Comparative Biochemistry and Physiology, Part A 152 (2009) 518–523

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation

Joshua B. Benoit [a,*], Giancarlo Lopez-Martinez [a], Michael A. Elnitsky [b,c], Richard E. Lee Jr. [b], David L. Denlinger [a]

[a] Department of Entomology, The Ohio State University, 318 West 12th Avenue, Columbus, OH, 43210, USA
[b] Department of Zoology, Miami University, Oxford, OH, 45056, USA
[c] Department of Biology, Mercyhurst College, Erie, PA, 16546, USA

ARTICLE INFO

Article history:
Received 1 October 2008
Received in revised form 11 December 2008
Accepted 12 December 2008
Available online 24 December 2008

Keywords:
Cross tolerance
Cold
Heat
Desiccation rates
Antarctica
Midge

ABSTRACT

Larvae of the Antarctic midge, *Belgica antarctica* (Diptera: Chironomidae), are frequently exposed to dehydrating conditions on the Antarctic Peninsula. In this study, we examined how rates and levels of dehydration alter heat and cold tolerance and how these relate to levels of trehalose within the insect. When dehydrated, larvae tolerated cold and heat stress more effectively, although resistance to cold was more pronounced than heat resistance. Slow dehydration was more effective than rapid dehydration in increasing temperature tolerance. Severe dehydration (50% reduction in water content) caused a much greater increase in temperature tolerance than did mild dehydration (e.g. 10% water loss). Larvae severely dehydrated at a slow rate (98% RH) were more temperature tolerant than those dehydrated quickly (0 or 75% RH). These results indicate that the slower dehydration rate allows the larvae to more effectively respond to reduced water levels and that physiological adjustments to desiccation provide cross tolerance to cold and heat. Levels of trehalose increased during dehydration and are likely a major factor increasing subsequent cold and heat resistance. This hypothesis was also supported by experimental results showing that injection of trehalose enhanced resistance to temperature stress and dehydration. We conclude that changes in temperature tolerance in *B. antarctica* are linked to the rate and severity of dehydration and that trehalose elevation is a probable mechanism enhancing this form of cross tolerance.

1. Introduction

The Antarctic midge, *Belgica antarctica*, spends the austral winter either frozen or in a state of cryoprotective dehydration (Elnitsky et al., 2008). Interestingly, even when frozen, exposure to −15 °C or below can induce mortality (Lee et al., 2006). During winter, larvae are likely in vapor pressure equilibrium with their environment, thus severe dehydration is unlikely (Elnitsky et al., 2008). These winter larvae are also protected within their harborages by snow and ice, with temperatures rarely reaching −15 °C (Schulte et al., 2008). Thus, some of the most stressful periods for these midges may not be during the winter but during the short austral summer (2–3 months) when larvae are exposed to high daytime temperatures that can exceed 25 °C (Schulte et al., 2008), as well as cold nights and desiccating conditions (Benoit et al., 2007). Larvae and adults are present during the summer, but adults live for only two weeks, while larvae require two years to complete their development (Sugg et al., 1983). Therefore, larvae are more likely to be exposed to periods of stress during their long development than are the short-lived adults.

Recently, we demonstrated that cold and desiccation tolerance in larvae of *B. antarctica* increased following dehydration (Hayward et al., 2007; Elnitsky et al., 2008), an observation also noted in many other insects (Hadley, 1994; Bayley et al., 2001). Cross tolerance between cold and dehydration is the likely consequence of similar physiological responses generated by these two types of stress (Ring and Danks, 1994, 1998). Particularly, during dehydration many insects synthesize additional trehalose and other osmolytes, which likely serve to protect membranes and proteins during subsequent cold exposure (Benoit et al., 2007). Few studies have focused on cross tolerance between dehydration and heat, but as with cold, the presence of excess osmolytes, particularly trehalose, may prevent protein denaturation during heat stress (Bowler, 2005). Of interest for *B. antarctica* is whether this cross tolerance between dehydration and temperature stress is influenced by different levels and rates of dehydration and whether dehydration can improve heat tolerance.

In this study, we report that dehydration enhances cold and heat tolerance in larvae of *B. antarctica*, and that both the level and rate of dehydration impact the level of tolerance attained. We provide

* Corresponding author. Tel.: +1 614 247 5093; fax: +1 614 292 2180.
E-mail address: benoit.8@osu.edu (J.B. Benoit).

1095-6433/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2008.12.009

evidence that elevation of trehalose is a key metabolite contributing to this form of cross tolerance. We propose that slow dehydration boosts synthesis of trehalose and is an important mechanism for enhancing temperature tolerance of these midge larvae during the austral summer.

2. Materials and methods

2.1. Insects

Larvae (third and fourth instars) of *B. antarctica* (Diptera: Chironomidae) were collected on Cormorant Island near Palmer Station, Antarctica (64°45′S, 64°04′ W) in January 2006 and 2007, in the substrate adjacent to penguin rookeries. Larvae were stored in the substrate at 4 °C until immediately before use in these experiments (Lopez-Martinez et al., 2008; 2009). At the onset of the experiments, larvae were sorted from the substrate in ice-cold water and blotted dry on paper towels.

2.2. Dehydration

Different relative humidities were used to generate different rates of drying in the desiccation experiments. The relative humidities were established using salt solutions (Winston and Bates, 1960) and verified with the use of a hygrometer (±2.0% RH, Thomas Scientific, Philadelphia, PA, USA). Complete dryness was attained with calcium sulfate (1.4×10^{-4}% RH, Benoit et al., 2005). Larvae were held in mesh-covered chambers above the solutions used to generate 0% RH ($CaSO_4$), 75% RH (NaCl), 98% RH (K_2SO_4), and 100%RH (double-distilled water). Specimens were used in experiments after 5, 10, 20, 35 and 50 ±1.5% losses of water content at 4 °C. Mass changes were monitored with an electrobalance (CAHN 25 Ventron Co., Cerritos, CA, USA; precision of ±0.2 μg SD and accuracy of ±6 μg at 1 mg).

