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Osmoregulation and salinity tolerance in the Antarctic midge, *Belgica antarctica*: seawater exposure confers enhanced tolerance to freezing and dehydration

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SUMMARY

Summer storms along the Antarctic Peninsula can cause microhabitats of the terrestrial midge *Belgica antarctica* to become periodically inundated with seawater from tidal spray. As microhabitats dry, larvae may be exposed to increasing concentrations of seawater. Alternatively, as a result of melting snow or following rain, larvae may be immersed in freshwater for extended periods. The present study assessed the tolerance and physiological response of *B. antarctica* larvae to salinity exposure, and examined the effect of seawater acclimation on their subsequent tolerance of freezing, dehydration and heat shock. Midge larvae tolerated extended exposure to hyperosmotic seawater; nearly 50% of larvae survived a 10-day exposure to 1000 mOsm kg^{-1} seawater and ~25% of larvae survived 6 days in 2000 mOsm kg^{-1} seawater. Exposure to seawater drastically reduced larval body water content and increased hemolymph osmolality. By contrast, immersion in freshwater did not affect water content or hemolymph osmolality. Hyperosmotic seawater exposure, and the accompanying osmotic dehydration, resulted in a significant correlation between the rate of oxygen consumption and larval water content and induced the *de novo* synthesis and accumulation of several organic osmolytes. A 3-day exposure of larvae to hyperosmotic seawater increased freezing tolerance relative to freshwater-acclimated larvae. Even after rehydration, the freezing survival of larvae acclimated to seawater was greater than freshwater-acclimated larvae. Additionally, seawater exposure increased the subsequent tolerance of larvae to dehydration. Our results further illustrate the similarities between these related, yet distinct, forms of osmotic stress and add to the suite of physiological responses used by larvae to enhance survival in the harsh and unpredictable Antarctic environment.

Key words: osmotic stress, dehydration, cold-hardiness, Antarctica, Chironomidae.

INTRODUCTION

Insects tolerant of seawater submergence can be classified as either osmoregulators or osmoconformers (Bradley, 1987). During exposure to high salinity, osmoregulators maintain the hemolymph at a concentration hyposmotic to the external medium. This strategy is common among dipteran larvae, including saline-water mosquitoes of the genus *Aedes* (for a review, see Bradley, 1987) and chironomids (Neumann, 1976; Kokkin, 1986). Larvae in saline water constantly lose water to their environment and must counter such water loss by actively 'drinking' the external medium. To prevent a substantial accumulation of ions that may be detrimental to protein function (Somero and Yancey, 1997), larvae must also excrete a concentrated 'urine.' Alternatively, osmoconformers maintain water balance by equilibrating the osmotic pressure of the body fluids with that of the saline environment. Indeed, other genera of mosquitoes [*Culex* (Garrett and Bradley, 1987); *Culiseta*, *Deinocerites* (Bradley, 1994)] and insect orders that contain euryhaline species [e.g. *Chironomus* sp. and a dragonfly nymph *Enallagma clausam* (Stobbart and Shaw, 1974)] osmoconform while inhabiting saline environments. Garrett and Bradley (Garrett and Bradley, 1987) demonstrated that larvae of the mosquito *Culex tarsalis* osmoconform by accumulating high concentrations of proline and trehalose. It is thought that accumulation of these organic osmolytes, as opposed to inorganic ions (predominantly Na^+ and Cl^-), preserves the function of enzymes despite an increase in osmolality (Somero and Yancey, 1997).

Most insects do not tolerate prolonged seawater submergence, and at least for most larval dipterans, the upper limit of salinity tolerance is equal to the initial osmotic concentration of the hemolymph (Bayly, 1972). Exposure to hyperosmotic saline results in an inability to osmo- and ionoregulate. However, physiological responses to salinity exposure that ameliorate this hyperosmotic and ionic stress (e.g. accumulation of organic osmolytes, reduced rate of water loss, etc.) may extend survival time.

Belgica antarctica Jacobs (Diptera: Chironomidae) is the southern-most free-living holometabolous insect, being sporadically dispersed, but locally abundant, on the west coast of the Antarctic Peninsula. Detailed accounts of the life-history and ecology of this terrestrial, Antarctic midge are provided by Convey and Block (Convey and Block, 1996), Sugg et al. (Sugg et al., 1983), Usher and Edwards (Usher and Edwards, 1984), and references cited therein. Briefly, its two-year life cycle includes four larval stages and over-wintering may occur in any instar. Larvae are freeze tolerant to approximately –15°C (Baust and Lee, 1981; Lee et al., 2006), and are extremely tolerant of desiccation; larvae survive the loss of ~70% of their total body water (Benoit et al., 2007). During freezing and desiccation, larvae are necessarily challenged with extensive osmotic stress, as solutes become concentrated in the remaining extracellular body water. Such tolerance suggests the larvae have a well developed ability to maintain a critical minimum volume necessary for cell survival and the maintenance of metabolic function when faced with osmotic stress.

During summer, Antarctic storms can result in terrestrial microhabitats of *B. antarctica* becoming periodically inundated with seawater from tidal spray (Baust and Lee, 1987) (M.A.E., personal observation). Subsequently, as microhabitats dry as a result of evaporation, larvae may be exposed to increasing concentrations of seawater. By contrast, as a result of melting snow or following rain, larvae may be immersed in freshwater for extended periods. Therefore, the physiological tolerance and response of larvae to such osmotic perturbations are probably critical for survival.

