Thus, the rate of rehydration has a significant influence on desiccation survival.

Changes in osmolality and mass during rehydration

Osmolality and mass changes were monitored in larvae following 1 day (Fig. 3A,B) or 5 days (Fig. 3C,D) of desiccation at 98.2% RH. Rehydration for 1 h in water was sufficient to restore both osmolality (Fig. 3A) and mass (Fig. 3B) of larvae desiccated for 1 day, with rehydrated values not significantly different from controls (two-tailed *t*-test; $P=0.19$ and 0.12, respectively). The osmolality of body fluids in larvae desiccated for 5 days (Fig. 3C) was also restored after 1 h in water (two-tailed *t*-test; $P=0.12$). One hour of rehydration was not sufficient to restore the original mass in larvae desiccated for 5 days, however, with a significant difference remaining between the fresh mass and rehydrated mass (two-tailed *t*-test; $P<0.05$). After 1 day of rehydration (Fig. 3D) the difference between fresh mass and rehydrated mass was not significant for 5-day desiccated samples (two-tailed *t*-test; $P=0.07$).

Effects of dehydration on freezing tolerance

Desiccation for 2 days at 98.2% RH dramatically increased the freezing tolerance of *B. antarctica* larvae: 100% of the desiccated larvae survived 3 days at –10°C, whereas <10% of the undesiccated larvae survived 2 days at –10°C (Fig. 4). None of the undesiccated larvae survived a 15-min exposure to –15°C, whereas 10% of the desiccated larvae survived this treatment (data not shown).

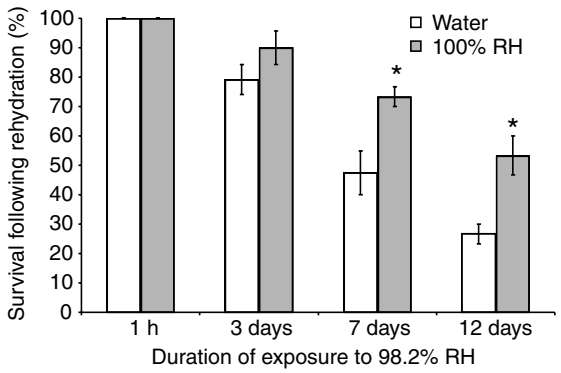

Fig. 2. Survival of desiccated larvae following rehydration by either submergence in water or exposure to 100% relative humidity (RH). Larvae were desiccated at 98.2% RH for different durations and then rehydrated for 1 day. *Significant difference ($P<0.05$) between treatments at a given time point. Values shown are means ± s.e.m. for three replicates of 10 individuals.

The mean SCP value for undesiccated control larvae (±s.e.m.) was –8.6±0.9°C ($N=11$; range=–3.8 to –11.9°C; two individuals recorded SCP values below –10°C). The mean (±s.e.m.) SCP value of desiccated larvae was –9.3±0.4°C ($N=8$; range=–7.9 to –11.3°C, with two individuals recording SCP values below –10°C). SCP values were not significantly different between treatments (two-tailed *t*-test; $P=0.52$).

Hsp expression

Transcripts encoding Hsp70, Hsp90 and a small Hsp were already upregulated in the midge larvae, and desiccation failed to further upregulate these transcripts, regardless of the severity of desiccation stress (Fig. 5).

Discussion

Bayley and Holmstrup highlighted the importance of assessing the desiccation tolerance of edaphic invertebrates under ‘realistic’ soil humidity conditions, i.e. those approaching the wilting point of plants (98.9% RH) (Bayley and Holmstrup, 1999). Yet even recent studies of polar terrestrial invertebrates persist in assessing desiccation tolerance under conditions below 5% RH (e.g. Sinclair et al., 2006). The ecological relevance of measuring desiccation tolerance under such extreme conditions is at best questionable but, more importantly, is likely to mask subtle physiological responses that may occur under buffered moisture conditions typically found in the soil environment. Based on the data presented here, *B. antarctica* exhibits the most extreme desiccation tolerance ever recorded in a polar insect. However, given the preponderance of soil organisms with high cuticular permeability at polar latitudes, similar physiological attributes may be widespread when assessed under appropriate humidity conditions.