To compare water loss between the three relative humidities (0, 75 and 98% RH), rates of water loss were determined according to Wharton (1985). Briefly, changes in water mass were monitored every 2 h for 8 h at each relative humidity at 4 °C and plotted according to the equation $m_t = m_0 e^{-kt}$, where m_t denotes the water mass at any time t, m_0 is the initial water mass, and $-k$ is the water loss rate. The slope of ln (m_t/m_0) vs. time denotes the water loss rate (k) and can be expressed as a %/h (Wharton, 1985). Three replicates of 10 individuals each were used to determine the water loss rate at each relative humidity.

2.3. Exposure to low and high temperature

To determine if dehydration alters survival of larvae during temperature stress, groups of five individuals were transferred to 2.0 mL Eppendorf tubes. The tubes were transferred from 4 °C to refrigerated baths at −15, −10, 4 (control, representing average January air temperatures at Palmer Station) 20 or 30 °C for 3 h, to match treatments used in previous studies of this insect (Lee et al., 2006; Rinehart et al., 2006). Following these temperature exposures, larvae were removed, placed at 4 °C and 100% RH, and survival was assessed after 48 h by observation of movement after tactile stimulation. Three replicates of 20 individuals were used for each experiment.

2.4. Trehalose concentration and injection into larvae

Trehalose concentrations were determined according to Parrou and François (1997) with modifications by Benoit et al. (2007). Groups of five larvae were homogenized in 500 μL sodium sulfate (2% w/v) and centrifuged at 3000 *g*. Supernatant (1 μL) was combined in a microcentrifuge tube with 50 μL distilled water and boiled for 2 min. After cooling, 50 μL of 0.8 mg/mL amyloglucosidase solution (Sigma) was mixed with the sample and incubated overnight at 23 °C. Controls to establish the natural glucose levels were prepared by the same methods, except 50 μL distilled water was added in place of the amyloglucosidase solution. Glucose concentration was measured colorimetrically at 450 nm (Sigma kit no. 510-A). Trehalose concentrations within the larvae were tested after a loss of 20, 35 and 50% of their water content after exposure to 0, 75 and 98% RH. Controls were fully-hydrated larvae.

To determine if increased trehalose levels enhanced cold and heat tolerance, larvae were injected with trehalose solutions and then exposed to different temperature regimes. To do so, two different concentrations of trehalose were generated in Coast's solution (Coast and Krasnoff, 1988; Teets et al., 2008): 0.3 μg/μL and 0.6 μg/μL. Larvae were held at 0% RH and 4 °C, a 1.5 μL finely-drawn glass capillary was inserted into the larvae, and over a 6–10 h period, as the larvae lost water, the solution was drawn into the body. This slow infusion prevented the larvae from becoming overhydrated due to a single large injection volume. Larvae were allowed to recover for 6 h at 100% RH and 4 °C and injected again with 1.5 μL in the same manner. Controls were larvae injected with only Coast's solution. After injection, larvae were stored at 4 °C and 100% RH for recovery. Survival was assessed after 24 h, and trehalose concentrations were determined. Cold and heat tolerance were determined for injected larvae held at −15 °C or 30 °C for 3 h. Desiccation tolerance was assessed by measuring water loss rates at 0% RH as described above.

2.5. Statistics

Survival following different temperature exposures was measured using two-way analysis of variance (ANOVA) after testing for parametric assumptions. Percentages were arcsin-transformed prior to analysis, and regression lines for water loss rates were analyzed by testing the equality of slopes. Trehalose concentrations were compared with analysis of variance and Bonferroni–Dunn tests. Survival following trehalose injection was analyzed in comparison to controls injected with Coast's solution, using two-way ANOVA and Dunnett's tests. If data were not parametric, log-transformation was used for normalization. Data are presented as means±SE. Significance was set at $P<0.05$ (Sokal and Rohlf, 1995).

3. Results

3.1. Dehydration

The water pool (water mass and percent water mass) of larvae used in this study was similar to that noted in our previous studies (Benoit et al., 2007; Hayward et al., 2007). For all groups, the initial weight/larva was approximately 0.9–1.0 mg, with water content of 75% and dry mass of 0.25–0.30 mg. These values indicate that samples tested in this study were a combination of third and fourth instars (Benoit et al., 2007; Hayward et al., 2007). Identical water mass to dry mass ratios were noted between the treatment groups before the experiments, and dry mass correlated with water mass, indicating that size differences can be ruled out as a factor influencing survival after cold and heat exposure (Wharton, 1985; Benoit et al., 2005).

Larvae tolerated a loss of up to 50% of their water content with little mortality (less than 5% in all cases). There were substantial differences between the time required to lose water at the three relative humidities, which was due to differences in rates of water loss at different relative humidities (Fig. 1A). At 0% RH, the larvae lost 50% of their water in 4–5 h at a rate of 12–13%/h (Fig. 1B). Higher relative humidities resulted in much slower dehydration to reach the 50% point: 24 h at 75% RH (2–2.5%/h) and 5 d at 98% RH (0.4–0.5%/h) (Fig. 1A,B; ANOVA; $P<0.05$). Thus, larvae required a significantly longer time to dehydrate at 75% RH (5×) and at 98% RH (30×) than at 0% RH.