A previous study of *B. antarctica* found >95% survival of larvae following a 7-day submergence in 0.5 mol l^{-1} NaCl (Baust and Lee, 1987). However, the osmotic response to such salinity and the tolerance of more severe hyperosmotic stress is unknown. Therefore, the purpose of the present study was to assess the salinity tolerance and acute osmotic response of larval *B. antarctica*, including changes in water content, body fluid osmotic pressure, and osmolyte accumulation during salinity stress. Additionally, we characterized the physiological effects of seawater exposure on the rate of oxygen consumption and investigated the effect of a brief seawater acclimation on the subsequent tolerance of freezing, desiccation and heat shock.

MATERIALS AND METHODS

Source of insects

Substratum containing larval *B. antarctica* was collected from sites near penguin rookeries on Cormorant and Humble Islands, near Palmer Station on the Antarctic Peninsula (64°46′S, 64°04′W) in January and February 2006 and 2007. Samples were stored at 4°C (0 h:24 h L:D) in moist native substratum prior to use. Larvae were then handpicked from the substrate on ice-cold water and held in freshwater at 4°C for 12–24 h to ensure clearance of the gut [mean gut clearance ~6 h (Baust and Edwards, 1979)] and to standardize body water content prior to use. Only fourth instar larvae were used for all experiments.

Osmoregulation and salinity tolerance

To assess the osmotic response and salinity tolerance of *B. antarctica*, larvae were exposed to various concentrations of seawater. The salinity tolerance of larvae was tested during exposure to freshwater [~0 mOsm kg^{-1} (range 3–12 mOsm kg^{-1})], isoosmotic [~400 mOsm kg^{-1} (range 394–408 mOsm kg^{-1})] and hyperosmotic [~1000 (range 993–1012 mOsm kg^{-1}), ~1500 (range 1496–1508 mOsm kg^{-1}) and ~2000 mOsm kg^{-1} (range 1992–2017 mOsm kg^{-1})] solutions of seawater. The desired seawater solutions were produced by either dilution or concentration, *via* evaporation, of pure seawater collected adjacent to Palmer Station. Groups of 10 larvae were placed in open-top, 1.6 ml microcentrifuge tubes and submerged in ~1 ml of the test seawater solution. Larvae were subsequently placed at 4°C and samples removed on days 0, 1, 3, 6 and 10 for assessment of survival and measurement of total body water content (WC) and hemolymph osmolality. Five groups of 10 larvae were removed for survival assessment and individuals displaying spontaneous movement were deemed to have survived. The WC of individual larvae was assessed gravimetrically from measurements (to the nearest 1 μg; Cahn C-31 electrobalance, Ventron, Cerritos, CA, USA) of fresh mass at the time of sampling and dry mass (DM) after drying to constant mass at 65°C. Osmolality determinations were made using a vapor pressure depression technique (Holmstrup and Sømme, 1998). Groups of five larvae were placed in a sample holder and quickly crushed with a Teflon rod to expose the body fluids. Samples were then allowed to equilibrate for 30 min following placement within a C-52 sample chamber (Wescor, Logan, UT, USA). The osmolality of the sample was measured using a Wescor HR-33T Dew Point Microvoltmeter (Wescor, Logan, UT, USA) operated in the dew point mode. Only surviving larvae were used for measurement of WC and osmolality.

Osmolyte analysis

The role of osmolyte accumulation during osmotic stress was also examined. Glycerol, trehalose, and glucose analyses were performed on larvae following exposure to seawater (~1000 mOsm kg^{-1}) at 4°C. A group of control larvae was maintained in freshwater (~0 mOsm kg^{-1}) at 4°C until termination of the experiment (day 6). On days 1, 3 and 6, groups of ~25 larvae were weighed and immediately frozen at –80°C until whole body concentrations of osmolytes were determined. Larvae were homogenized in 1 N perchloric acid and neutralized with an equal volume of 1 N potassium hydroxide prior to determining osmolyte content. Glycerol concentrations were determined enzymatically as described by Holmstrup et al. (Holmstrup et al., 1999). Trehalose content was determined following digestion with trehalase as described by Chen et al. (Chen et al., 2002). Glucose concentration was determined using the glucose oxidase procedure (no. 510; Sigma, St Louis, MO, USA).

Oxygen consumption

Recently, Benoit et al. (Benoit et al., 2007) reported a reduced rate of oxygen consumption of *B. antarctica* larvae following desiccation. Therefore, the effect of osmotic stress, and the accompanying changes in WC, on the rate of oxygen consumption of the midge larvae was assessed following submergence in seawater (~1000 mOsm kg^{-1}) at 4°C. Groups of 10 larvae were acclimated in open-top, 1.6-ml microcentrifuge tubes containing ~1.0 ml of either seawater or freshwater. Daily, five groups of 10 larvae were removed for measurement of oxygen consumption by closed-system respirometry using an Instech Fiber Optic Oxygen Monitor (Model 110; Instech Laboratories, Plymouth Meeting, PA, USA). Larvae were placed in the FOXY chamber (Instech Laboratories, Plymouth Meeting, PA, USA) along with 1.0 ml of the acclimation solution. Larvae were allowed to equilibrate within the chamber for ~10 min, prior to recording changes in dissolved oxygen concentration for the subsequent 12 min using OOISensors Software (Instech Laboratories, Plymouth Meeting, PA, USA). The rate of oxygen consumption per unit time was calculated using the slope calculator on the OOISensors Software. At the end of each trial, larvae were removed from the chamber, blotted dry, and weighed (to nearest 0.01 mg). The WC of larvae was determined following drying to constant mass at 65°C. Oxygen consumption was expressed as μl O_2 g^{-1} DM h^{-1}. Prior to all measurements, the oxygen monitoring system was calibrated using solutions of 0% oxygen concentration (200 μmol l^{-1} sodium hydrosulfite) and a saturated oxygen solution at 4°C.

