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

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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 dehydration alter heat and cold tolerance and how these relate to levels of trehalose within the insect. When 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.

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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 harborage 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
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.

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; ANOVA; P<0.05). Thus, larvae required a significantly longer time to dehydrate at 75% RH (5x) and at 98% RH (30x) than at 0% 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 larva 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. 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.]

![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.]

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 \( \mu \text{g} \) (1.5 \( \mu \text{L} \) injected twice, 0.3 \( \mu \text{g/\muL} \)) of trehalose experienced a 3.9±0.2 \( \mu \text{g/mg dry mass (DM)} \) increase in trehalose, and those treated with 1.8 \( \mu \text{g} \) (1.5 \( \mu \text{L} \) injected twice, 0.6 \( \mu \text{g/\muL} \)) increased their levels to 8.6±0.3 \( \mu \text{g/mg DM} \). Controls injected with Coast’s solution only had an increase of 1.8±0.3 \( \mu \text{g/mg dry mass (DM)} \). 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 \( \mu \text{g/mg DM} \) 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 \( \mu \text{g/mL} \) 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 \( \mu \text{g trehalose/mg DM} \) were better able to tolerate exposure to −15 °C and 30 °C (Fig. 6; ANOVA, \( P < 0.05 \)). In all cases, an injection 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*.
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 Samme, 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 improved cold tolerance (Hayward et al., 2007; Benoit et al., 2007). Thus, we conclude 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, metallothioinein, and cytochrome P450 monoxygenase) 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 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.

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.

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.

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