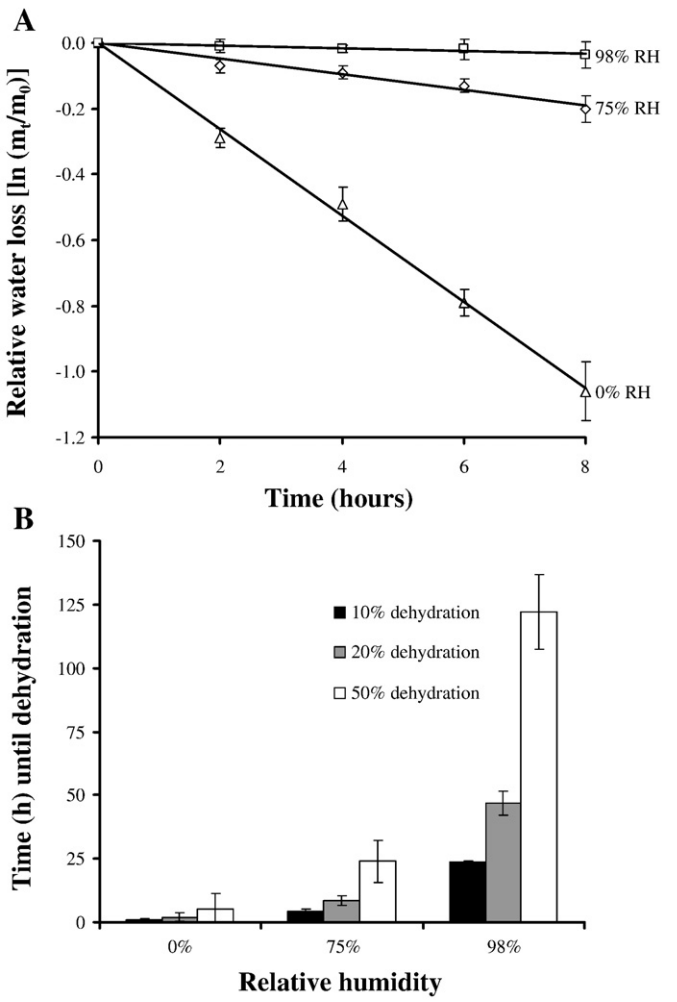

Fig. 1. Water loss in *B. antarctica* at three different relative humidities, 0, 75 and 98% RH at 4 °C. (A) Proportion of water mass lost over 8 h. The slope of the line is the water loss rate (integumental plus respiratory). Each point represents the mean±SE of 3 replicates of 10 individuals each. (B) Time until 10, 20, and 50% dehydration is reached by *B. antarctica* larvae when held at 0, 75, and 98% RH.

3.2. Cold tolerance following dehydration

When larvae were dehydrated, they were better able to tolerate exposure to sub-zero temperatures (Fig. 2). For fully-hydrated larvae, survival after a 3 h exposure at −10 °C dropped approximately 30% in comparison to the controls, and at −15 °C survival declined by 65% (Fig. 2). Survival for larvae that lost ≥20% of their water pool at −10 °C was similar to control larvae exposed to 4 °C (Fig. 2; ANOVA; $P>0.05$). A significant increase in survival was noted at −15 °C after a 20% water loss for larvae held at 98% RH (ANOVA; $P<0.05$), but not at 0 or 75% RH (ANOVA; $P>0.05$). A 50% reduction in water content increased survival by at least 30% in all treatment groups exposed to −15 °C (Fig. 2), which was significantly higher than the survival rate of fully-hydrated larvae (ANOVA; $P<0.05$).

Differences did occur when larvae were dehydrated at different rates to reach a 50% reduction in water content (Fig. 2). When exposed to 98% RH, larvae required nearly 5 d to lose 50% of their water content, and survival exceeded 90% after exposure to −15 °C, a value that was >60% higher than observed in fully-hydrated larvae (Fig. 2C; ANOVA; $P<0.05$) and was not significantly different from survival of control larvae held at 4 °C (Fig. 1C; ANOVA; $P>0.05$). Survival after losing 50% of their water at 98% RH followed by exposure to −15 °C was significantly higher than the protection imparted by a rapid 50% water loss (6 h) caused by exposure to 0% RH (Fig. 2A,C; ANOVA; $P<0.05$). Thus, prior dehydration increased cold tolerance of *B. antarctica* larvae, and a slow rate was more effective in improving survival at subzero temperatures than rapid dehydration.

3.3. Heat tolerance following dehydration

Larvae of *B. antarctica* are extremely sensitive to heat exposure (Rinehart et al., 2006); individuals survive only a few hours at temperatures above 25 °C. As with cold exposure, dehydration improved the ability of larvae to tolerate exposure to heat (Fig. 3). Survival was similar for fully hydrated larvae exposed to 20 °C for 3 h compared to controls held at 4 °C (Fig. 3; ANOVA; $P>0.05$). At 30 °C only 20–35% of the fully-hydrated larvae survived (Fig. 3). Losing 20% of their water content at 98% RH significantly increased survival of larvae dehydrated at 4 °C (Fig. 3C; ANOVA, $P<0.05$), but no differences were noted between fully-hydrated individuals and those dehydrated