Effects of seawater exposure on the tolerance of freezing, heat shock and desiccation

Hayward et al. (Hayward et al., 2007) recently demonstrated that a mild desiccation stress conferred cross tolerance to freezing in *B. antarctica* larvae. Therefore, we assessed the effect of a mild osmotic stress on the subsequent tolerance of freezing, heat shock and desiccation by exposing larvae for 3 days to seawater (~1000 mOsm kg^{-1}) at 4°C prior to tests of environmental stress tolerance. In addition, to test whether seawater acclimation conferred increased tolerance independent of reductions in body water content, a group of larvae was acclimated to seawater for 3 days followed

by a 24-h rehydration in freshwater prior to assessment of stress tolerance. A control group of larvae was maintained in freshwater (~0 mOsm kg^{-1}) at 4°C for 3 days prior to measurement of stress tolerance. All larvae were acclimated to either seawater or freshwater as described above.

Immediately before tests of freeze tolerance, larvae were removed from their respective treatment and placed in microcentrifuge tubes containing 100 µl of fresh water (to standardize the amount of ice formation within the freezing media). Larvae were subsequently placed directly at the target temperature (–10, –12, –15 or –20°C) and allowed to freeze for 6 h. As larvae have a limited resistance to inoculative freezing (Elnitsky et al., 2008), the freezing of the body fluids probably occurred at a high subzero temperature, but was not controlled or monitored. Larvae were allowed to thaw/recover for 24 h at 4°C before survival assessment. For tests of heat shock tolerance, larvae were placed directly at 30°C and five groups of 10 larvae removed at 0.5 h intervals to assess survival. Larvae were permitted to recover for 24 h at 4°C before survival assessment. Finally, for tests of desiccation tolerance, groups of five larvae were removed from their respective treatment, blotted dry and placed within mesh-covered cages (20 µm mesh size), which allowed the free movement of water vapor. Cages containing larvae were in turn placed on a dry platform within 3.8 l glass desiccators containing 500 ml of either 31.6 g l^{-1} or a saturated solution of NaCl. The air inside the closed system quickly equilibrated with the salt solution (following Raoult's Law) to create a relative humidity (RH) of either 98.2% or 75.0%, respectively. At 1- to 2-day intervals, 20 individuals were removed for assessment of WC and five groups of 10 individuals removed for assessment of survival. Water content was measured gravimetrically as described above. Larvae were allowed to rehydrate for 24 h at 100% RH at 4°C before survival was assessed. For all tests of tolerance of environmental stressors, individuals displaying spontaneous movement were deemed to have survived.

Statistical analysis

Changes in survival, WC and osmolality over the course of the saline or freshwater exposure were analyzed with two-way (treatment × time along with their interaction) analysis of variance (ANOVA) following a test of parametric assumptions. Where significant treatment effects were observed, the Student–Newman–Keuls (SNK) comparison was used to test for significant differences over time. Linear regression analysis was used to evaluate the relationship between the rate of oxygen consumption during acclimation to seawater and WC. Mean sugar and polyol concentrations of larvae were compared with one-way ANOVA and Bonferroni–Dunn tests. Survival following tests of tolerance to other environmental stressors was analyzed relative to the freshwater (control) acclimation with two-way ANOVA and Dunnett's test. Survival data were arcsin-square root transformed prior to analysis. Data not meeting parametric assumptions were log transformed to correct for non-normality or heteroscedasticity. All data are presented as mean ± 1 s.e.m. with statistical significance set at $P<0.05$.

RESULTS

Osmoregulation and salinity tolerance

The survival of larval *B. antarctica* exposed to solutions of hyperosmotic seawater was significantly (treatment × time interaction, $F_{25,120}=16.23$; $P<0.0001$) affected by both the time of exposure and the strength of the seawater solution (Fig. 1A); survival declined with submergence time and the concentration of seawater. Nevertheless, greater than 75% of larvae survived