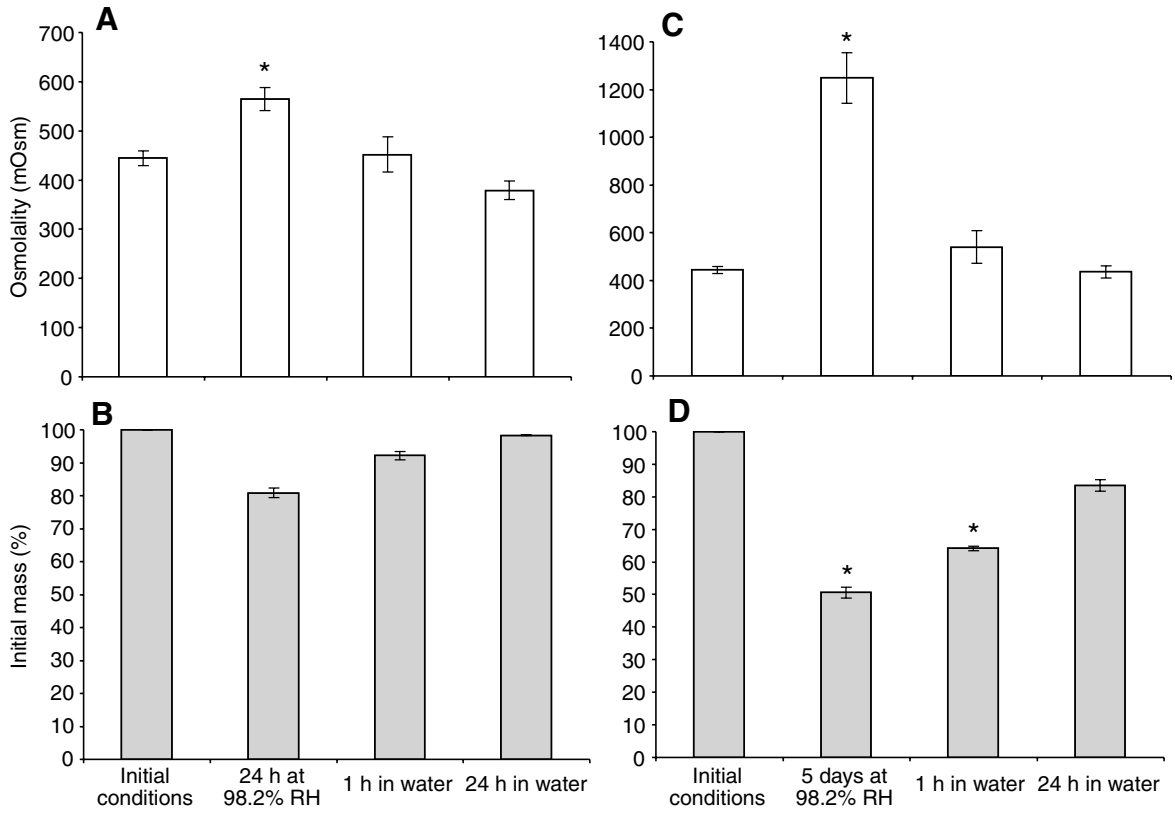

Fig. 3. Changes in (A) osmolality (mOsm) and (B) mass (represented as a percentage of the initial fresh mass) during desiccation at 98.2% relative humidity (RH) for 24 h, followed by 1 h and 24 h of rehydration in water. C and D show changes in osmolality and mass, respectively, after 5 days of desiccation at 98.2% RH, followed by 1 h and 24 h of rehydration in water. Initial conditions refer to osmolality and mass values prior to desiccation. *Significant difference (P<0.05) from initial conditions. Values shown are means ± s.e.m. for at least three replicates of 10 individuals.