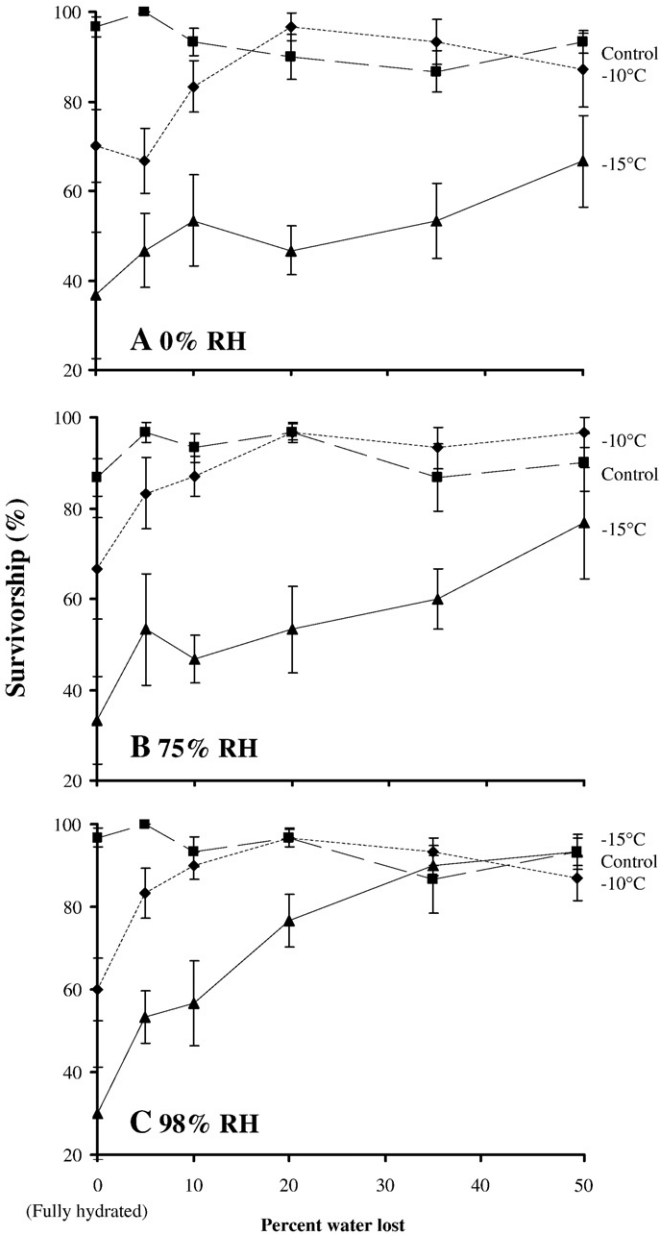

Fig. 2. Survival of *B. antarctica* larvae after losing various percentages of water at different relative humidities (A, 0% RH; B, 75% RH; C, 98% RH) and then exposed to subzero temperatures (−15 or −10 °C for 3 h). Control larvae were held at 4 °C. Measurements represent the mean±SE of 3 samples of 20 individuals each.

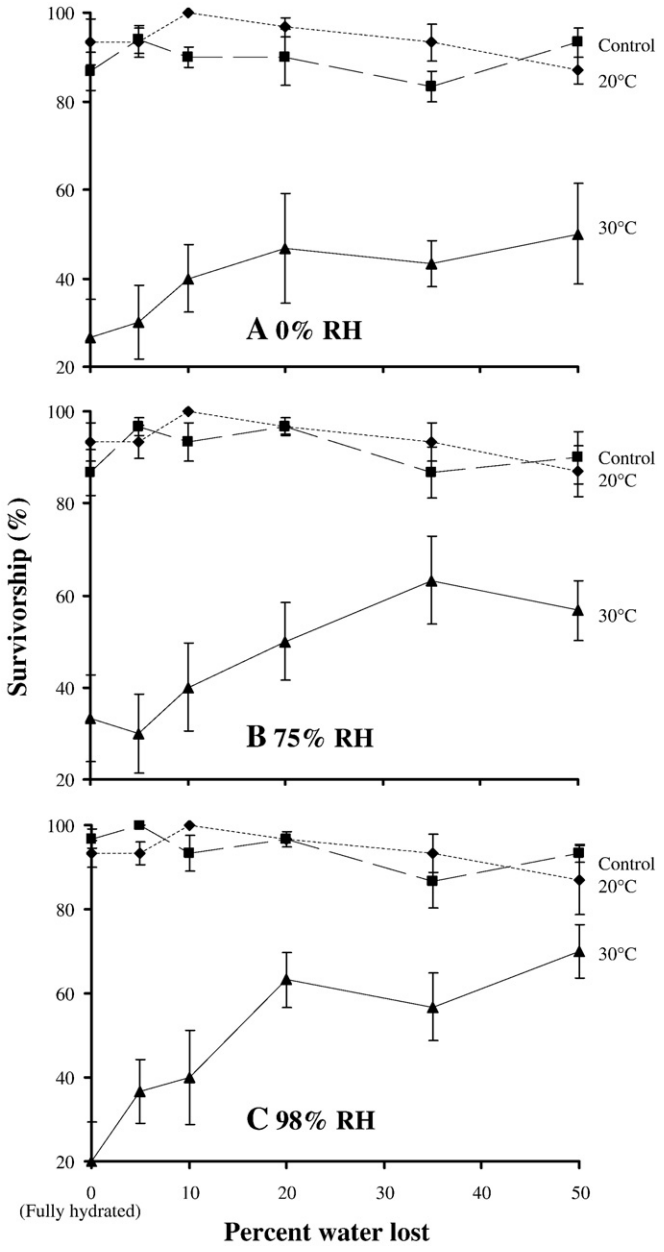

Fig. 3. Survival of *B. antarctica* larvae after losing various percentages of water at different relative humidities (A, 0% RH; B, 75% RH; C, 98% RH) and then exposed to high temperatures (20 or 30 °C for 3 h). Control larvae were held at 4 °C. Measurements represent the mean±SE of 3 samples of 20 individuals each.

at 0 or 75% RH, then exposed to 30 °C (Fig. 3A,B; ANOVA, $P>0.05$). At all relative humidities tested, dehydration improved the ability of the larvae to tolerate heat (Fig. 3), but slow dehydration (98% RH for 5 days) was most effective in generating heat resistance, as indicated by a 3.5-fold increase in survival compared to the fully-hydrated larvae (Fig. 3C; ANOVA, $P<0.05$). Rapid dehydration at 0 and 75% RH to a 50% reduction in water content only increased survival by approximately two-fold. We conclude that dehydration, particularly at a slow, controlled rate, improved high temperature tolerance of *B. antarctica*.