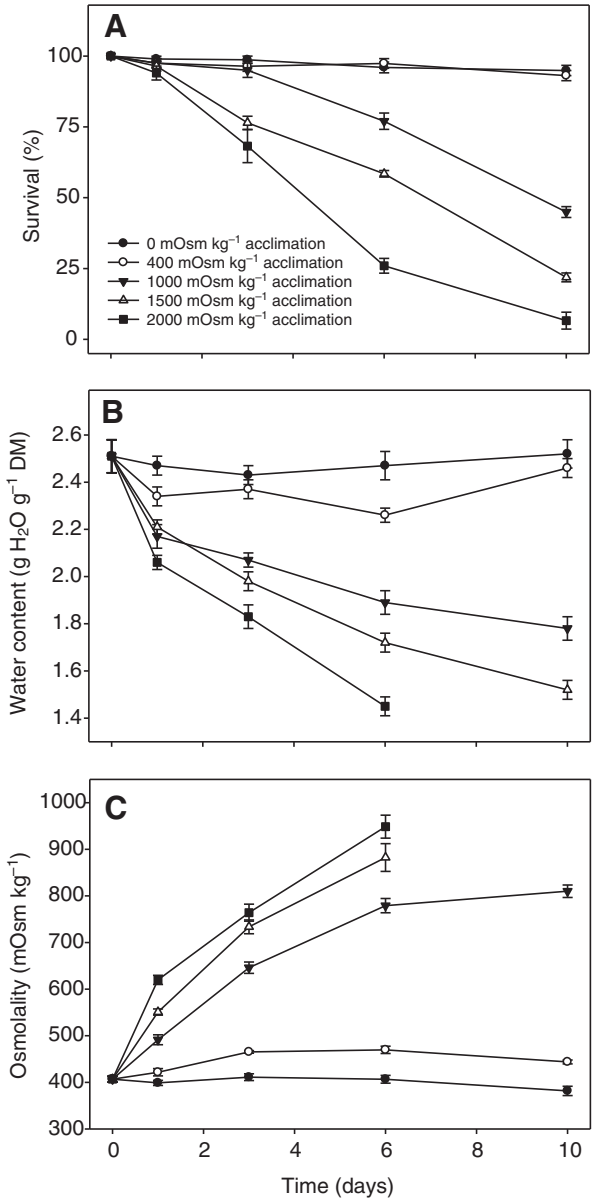

Fig. 1. (A) Survival (N=5 groups of 10 larvae), (B) water content (N=25–30), and (C) hemolymph osmolality (N=6) of *Belgica antarctica* larvae exposed to various concentrations of seawater or to freshwater (~0 mOsm kg^{-1}). Values are means ± 1 s.e.m.

exposure in pure seawater (~1000 mOsm kg^{-1}) for 6 days, while ~45% survived to the termination of the experiment (day 10). Even after 6 days, >50 and 25% of larvae survived exposure to ~1500 and 2000 mOsm kg^{-1} seawater, respectively. Nearly all larvae exposed to freshwater or isoosmotic seawater (~400 mOsm kg^{-1}) survived to day 10 of treatment.

Exposure of larvae to hyperosmotic seawater resulted in significant (treatment × time interaction, $F_{24,643}=12.87$; $P<0.0001$) reductions of WC (Fig. 1B). The WC of larvae exposed to ~1000 mOsm kg^{-1} seawater declined significantly up to day 6 and then stabilized over the remainder of the experiment. By day 10, the WC of larvae exposed to ~1000 mOsm kg^{-1} was reduced by ~30% to 1.78±0.05 g H_2O g^{-1} DM. Similarly, by day 6, the WC of larvae exposed to ~1500 and 2000 mOsm kg^{-1} seawater was significantly reduced by ~32 and 43%, respectively. The WC of

larvae exposed to 400 mOsm kg^{-1} seawater varied slightly, but significantly (one-way ANOVA, $F_{4,127}$=2.97; P=0.016), throughout the experiment, but by day 10 did not differ from control larvae maintained in freshwater. Freshwater submergence did not affect larval WC over the course of the experiment.

The reduction of WC during exposure of larvae to hyperosmotic seawater solutions necessarily resulted in significant (treatment × time interaction, $F_{23,133}$=34.16; P<0.0001) increases in hemolymph osmolality over the course of the experiment (Fig. 1C). The osmolality of larvae exposed to ~1500 and 2000 mOsm kg^{-1} seawater increased by ~2.2 and 2.3-fold, respectively, by day 6. Similarly, the hemolymph osmolality of larvae exposed to ~1000 mOsm kg^{-1} seawater nearly doubled by day 6 before leveling off throughout the remainder of the experiment. The hemolymph osmolality of larvae exposed to isoosmotic seawater also increased slightly, but significantly (one-way ANOVA, $F_{4,25}$=5.67; P=0.0031), over the course of the exposure. Submergence in freshwater did not affect hemolymph osmolality over the 10-day exposure.

Osmolyte accumulation

The reductions of WC as a result of exposure to hyperosmotic seawater were insufficient to account for the observed increase in hemolymph osmolality (Table 1). As osmolyte synthesis in *B. antarctica* larvae has been documented in response to dehydration (Benoit et al., 2007), we measured the concentrations of several osmolytes during osmotic stress. Exposure to hyperosmotic seawater induced the *de novo* synthesis of osmolytes in *B. antarctica* larvae. Glucose and trehalose concentrations increased approximately threefold over the course of a 6-day exposure in ~1000 mOsm kg^{-1} seawater. Similarly, by day 6 the concentration of glycerol increased more than fourfold to nearly 10 μg mg^{-1} DM. The osmolyte concentration of control larvae maintained in freshwater was not affected over the course of the experiment.

Oxygen consumption

Exposure to seawater, and the accompanying osmotic dehydration, resulted in a significant correlation (R^2=0.822; P<0.001; N=15) between the rate of oxygen consumption and larval WC (Fig. 2). In general, lower rates of oxygen consumption were found in larvae having a lower WC. The rate of oxygen consumption ranged from 0.32–0.68 μl g^{-1} DM h^{-1} and the linear regression model suggested that larvae having the lowest WC had rates of oxygen consumption that were ~50% below those of fully hydrated larvae.

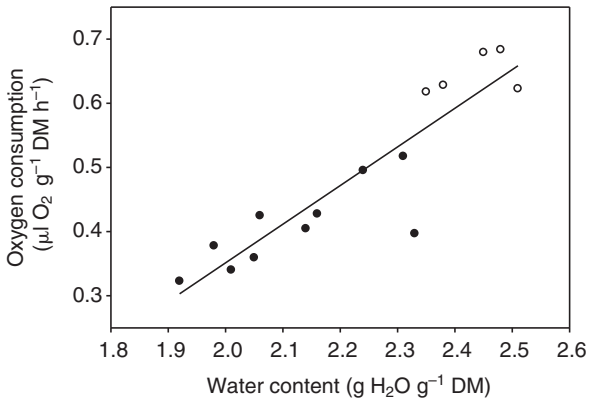

Fig. 2. Relationship between total body water content and the rate of oxygen consumption of *Belgica antarctica* larvae (y=–0.853+0.602x; N=15; R^2=0.822; P<0.001). Open circles denote groups of larvae acclimated to freshwater, and closed circles denote larvae osmotically dehydrated in seawater (~1000 mOsm kg^{-1}).