The slow rate of desiccation recorded in *B. antarctica* at 98.2% RH significantly increased survival rates above those of larvae desiccated at 75% RH, and enabled more than 50% of animals to survive the loss of >75% of their OAW. Survival was further enhanced by rehydration at 100% RH rather than direct contact with water (Fig. 2). Under similar desiccating conditions, certain species of Collembola accumulate polyols and sugars to actively combat water loss (Bayley and Holmstrup, 1999; Holmstrup et al., 2001), and it seems reasonable to assume that similar metabolites contribute to the enhanced survival of desiccated *B. antarctica* larvae. Furthermore, an accumulation of metabolites under slow desiccation (this time acting as cryoprotectants) could explain the dramatically increased freeze tolerance of desiccated *B. antarctica* larvae at –10 and –15°C.

Interestingly, the initial OAW content of *B. antarctica*, 2.3 g water g^{-1} DM (Fig. 1D), was more than double that recorded in *F. candida* prior to desiccation (~1 g water g^{-1} DM) (Bayley and Holmstrup, 1999). This, in itself, may represent a desiccation tolerance mechanism in the polar insect, and perhaps explains why *B. antarctica* larvae were able to lose such a significant proportion of their OAW with only limited mortality. Increased body water content has been identified as a selected characteristic in desiccation-tolerant lines of *Drosophila melanogaster* (Gibbs, 2002), and although a high OAW content may represent a risk to freeze-avoiding species at high latitudes, it is unlikely to pose a problem to the freeze-tolerant *B. antarctica*. Another possible adaptation to an arid environment was noted in the coiling and aggregative behaviour of *B. antarctica* larvae during desiccation; animals desiccated in groups of 10 tended to entangle into a 'ball' of larvae as they became more dehydrated, and even individual larvae coiled their bodies upon desiccation, perhaps as a strategy to reduce surface area to volume ratio. Interestingly, we also observed this strategy operating in the field, where larvae were found in dense aggregations at dry sites but were more evenly dispersed in wet habitats. One problem with desiccating multiple larvae, however, is that subtle differences in this coiling behaviour could produce slightly different rates of water loss. This could explain the somewhat inconsistent slope of the desiccation curve noted at 98.2% RH (Fig. 1A).

The osmotic pressure of *B. antarctica* body fluids after 12 h of desiccation at 98.2% RH (Fig. 1D) was approximately –12.5 bar. After 120 h (5 days), when they have four times less OAW, their water potential deficit should be approximately

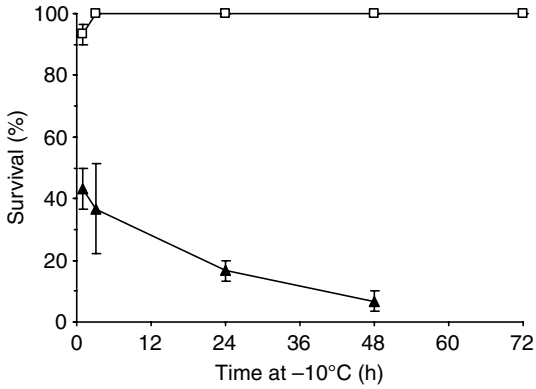

Fig. 4. Effect of a 48-h desiccation pretreatment at 98.2% relative humidity (RH) (squares) on cold tolerance at –10°C. Control (triangles). Values shown are means ± s.e.m. for at least three replicates of 10 individuals.

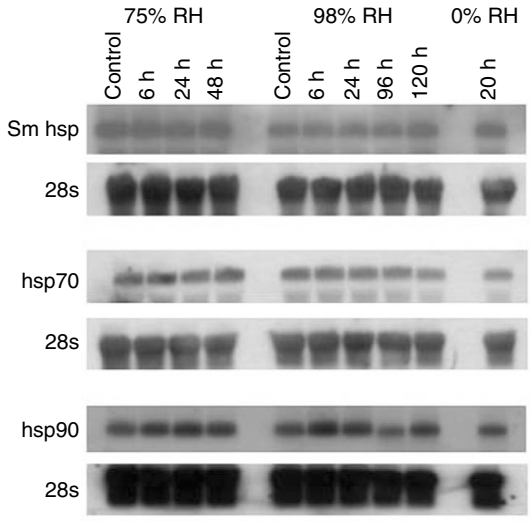

Fig. 5. Expression of the small *hsp*, *hsp70* and *hsp90* transcripts in fourth instar *Belgica antarctica* larvae in response to desiccation. Lanes represent RNA samples from larvae desiccated for different durations at 0, 75 and 98.2% relative humidity (RH). 28s ribosomal RNA was used as a control to confirm equal sample loading. Each sample was run in triplicate.