3.4. Effect of trehalose on cold, heat and dehydration tolerance

During dehydration, levels of trehalose increased when larvae were dried at 75 and 98% RH, but failed to do so at 0% RH (Fig. 4A). The highest increase of trehalose occurred when larvae were held at 98% RH, and trehalose increased as the level of dehydration increased up to a 50% loss of water (Fig. 4A). Additionally, a loss of 20% water prompted an increase of trehalose at 98% RH (Fig. 4; ANOVA, $P<0.05$) but not at 0 or 75% RH (Fig. 4; ANOVA, $P>0.05$). When time differences between dehydration at 0, 75 and 98% RH were used in the analysis, it was apparent that more trehalose synthesis had occur at 98% RH, most likely because the larvae experienced a longer period of dehydration at the higher RH (Fig. 4B). Thus, slower dehydration yielded higher concentrations of trehalose, and if larvae dehydrated too quickly, they apparently did not have adequate time to synthesize trehalose (*e.g.* 0% RH, Fig. 4A,B).

Larvae injected with trehalose were more tolerant of temperature stress and dehydration than control larvae (Figs. 5 and 6). Individuals injected with 0.9 μg (1.5 μL injected twice, 0.3 μg/μL) of trehalose experienced a 3.9±0.2 μg/mg dry mass (DM) increase in trehalose, and those treated with 1.8 μg (1.5 μL injected twice, 0.6 μg/μL) increased their levels to 8.6±0.3 μg/mg DM. Controls injected with Coast's solution only had an increase of 1.8±0.3 μg/mg dry mass (DM). Thus, injection of trehalose did indeed elevate trehalose levels within the larvae. When dehydrated at 0% RH, larvae showed an increase of 3.9±0.2 μg/mg DM which did not reduce the water loss rate when compared to the controls (Fig. 5; ANOVA, $P>0.05$), but an increase of 8.6±0.3 μg/mL resulted in a reduction in the water loss rate from 13.5±0.3%/h to 10.2±0.4%/h (Fig. 5; ANOVA $P<0.05$). Similar increases in tolerance were noted for heat and cold (Fig. 6). Larvae with an increase of 8.6±0.3 μg trehalose/mg DM were better able to tolerate exposure to −15 °C and 30 °C (Fig. 6; ANOVA, $P<0.05$). In all cases, an injection

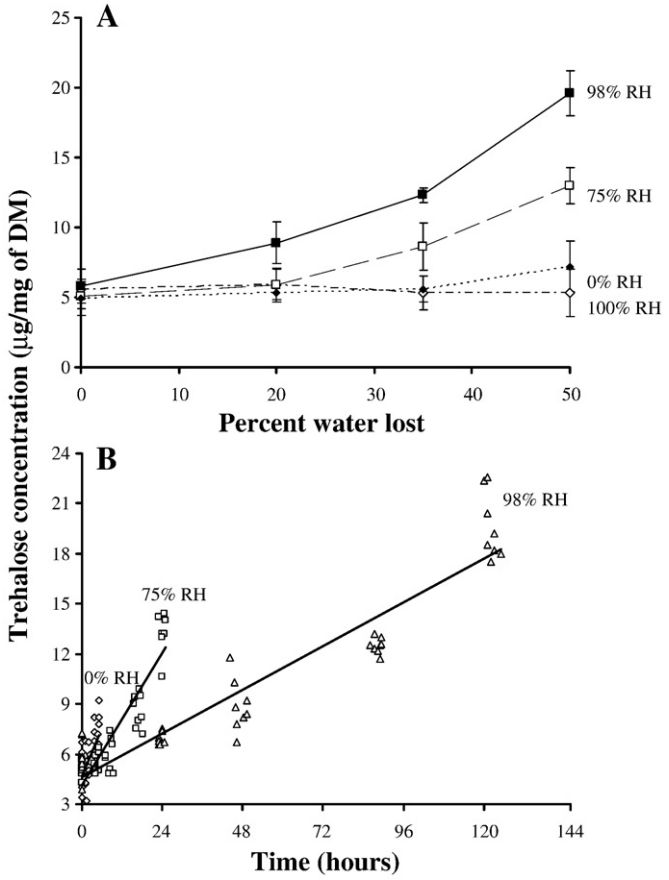

Fig. 4. Levels of trehalose in *B. antarctica* larvae during dehydration at 0, 75 and 98% RH. Individuals held at 100% RH represent continually-hydrated controls (0% water loss). (A) represents levels of trehalose after dehydration at four different RHs, and (B) represents the levels of trehalose as a function of time. DM indicates dry mass. Mean±SE of 8 measurements.

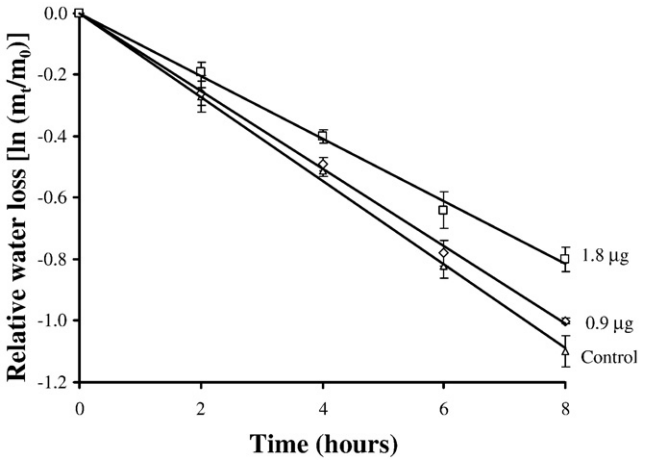

Fig. 5. Water loss in *B. antarctica* larvae after injection of 0.9 or 1.8 µg of trehalose in Coast's solution. Controls represent the results from larvae injected only with Coast's solution; rates observed in these controls were indistinguishable from rates observed in non-injected controls (data not shown). Each point represents the mean±SE of 24 individuals.

of Coast's solution did not enhance tolerance to dehydration, cold or heat exposure (Figs. 5 and 6; ANOVA, $P>0.05$).