Effects of seawater exposure on the tolerance of other environmental stressors

Relative to the freshwater controls, a 3-day exposure to ~1000 mOsm kg^{-1} seawater significantly increased (two-way ANOVA treatment effect, $F_{2,48}$=403.74; P<0.0001) the freeze tolerance of *B. antarctica* larvae (Fig. 3). Nearly all larvae survived freezing for 6 h at –10°C. However, of the larvae acclimated for 3 days to freshwater, less than 65% survived freezing at –12°C and <15% survived freezing for 6 h at –15°C. By contrast, nearly 95% and 55% of larvae acclimated to 1000 mOsm kg^{-1} seawater survived freezing at –12 and –15°C, respectively. Even when frozen at –20°C, nearly 15% of seawater-acclimated larvae survived. This contrasts with freshwater acclimation, after which no larvae survived freezing for 6 h at –20°C. Additionally, the freeze tolerance of seawater-acclimated larvae rehydrated for 24 h, during which their WC was

Table 1. Estimated osmotic contribution of initial osmolytes in the hemolymph and osmolytes produced during exposure of *Belgica antarctica* larvae to hyperosmotic seawater (~1000 mOsm kg^{-1})

	Seawater (1000 mOsm kg^{-1})				Control (freshwater)
Days of exposure	0	1	3	6	6
Osmolality (mOsm kg^{-1})	407±7^{a}	491±10^{b}	646±12^{c}	779±15^{d}	391±10^{a}
Total body water content (g H_2O g^{-1} DM)	2.51±0.07^{a}	2.17±0.05^{b}	2.07±0.03^{b}	1.89±0.05^{c}	2.46±0.06^{a}
Osmotically active water (OAW) content* (g H_2O g^{-1} DM)	2.04	1.72	1.63	1.46	1.99
Loss of OAW (%)	–	15.7	21.1	28.5	–
Osmotic contribution of original solutes due to loss of OAW (mOsm kg^{-1})	–	480	516	569	–
Osmolyte concentration (μg mg^{-1} DM)					
Glycerol	2.17±0.27^{a}	2.96±0.46^{a}	6.87±0.58^{b}	9.56±0.46^{c}	1.94±0.31^{a}
Glucose	3.75±0.34^{a}	4.71±0.42^{a}	8.30±0.48^{b}	12.68±0.54^{c}	3.82±0.28^{a}
Trehalose	4.91±0.31^{a}	6.62±0.36^{b}	9.11±0.41^{c}	14.38±0.53^{d}	4.23±0.37^{a}
Osmotic contribution of synthesized osmolytes† (mOsm)	–	11	54	108	–
Total explainable osmotic pressure (%)	–	~100	88.2	86.9	–

Values are mean ± s.e.m. Different letters indicate significant differences between days of exposure (ANOVA; Bonferroni-Dunn test).

*OAW was calculated from Worland et al. (1998). [(OIW) = 0.069(TBW) + 0.3, where OIW is osmotically inactive water content, TBW is total body water content and is the sum of OIW and OAW].

†Assuming that osmolytes are only dissolved in OAW.

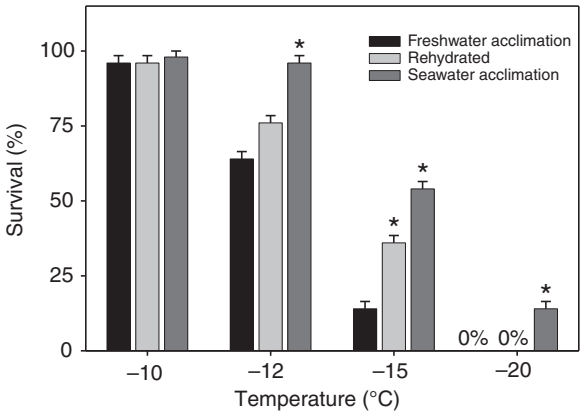

Fig. 3. Effect of acclimation to seawater on the freeze tolerance of *Belgica antarctica* larvae. Larvae were acclimated to either seawater (~1000 mOsm kg^{-1}) or freshwater (~0 mOsm kg^{-1}) for 3 days prior to assessment of freeze tolerance. A third group of larvae (rehydrated) were acclimated to seawater for 3 days followed by rehydration for 24 h in freshwater. Larvae were frozen in groups of 10 individuals in ~100 µl of freshwater for 6 h. Values are means ± 1 s.e.m. of five groups of 10 larvae. Asterisks denote a significant difference relative to the freshwater (control) treatment (ANOVA, Dunnett's test, $P<0.05$).