–50 bar, but instead the value was –33 bar. As relatively few cells were ruptured when determining osmolality, extracellular fluids, especially the haemolymph, predominantly contribute to this value. Thus, osmolytes must have been removed from these extracellular fluids. The dry mass of samples did not significantly change during the desiccation treatment, indicating that there was no overall gain or loss of metabolites and/or osmolytes during the desiccation treatment. Instead, osmolytes may have been redistributed, for example, to intracellular compartments. The acquisition of sugars in the cytoplasm of cells is thought to be a fundamental component of successful anhydrobiosis (Crowe et al., 2002), and presumably would also contribute to less extreme examples of desiccation tolerance. The intracellular partitioning of these and other compounds may also play a crucial role during desiccation (Oliver et al., 2002).

For *B. antarctica*, a reduced concentration of osmolytes in the haemolymph would mean that, upon rehydration, less water is required than that lost to return the haemolymph osmolality to its predesiccated value – providing, of course, that rehydration occurred at a faster rate than osmolyte transfer back out of the cells. This idea appears to be supported by the data presented in Fig. 3, in which the osmolality of body fluids returns to predesiccated levels within 1 h of rehydration, despite the fact that larvae have not yet regained all the water lost from desiccation, i.e. their mass was significantly different from that prior to desiccation. These data, therefore, suggest that osmolytes may be redistributed during the desiccation process, and that intracellular rehydration occurs more slowly than extracellular rehydration.

The active removal of osmolytes from the haemolymph to intracellular compartments could be advantageous in many respects. This strategy would reduce the initial rate of rehydration upon contact with water, which is known to affect survival (Fig. 2) (Bayley and Holmstrup, 1999). An increased concentration of sugars and/or polyols is also likely to enhance cellular and membrane integrity during both desiccation and freezing (Crowe et al., 1984; Crowe et al., 1992; Sano et al., 1999; Takagi et al., 2000). This could explain the increased cold tolerance of desiccated samples (Fig. 4). Removal of osmolytes from the haemolymph is also consistent with the idea that a freeze-tolerant organism should not have a low SCP value. In this regard, it should be noted that the SCP values in samples desiccated for 48 h were only slightly reduced, compared with controls, an observation that is somewhat surprising given the increase in osmotic pressure during this period. However, it is possible that other mechanisms, possibly the synthesis of ice nucleators, contribute to maintaining a high SCP; indeed, maintaining a high SCP would be made easier if osmolytes and/or cryprotectants were removed.

A link between enhanced cold tolerance following desiccation has long been established (Ring and Danks, 1994; Ring and Danks, 1998) and, in *B. antarctica*, 48 h at 98.2% RH resulted in a dramatic increase in survival at –10 and –15°C, relative to undesiccated controls (Fig. 4). This result concurs with Bayley et al. (Bayley et al., 2001), who found that 7 days at 98.2% RH significantly increased the cold tolerance of *F. candida*, although, unlike the collembolan, Hsps appear not to contribute to this response. In *B. antarctica* larvae, *hsp70*, *hsp90* and a small *hsp* gene transcripts are constitutively expressed and cannot be further upregulated by exposure to either high or low temperature (Rinehart et al., 2006). This pattern of expression is similar to that observed in the overwintering diapause of some temperate-zone species, e.g. the flesh fly *Sarcophaga crassipalpis*, in which *hsp70* and small *hsps* are expressed throughout pupal diapause, but remain unresponsive to temperature stress (Yocum et al., 1998; Rinehart et al., 2000; Hayward et al., 2005). Interestingly, these *hsp* transcripts are also unresponsive to desiccation stress

during diapause in *S. crassipalpis* (Hayward et al., 2004). Thus, Hsps appear to contribute to an underlying enhanced stress tolerance of the midge larvae (Rinehart et al., 2006), but are not further upregulated in response to cold or desiccation.