4. Discussion

The rate at which dehydration occurred was extremely important in allowing larvae to adapt physiologically to changes in their internal water pool. Slow dehydration occurring near water vapor saturation, particularly above 98% RH (Hayward et al., 2007; Benoit et al., 2007), appeared to be optimal for allowing larvae sufficient time to respond physiologically to dehydration stress. The rate of dehydration altered dehydration tolerance: larvae dried at 0% RH tolerated a loss of 72% of their water content, while larvae dried much more slowly at 98% RH tolerated a loss of 76% of their water content (Benoit et al., 2007). This difference between dehydration regimens seems to be particularly important for chironomid larvae; similar observations have been noted in *Polypedilum vanderplanki* (Kikawada et al., 2005) and other chironomid larvae (Suemoto et al., 2004). Thus, the rate of dehydration is an important factor when assessing dehydration-induced mortality in midge larvae and presumably other insects as well. This is supported by the expression patterns of numerous genes noted by Lopez-Martinez et al. (2008; 2009); rapid dehydration (75% RH) yielded a strong upregulation of many stress-related genes, but slow dehydration did not cause as great a change in expression. Although dehydration at 0, 75, and 98% RH all led to a removal of 50% of the larval water content, attaining this loss at 98% RH was less stressful by allowing the larvae more time to respond.

The effects of desiccation on cold tolerance are well documented (Holmstrup and Sømme, 1998; Ring and Danks, 1998; Zachariassen et al., 2008). In a previous study on *B. antarctica* (Hayward et al., 2007), larvae dehydrated slowly at 98.2% RH increased their tolerance to −10 °C. Similar cross-tolerance effects were noted in the collembolan *Folsomia candida*, where periods of slow dehydration improved cold tolerance (Bayley et al., 2001). Also, the most cold-resistant insect is a chironomid, *P. vanderplanki*, that, during complete dehydration, can tolerate extremely low temperatures (−270 °C, Hinton, 1960) since a lack of water minimizes damage from freezing (Ring and Danks, 1994; Convey, 2000). Additionally, larvae of *B. antarctica* can undergo cryoprotective dehydration (Elnitsky et al., 2008), a slow dehydration that occurs when a supercooled organism loses water in the presence of ice due to the vapor pressure gradient (Holmstrup and Sømme, 1998). In our current study, we show that dehydration-induced cross tolerance to cold is extremely dependent on the rate and level of dehydration. The greatest increase in cold tolerance was observed when larvae were dehydrated slowly (98% RH) until attaining a loss of 50% of their water. A faster dehydration caused by exposure to 0 and 75% RH also increased cold tolerance, but the effect was not as pronounced. Thus, we conclude that cold tolerance for *B. antarctica* was affected both by the rate and level of dehydration.

The relationship between dehydration and heat tolerance is less studied but has been demonstrated in a few other insects, including chironomids (Hinton, 1960; Bowler, 2005; Watanabe et al., 2002). As with cold tolerance, the most heat-resistant organism is *P. vanderplanki*, a species that when completely dehydrated has a cross tolerance to extremely high temperatures (>100 °C; Hinton, 1960). For *B. antarctica*, the increased cross tolerance to heat following dehydration was significant, but not nearly as dramatic as in the anhydrobiotic midge, *P. vanderplanki*. Constant exposure of *B. antarctica* to temperatures above 20 °C caused death within 5 days, and at 30 °C nearly 50% of the larvae died within 3 h (Rinehart et al., 2006). A recent study reported daytime temperatures of 20–25 °C in the microhabitat where *B. antarctica* resides (Schulte et al., 2008). Field collections during these warm periods contained many larvae that were already dehydrated, with their water content reduced by 15–30% (Benoit et al., 2007), indicating that dehydration-induced heat tolerance may be important for larval survival during the austral summer.

The increased cold and heat tolerance generated by dehydration is likely prompted by a combination of factors (Denlinger and Yocum, 1998; Clark and Worland, 2008). Heat shock proteins (Hsps) may be involved: at least three Hsps (smHsp, Hsp70, and Hsp90) are expressed at all times by larvae of *B. antarctica* (Rinehart et al., 2006; Hayward et al., 2007), and Hsp70 is further upregulated during both slow and rapid dehydration and in response to high temperature (Lopez-Martinez et al., 2009). Genes encoding four antioxidant and detoxification enzymes (catalase, superoxide dismutase, metallothionein, and cytochrome *P450* monooxygenase) are also elevated in response to dehydration (Lopez-Martinez et al., 2008; 2009); the proteins encoded by these genes act to remove harmful substances, particularly oxygen free radicals (Franca et al., 2005, 2007). Thus, upregulation of these genes during dehydration may enhance resistance to subsequent temperature stress, particularly the ability to resist and repair oxidative damage.