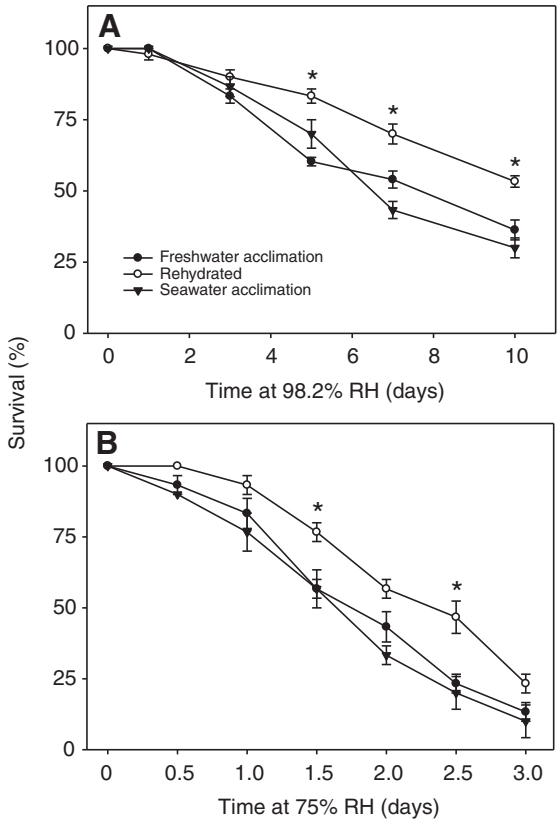

Fig. 4. Effect of seawater acclimation on the desiccation tolerance of *Belgica antarctica* larvae to 98.2% RH (A) or 75.0% RH (B) and 4°C. Larvae were acclimated to either seawater (~1000 mOsm kg^{-1}) or freshwater (~0 mOsm kg^{-1}) for 3 days prior to assessment of desiccation tolerance. Rehydrated larvae were acclimated to seawater for 3 days and then allowed to rehydrate for 24 h in freshwater prior to desiccation. Values are means ± 1 s.e.m. of five groups of 10 larvae. Asterisks denote a significant difference relative to the freshwater (control) treatment (ANOVA, Dunnett's test, $P<0.05$).

restored to pre-acclimation levels, was significantly greater than that of freshwater-acclimated larvae (Fig. 3). Seawater exposure followed by rehydration increased survival by 18% and 22% following freezing at –12 and –15°C, respectively, relative to survival of freshwater-acclimated larvae.

Similarly, exposure to ~1000 mOsm kg^{-1} seawater significantly affected the subsequent desiccation tolerance of *B. antarctica*, however, survival as a function of time was increased only in larvae rehydrated prior to desiccation (Fig. 4A,B). At both 98.2% (two-way ANOVA treatment effect, $F_{2,84}=6.38$; $P=0.0028$) and 75.0% RH (two-way ANOVA treatment effect, $F_{2,84}=4.26$; $P=0.017$), the survival of rehydrated larvae was significantly higher than that of the control, freshwater-acclimated larvae. The desiccation tolerance of larvae acclimated for 3 days to seawater did not differ from that of freshwater-acclimated larvae. However, this result may have been simply due to the reduced WC of the seawater-acclimated larvae (~2.0 *versus* ~2.5 g H_2O g^{-1} DM for freshwater-acclimated larvae; see Fig. 1). This is supported by the observation that at any given WC during desiccation at 75.0% RH the survival of both the seawater-acclimated and rehydrated groups was greater than that of the freshwater-acclimated larvae (i.e. seawater-acclimated larvae tolerated a greater loss of body water during dehydration).

The increased survival of freezing and desiccation following seawater acclimation, however, was contrary to the response of the larvae to heat shock (Fig. 5). Survival of heat shock at 30°C declined rapidly with exposure time, such that less than 75% and 25% of freshwater-acclimated larvae survived a 1.5 h and 2.5 h heat shock, respectively. However, relative to freshwater acclimation, the survival of seawater-acclimated larvae was significantly (two-way ANOVA treatment effect, $F_{2,84}=10.23$; $P<0.0001$) lower at all exposure times. The survival of larvae rehydrated following the 3-day seawater acclimation did not differ from that of freshwater-acclimated larvae.

DISCUSSION

Larval *B. antarctica* must endure potentially severe osmotic perturbation, as they are regularly challenged by a variety of environmental conditions that generate hydric and osmotic stress. On the Antarctic Peninsula, the freeze-tolerant larvae may be faced with subzero temperatures throughout the year. Freezing of the body fluids results in cellular dehydration, whereby water is osmotically drawn from intracellular stores to the now concentrated extracellular fluids (for a review, see Lee, 1991). Similarly, larvae may be exposed to severely desiccating conditions as microhabitats dry because of the vagaries of summer precipitation, elevated temperature, wind and insolation. Desiccation may also occur during winter, as freezing of the surrounding soil solution establishes an osmotic gradient for water loss from the yet unfrozen body fluids of the larvae (Elnitsky et al., 2008). Because microhabitat sites may be occasionally immersed in increasing concentrations of seawater from Antarctic storms, we investigated the tolerance and physiological response of the larvae to hyperosmotic seawater exposure. Together with previous investigations (Baust and Edwards, 1979; Baust and Lee, 1987; Hayward et al., 2007; Benoit et al., 2007; Michaud et al., 2008), our results demonstrate the midge larvae's impressive tolerance of osmotic stress. This inherent tolerance is facilitated by a variety of physiological responses (e.g. osmolyte accumulation) induced by the osmotic challenge, that probably also contribute to the observed enhanced tolerance to freezing and dehydration. These

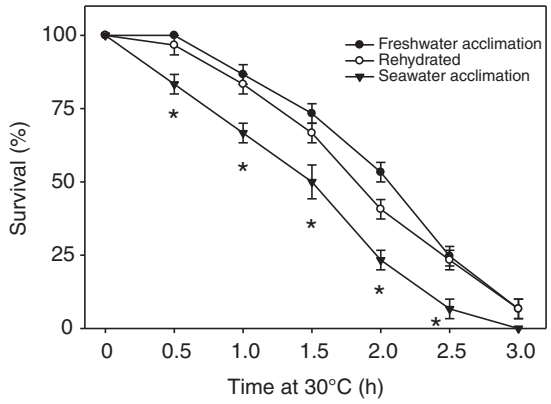

Fig. 5. Effect of seawater acclimation on the heat shock tolerance (time at 30°C) of *Belgica antarctica* larvae. Larvae were acclimated to either seawater (~1000 mOsm kg^{-1}) or freshwater (~0 mOsm kg^{-1}) for 3 days prior to assessment of heat shock tolerance. Rehydrated larvae were acclimated to seawater for 3 days and then allowed to rehydrate for 24 h in freshwater prior to heat shock. Values are means ± 1 s.e.m. of five groups of 10 larvae. Asterisks denote a significant difference relative to the freshwater (control) treatment (ANOVA, Dunnett's test, $P<0.05$).

results are discussed below in the context of our current knowledge of this and other species' tolerance and response to osmotic stress.