The increased cold tolerance noted in desiccated *B. antarctica* larvae was not the result of a reduced SCP, as these values were not significantly different between desiccated samples (–9.3±0.4°C) and controls (–8.6±0.9°C). Furthermore, as values remained above –10°C, larvae presumably froze at some point during the 3-day cold treatments. This work therefore represents the first evidence of gradual desiccation increasing the freezing tolerance of a polar arthropod. This strategy is quite different from cryoprotective dehydration, employed by certain Collembola and earthworm cocoons (Holmstrup and Westh, 1995; Holmstrup and Sømme, 1998), in which water loss, and an associated drop in SCP, continues until water potential equilibrium between the organism and ice is attained (Zachariassen, 1991; Lundheim and Zachariassen, 1993; Holmstrup et al., 2002b), preventing the animal from freezing.

In the anhydrobiotic nematode *Aphelenchus avenae*, ice formation does not occur below a water content of 0.3 g water g^{-1} DM (Crowe et al., 1983), whereas for *Artemia* cysts the value is 0.6 g water g^{-1} DM (Crowe et al., 1981). After 48 h at 98.2% RH, the group for which cross-tolerance was assessed, the water content of *B. antarctica* larvae was >2 g water g^{-1} DM. The lowest water content recorded at this humidity was 0.86 g water g^{-1} DM (Fig. 1C), suggesting that even the most desiccated larvae were still susceptible to freezing. Cross-tolerance was not assessed for this level of water loss, however, as there would have been compounding mortality factors resulting from both desiccation and cold stress. Indeed, cross-tolerance data for *F. candida* (Bayley and Holmstrup, 1999) was complicated somewhat by the fact that the desiccation treatment used resulted in some mortality, which may explain the relatively limited cross-tolerance to cold noted in this species (Bayley et al., 2001). That aside, the synthesis of polyols and sugars (Bayley and Holmstrup, 1999) and alterations in membrane phospholipid composition (Bayley et al., 2001) seem the most likely physiological mechanisms contributing to increased cold tolerance noted in *F. candida*.

It seems likely that similar mechanisms underpin the desiccation response of *B. antarctica*, but in this instance facilitate enhanced freezing tolerance. Limited data exists regarding the principal stress-response metabolites of *B. antarctica*. However, a preliminary analysis of desiccation-responsive metabolites, using Fourier Transform Infrared (FT-IR) spectroscopy and a discrimination function analysis, indicated that the polysaccharide region of the spectra is altered significantly in response to desiccation. A variety of polyhydric alcohols and sugars, including erythritol and trehalose, have already been identified in larvae of this species (Baust and Edwards, 1979; Baust and Lee, 1983) and represent excellent water replacement molecules that could facilitate increased desiccation and freezing tolerance (Holmstrup and Westh, 1995; Worland et al., 1998; Sano et al., 1999).

The capacity to tolerate prolonged periods of low moisture availability is of considerable adaptive significance to polar terrestrial organisms. Yet, despite the apparent harshness of high-latitude terrestrial habitats, the buffered moisture status of the soil substrate should not be disregarded. This study highlights the crucial importance of performing desiccation tolerance experiments under ecologically relevant humidity conditions. The extreme desiccation tolerance noted in *B. antarctica* at high relative humidities also suggests that this parameter should perhaps be reassessed in other polar terrestrial invertebrates. Our study lends yet further support to the idea that adaptations to desiccation stress promote enhanced cold tolerance, and provides the first evidence that gradual desiccation can enhance the lower limit of freeze tolerance in a polar arthropod. The slow rate of water loss, which occurs in permeable edaphic invertebrates at humidities approaching the wilting point of plants (e.g. 98.2% RH), is as relevant to polar species, as it is for temperate and tropical soil faunas. Furthermore, as demonstrated here, such conditions can facilitate the survival of extensive water loss, and permit the identification of more subtle desiccation and cold tolerance strategies.

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