Several metabolites are also altered during dehydration (Michaud et al., 2008), particularly trehalose (Benoit et al., 2007; Goyal et al., 2005). In our current and previous studies of *B. antarctica*, the increase of trehalose during dehydration was dependent on the rate

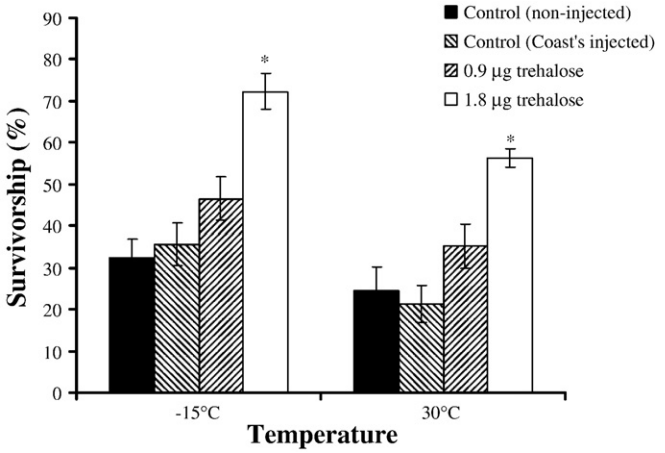

Fig. 6. Cold and heat tolerance of *B. antarctica* larvae after injection of 0.9 or 1.8 µg of trehalose in Coast's solution. Controls include non-injected individuals and those injected with Coast's solution. Larvae were exposed to −15 or 30 °C for 3 h. *, indicates a significant difference from all other treatments (ANOVA; $P<0.05$). Each point represents the mean±SE of 3 samples of individuals.

of dehydration (Benoit et al., 2007). In this study, we showed that if larvae were dehydrated too quickly (0% RH), they failed to produce additional trehalose and were less resistant to subsequent stress exposure than counterparts dehydrated at 75% and 98% RH. We suspect that this is due primarily to trehalose synthesis under slow dehydration. Additionally, by injecting exogenous trehalose into the larvae, we verified that trehalose is a key factor that increases subsequent resistance to dehydration, cold and heat. Injecting trehalose into *Sarcophaga bullata* pupae did not increase stress tolerance (Yoder et al., 2006), but this could be attributed to species differences or differences in the amount of trehalose injected. For *P. vanderplanki*, trehalose synthesis only occurs at a certain level of dehydration, and a slowly-dehydrated larva has more time to generate trehalose than individuals undergoing rapid dehydration before damage-inducing levels of dehydration occur (Kikawada et al., 2005). As dehydration progresses, the excess trehalose acts to replace water, which prevents cellular damage and unwanted protein interactions (Sano et al., 1999; Goyal et al., 2005). Trehalose may act to stabilize phospholipids in the plasma membrane (Crowe et al., 1992), a common location for cold-induced injury (Cossins, 1994; Michaud and Denlinger, 2006). During heat stress, the main function of trehalose seems to be stabilization of heat-sensitive proteins (Bowler, 2005), a feature that may be particularly beneficial when warm and dry periods co-occur. Thus, the increased concentrations of trehalose during slow dehydration are likely to be a major factor involved in cross tolerance to temperature stress.

The changing environment on the Antarctic Peninsula requires high tolerance for multiple types of stress. We add to this understanding by establishing that dehydration provides the larvae with a general tolerance to both high and low temperature. This cross tolerance is likely a result of multiple factors including changes in proteins, membrane composition, and a shift in metabolites, such as trehalose. Based on this study and previous work on *B. antarctica* (Hayward et al., 2007; Elnitsky et al., 2008), pre-exposure to dehydration may be an important mechanism to prevent temperature-induced mortality in this Antarctic insect.

Acknowledgements

This research was funded by the NSF grants OPP-0337656 and OPP-0413786. We thank the staff at Palmer Station for their excellent support.

References

Bayley, M., Peterson, S.O., Knigge, T., Köhler, H.R., Holmstrup, M., 2001. Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. J. Insect Physiol. 47, 1197–1204.

Benoit, J.B., Yoder, J.A., Rellinger, E.J., Ark, J.T., Keeney, G.D., 2005. Prolonged maintenance of water balance by adult females of the American spider beetle, *Mezium affine* Boieldieu, in the absence of food and water resources. J. Insect Physiol. 51, 565–573.

Benoit, J.B., Lopez-Martinez, G., Michaud, M.R., Elnitsky, M.A., Lee Jr., R.E., Denlinger, D.L., 2007. Mechanisms to reduce dehydration stress in larvae of the Antarctic midge, *Belgica antarctica*. J. Insect Physiol. 53, 656–667.

Bowler, K., 2005. Acclimation, heat shock and hardening. J. Therm. Biol. 30, 125–130.

Clark, M.S., Worland, M.R., 2008. How insects survive the cold: molecular mechanisms—a review. J. Comp. Physiol. B. 178, 917–933.

Coast, G.M., Krasnoff, S.B., 1988. Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domesticus*, and their response to diuretic hormone. Physiol. Entomol. 13, 381–391.

Convey, P., 2000. How does cold constrain life cycles of terrestrial plants and animals? CryoLetters 21, 73–82.

Cossins, A.R., 1994. Temperature Adaptations of Biological Membranes. Portland Press, London, UK.

Crowe, J.H., Hoekstra, F.A., Crowe, L.M., 1992. Anhydrobiosis. Ann. Rev. Physiol. 54, 579–599.

Denlinger, D.L., Yocum, G.D., 1998. Physiology of heat sensitivity. In: Hallman, G.J., Denlinger, D.L. (Eds.), Temperature Sensitivity in Insects and Application in Integrated Pest Management. Westview Press, Boulder, pp. 7–53.

Elnitsky, M.A., Hayward, S.A.L., Rinehart, J.P., Denlinger, D.L., Lee Jr., R.E., 2008. Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic midge, *Belgica antarctica*. J. Exp. Biol. 211, 524–530.