Tolerance and physiological response to salinity

Terrestrial larvae of *B. antarctica* tolerated extensive osmotic dehydration when challenged by hyperosmotic seawater. Nearly 50% of the larvae survived a 10-day exposure in ~1000 mOsm kg^{-1} seawater, during which the total body water content of the larvae was reduced by ~30% to <1.80 g H_2O g^{-1} DM. Survival declined rapidly during exposure to higher seawater concentrations; however, even in ~2000 mOsm kg^{-1} seawater ~25% of larvae survived a 6-day exposure. As the larvae are known to tolerate an extensive loss of body water, survival during exposure to hyperosmotic seawater is probably not solely dependent on tolerance to dehydration. During desiccation in air, larvae survive the loss of nearly 70% of their body water to <1.0 g H_2O g^{-1} DM (Benoit et al., 2007). Instead, during seawater exposure, an incurred salt load from the external medium probably contributed to the observed mortality. Inorganic ions, and especially Na^+ and Cl^-, are well known to disrupt cellular activity by binding to and destabilizing proteins and nucleic acids (Somero and Yancey, 1997; Hochachka and Somero, 2002; Yancey, 2005). Together with the reduced body water content, such salt load probably results in a breakdown of cellular homeostasis that may limit salinity tolerance in *B. antarctica.* A similar failure to effectively osmoregulate was observed in another Antarctic invertebrate, the collembolan *Cryptopygus antarcticus*, during exposure to hyperosmotic seawater (Hawes et al., 2008).

The reduced water content of the larvae, and associated concentration of solutes in the remaining body fluids, necessarily contributed to the increased osmotic concentration (Table 1). However, water loss alone was insufficient to account for the observed increase in hemolymph osmolality. At day 6 of exposure to ~1000 mOsm kg^{-1} seawater, the reduction of body water could explain only ~70% of the observed osmotic concentration of the body fluids. Salinity exposure also induced the *de novo* synthesis and accumulation of several organic osmolytes, as glycerol, glucose and trehalose concentrations all increased three- to more than fourfold over the 6-day exposure. Assuming that the organic osmolytes were dissolved in the osmotically active fraction of the body water (Worland et al., 1998), such osmolyte accumulation could have contributed ~100 mOsm kg^{-1} (~13%) toward the observed osmolality at day 6 during exposure to seawater (Table 1). The larvae probably also incurred a significant accumulation of inorganic ions from the seawater medium that contributed to the osmolality; however, we cannot rule out the additional accumulation of other organic osmolytes.

The accumulation of organic osmolytes is a well known response of organisms to reduce osmotic stress (Hochachka and Somero, 2002). These compatible organic solutes are generally favored over the accumulation of inorganic ions because they limit the perturbation of macromolecules even at high concentrations (Yancey, 2001). In addition to their colligative effect in reducing the gradient for water loss, many organic solutes also have other physiological properties, such as stabilizing membranes and proteins during cell shrinkage (Crowe et al., 1984; Crowe et al., 1992; Sano et al., 1999), which preserve cellular function. Among arthropods, larvae of the euryhaline mosquito *C. tarsalis* accumulate high concentrations of proline and trehalose in response to increased environmental salinity (Garrett and Bradley, 1987; Patrick and Bradley, 2000). We previously demonstrated that larval *B. antarctica* accumulate significant concentrations of glycerol and trehalose in response to desiccation stress at high relative humidities in air (Benoit et al., 2007; Michaud et al., 2008), and glucose and trehalose at subzero temperatures in the presence of environmental ice (Elnitsky et al., 2008). In the present study, we observed a similar response to hyperosmotic seawater exposure. In all cases, the accumulation of organic osmolytes probably serves to slow and/or limit cellular dehydration while preserving metabolic function. This may be especially important during exposure to hyperosmotic seawater to extend survival time and allow the larvae to use other behavioral mechanisms to reduce further osmotic stress.

During submergence in freshwater (~0 mOsm kg^{-1}), the larvae maintained the total body water content and osmotic concentration of the body fluids at ~2.5 g H_2O g^{-1} DM and ~400 mOsm kg^{-1}, respectively. In isoosmotic seawater (~400 mOsm kg^{-1}), hemolymph osmolality increased slightly (~10%) over the 10-day exposure. This increase could not be accounted for by a simple concentration effect due to changes in WC, as by day 10 of the exposure the WC did not differ from that of larvae maintained in freshwater. Therefore, it suggests that these larvae too incurred an increased salt load from the external medium and/or the salinity exposure resulted in the accumulation of organic osmolytes.