Franca, M.B., Panek, A.D., Eleuterio, E.C.A., 2005. The role of cytoplasmic catalase in dehydration tolerance of *Saccharomyces cerevisiae*. Cell Stress Chaperones 10, 167–170.

Franca, M.B., Panek, A.D., Eleuterio, E.C.A., 2007. Oxidative stress and its effect during dehydration. Comp. Biochem. Physiol. A 146, 621–631.

Goyal, K., Walton, L.J., Browne, J.A., Burnell, A.M., Tunnacliffe, A., 2005. Molecular anhydrobiosis: identifying molecules implicated in invertebrate anhydrobiosis. Integ. Comp. Biol. 45, 702–709.

Hadley, N.F., 1994. Water Relations of Terrestrial Arthropods. Academic Press, New York.

Hayward, S.A.L., Rinehart, J.P., Sandro, L.H., Lee Jr., R.E., Denlinger, D.L., 2007. Slow dehydration promotes desiccation and freeze tolerance in the Antarctic midge, *Belgica antarctica*. J. Exp. Biol. 210, 836–844.

Hinton, H.E., 1960. A fly larva tolerates dehydration and temperatures of −270 °C to +102 °C. Nature 188, 336–337.

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

Kikawada, T., Minakawa, N., Watanabe, M., Okuda, T., 2005. Factors inducing successful anhydrobiosis in the African chironomid *Polypedilum vanderplanki*: significance of the larval tubular nest. Integ. Comp. Biol. 45, 710–714.

Lee Jr., R.E., Elnitsky, M.A., Rinehart, J.P., Hayward, S.A.L., Sandro, L.H., Denlinger, D.L., 2006. Rapid cold-hardening increases the freezing tolerance of the Antarctic midge, *Belgica antarctica*. J. Exp. Biol. 209, 399–406.

Lopez-Martinez, G., Benoit, J.B., Rinehart, J.P., Elnitsky, M.A., Lee Jr., R.E., Denlinger, D.L., 2009. Dehydration, rehydration, and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. J. Comp. Physiol. B (in revision).

Lopez-Martinez, G., Elnitsky, M.A., Benoit, J.B., Lee Jr., R.E., Denlinger, D.L., 2008. High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally linked expression of genes encoding superoxide dismutase, catalase, and heat shock proteins. Insect Biochem. Mol. Biol. 38, 796–804.

Michaud, M.R., Denlinger, D.L., 2006. Oleic acid is elevated in cell membranes during rapid cold hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. J. Insect Physiol. 52, 1073–1082.

Michaud, M.R., Benoit, J.B., Lopez-Martinez, G., Elnitsky, M.A., Lee Jr., R.E., Denlinger, D.L., 2008. Metabolomics reveals unique and shared changes in response to heat shock, freezing, and desiccation in the Antarctic midge, *Belgica antarctica*. J. Insect Physiol. 54, 645–655.

Parrou, J.L., François, J., 1997. A simplified procedure for rapid and reliable assay of both glycogen and trehalose in whole yeast cells. Anal. Biochem. 248, 186–188.

Rinehart, J.P., Hayward, S.A.L., Elnitsky, M.A., Sandro, L.H., Lee Jr, R.E., Denlinger, D.L., 2006. Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. Proc. Natl. Acad. Sci. U.S.A. 38, 14223–14227.

Ring, R.A., Danks, H.V., 1994. Desiccation and cryoprotection: overlapping adaptations. CryoLetters 15, 181–190.

Ring, R.A., Danks, H.V., 1998. The role of trehalose in cold-hardiness and desiccation. CryoLetters 19, 275–282.

Sano, F., Asakawa, N., Inoue, Y., Sakurai, M., 1999. A dual role for intracellular trehalose in the resistance of yeast cells to water stress. Cryobiology 39, 80–87.

Schulte, G.G., Elnitsky, M.A., Benoit, J.B., Denlinger, D.L., Lee Jr., R.E., 2008. Extremely large aggregations of collembolan eggs on Humble Island, Antarctica: a response to early season warming? Polar Biol. 31, 889–892.

Sokal, R.R., Rohlf, F.J., 1995. Biometry. W. H. Freeman, New York.

Suemoto, T., Kawai, K., Imabayashi, H., 2004. A comparison of desiccation tolerance among 12 species of chironomid larvae. Hydrobiologia 515, 107–114.

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

Teets, N.M., Elnitsky, M.A., Benoit, J.B., Lopez-Martinez, G., Denlinger, D.L., Lee Jr., R.E., 2008. Rapid cold-hardening in larvae of the Antarctic midge, *Belgica antarctica*: cellular cold-sensing and a role for calcium. Am. J. Physiol. 294, R1938–R1946.

Watanabe, M., Kikawada, T., Minagawa, N., Yukuhiro, F., Okuda, T., 2002. Mechanisms allowing an insect to survive complete dehydration and extreme temperatures. J. Exp. Biol. 205, 2799–2802.

Wharton, G.W., 1985. Water balance of insects. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 4. Pergamon Press, Oxford, pp. 565–603.

Winston, P.W., Bates, D.S., 1960. Saturated salt solutions for the control of humidity in biological research. Ecology 41, 232–237.

Yoder, J.A., Benoit, J.B., Denlinger, D.L., Rivers, D.B., 2006. Stress-induced accumulation of glycerol in the flesh fly, *Sarcophaga bullata*: evidence indicating anti-desiccant and cryoprotectant functions of his polyol and a role for the brain in coordinating the response. J. Insect Physiol. 52, 202–214.

Zachariassen, K.E., Li, N.G., Laugsand, A.E., Kristiansen, E., Pedersen, S.A., 2008. Is the strategy for cold hardiness is insects determined by their water balance? A study on two closely related families of beetles: Cerambycidae and Chrysomelidae. J. Comp. Physiol. B. 178, 977–984.