Reduced O_2 consumption during osmotic stress

Exposure to hyperosmotic seawater and the accompanying osmotic dehydration resulted in a significant positive correlation between the rate of oxygen consumption and the total body water content of *B. antarctica* larvae. Whether the reduction in the rate of oxygen consumption was compensated for by an increase in anaerobic metabolism is unknown. However, we previously reported a similar reduction of aerobic metabolism of larval *B. antarctica* following dehydration in air (Benoit et al., 2007). Such hypometabolism in response to dehydration is well known among arthropods and has been suggested as a mechanism to reduce respiratory water loss (Hadley, 1994). In our study, the reduced rate of oxygen consumption would not be expected to curtail water loss during submergence in hyperosmotic seawater. Osmoregulating mosquito larvae typically display increased rates of oxygen consumption during exposure to increased environmental salinity, presumably in an effort to ionoregulate (Bradley, 1987). However, in species unable

to effectively osmoregulate during hyperosmotic seawater exposure, rates of oxygen consumption generally decline with increased environmental salinity and salt load within the body fluids (Bradley, 1987; Plaut, 1999; Ordiano et al., 2005; Irwin et al., 2007). Together with the present results, this suggests that rather than an active physiological downregulation of metabolism in an effort to limit water loss, the reduced rate of oxygen consumption may be a passive response to osmotic and ionic perturbations of cellular function.

Osmotic dehydration, and any additional salt load from the external medium, incurred during the exposure to hyperosmotic seawater may have resulted in the observed hypometabolism because of the effects on protein function. In addition to the aforementioned effects of inorganic ions on protein stability, solute crowding, as a result of reduced body water, can also impose osmophobic and hydrophobic effects on macromolecules (Hochachka and Somero, 2002). Such osmo- and hydrophobicity have important effects on the assembly and folding of proteins and nucleic acid structures and, therefore, could result in reduced protein function and metabolic rate in the dehydrated state. Additionally, we cannot rule out a role for the accumulation of organic solutes [e.g. urea (Muir et al., 2007)] contributing to the observed reduction of oxygen consumption.

Effects of seawater exposure on the tolerance of other environmental stressors

Acclimation to hyperosmotic seawater (~1000 mOsm kg^{-1}) increased larval tolerance to freezing and dehydration, but reduced tolerance to high temperature. A link between cold tolerance and dehydration is now well established (Ring and Danks, 1994; Ring and Danks, 1998). At the cellular level, freezing and dehydration present a similar challenge of maintaining membrane integrity as water is osmotically removed from cellular stores. Furthermore, in many cases the physiological response(s), such as the accumulation of osmolytes, elicited by organisms to these related stressors is similar. Among arthropods, enhanced cold tolerance following dehydration is known from several taxa (Hadley, 1994). Similar to our present results, Hayward et al. (Hayward et al., 2006) recently documented enhanced freeze tolerance of *B. antarctica* following slow dehydration in air. The simple reduction of body water content would be expected to slow and reduce ice formation within the body fluids (Lee, 1991), and probably contributed to the enhanced survival of subsequent freezing. However, even after larvae were rehydrated their subsequent tolerance of freezing was increased, suggesting other physiological mechanisms, independent of reductions of body water content, were involved (see below).

In contrast to increased cold tolerance following hydric stress, enhanced desiccation tolerance is less well documented. However, a drought acclimation response that enhances subsequent tolerance of dehydration and low temperature is known from the soil-dwelling springtail *Folsomia candida* (Sjursen et al., 2001). When this collembolan was exposed to 98.2% RH for 6 days, survival of subsequent drought stress, to as low as 94% RH, and the tolerance of water loss were dramatically increased. We recently documented a similar drought acclimation response, analogous to the enhanced desiccation tolerance following acclimation to hyperosmotic seawater, in *B. antarctica* following desiccation at high relative humidities (Benoit et al., 2007). Accumulation of organic solutes appears to be a common component of the drought acclimation response (Sjursen et al., 2001; Benoit et al., 2007) (and present study), and probably contributes mechanistically, by protecting membranes and proteins, to the increased tolerance of dehydration. In *F. candida*, drought acclimation also results in a higher degree of unsaturation of membrane phospholipid fatty acids (Bayley et al., 2001; Holmstrup et al., 2002), a change that resembles membrane alterations seen in ectothermic animals acclimated to low temperature. Holmstrup et al. (Holmstrup et al., 2002) suggest such membrane desaturation may counter the increased packing of membrane lipids that occurs as water is removed from the cell during dehydration, thereby maintaining membrane fluidity and metabolic function. Such changes in *B. antarctica* during acclimation to seawater could help to explain their enhanced tolerance of desiccation and low temperature. Additionally, desaturation of phospholipid fatty acids may render the larvae more susceptible to heat shock, as cellular membranes may be more prone to phase transitions at high temperatures resulting in a loss of membrane integrity. It is also worth noting that as with *B. antarctica* larvae, *F. candida* experiences reduced tolerance to high temperatures following drought acclimation (Holmstrup et al., 2002).

Conclusions

In the harsh and highly variable environment of the Antarctic Peninsula, *B. antarctica* may be regularly challenged by osmotic stress, including freezing, dehydration and periodic submergence in hyperosmotic seawater. It has become apparent that these terrestrial larvae possess an impressive tolerance and rely upon a number of behavioral and physiological mechanisms to ameliorate such stress. During hyperosmotic seawater submergence, the inherent tolerance of osmotic stress probably allows the larvae to maintain metabolic function and escape to more favorable microhabitats. However, prolonged submergence in seawater presents a severe threat to survival, as a result of water loss and salt load from the external environment. Cellular water loss may be slowed and protein structure and function preserved by the accumulation of several organic osmolytes. These osmolytes might also contribute to the subsequent enhanced tolerance to freezing and desiccation following a brief seawater acclimation, and reflect a conserved physiological response to these related yet distinct forms of osmotic stress.

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