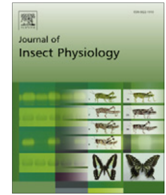
Journal of Insect Physiology 58 (2012) 498–505

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

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



Energetic consequences of repeated and prolonged dehydration in the Antarctic midge, *Belgica antarctica*

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ARTICLE INFO

Article history:
Available online 25 November 2011

Keywords:
Antarctic midge
Repeated dehydration
Energy reserves
Osmoprotectants

ABSTRACT

Larvae of the Antarctic midge, *Belgica antarctica*, routinely face periods of limited water availability in their natural environments on the Antarctic Peninsula. As a result, *B. antarctica* is one of the most dehydration-tolerant insects studied, surviving up to 70% loss of its body water. While previous studies have characterized the physiological effects of a single bout of dehydration, in nature larvae are likely to experience multiple bouts of dehydration throughout their lifetime. Thus, we examined the physiological consequences of repeated dehydration and compared results to larvae exposed to a single, prolonged period of dehydration. For the repeated dehydration experiment, larvae were exposed to 1–5 cycles of 24 h dehydration at 75% RH followed by 24 h rehydration. Each bout of dehydration resulted in 30–40% loss of body water, with a concomitant 2- to 3-fold increase in body fluid osmolality. While nearly 100% of larvae survived a single bout of dehydration, <65% of larvae survived five such cycles. Larvae subjected to multiple bouts of dehydration also experienced severe depletion of carbohydrate energy reserves; glycogen and trehalose content decreased with each successive cycle, with larvae losing 89% and 48% of their glycogen and trehalose, respectively, after five cycles of dehydration/rehydration. Larvae exposed to prolonged dehydration (99% RH for 10 d) had 26% less water, 43% less glycogen, and 27% less lipid content than controls, but did not experience any mortality. Thus, both repeated and prolonged dehydration results in substantial energetic costs that are likely to negatively impact fitness.

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1. Introduction

The Antarctic midge, *Belgica antarctica*, is the world's southernmost insect and the only insect endemic to Antarctica (Sugg et al., 1983). Found exclusively on the west coast of the Antarctica Peninsula and its surrounding islands, *B. antarctica* has a patchy distribution and is typically found in nutrient-rich areas associated with penguin and seal rookeries. The midge has a 2-year life cycle in which larvae feed during the brief austral summer and overwinter as any of four larval instars (Convey and Block, 1996). Apterous adults synchronously emerge for a brief period in the summer, during which they mate, oviposit, and die within 10 d.

Not surprisingly, larvae are extremely tolerant of a number of environmental stresses, including low temperature (Lee et al., 2006), dehydration (Benoit et al., 2007; Hayward et al., 2007), oxidative stress (Lopez-Martinez et al., 2008), and osmotic perturbations (Elnitsky et al., 2009). While Antarctic terrestrial organisms cope with a number of environmental insults, water availability is the primary factor governing the distribution of terrestrial organisms on the continent (Kennedy, 1993). For much of the year, water is frozen and therefore biologically unavailable. Even in the summer months, when liquid water is abundant, there can be spatial and temporal differences in microhabitat water availability (Benoit et al., 2007; Kennedy, 1993).

Thus, throughout its life cycle, *B. antarctica* is exposed to numerous periods of desiccating conditions. At ecologically relevant relative humidities, larvae of *B. antarctica* tolerate up to 70% loss of their body water, the highest level of dehydration tolerance measured in a polar arthropod (Hayward et al., 2007). Additionally, the high cuticular water permeability and dehydration tolerance of larvae allow them to use cryoprotective dehydration as a means to survive prolonged periods of subzero exposure (Elnitsky et al., 2008). In response to dehydration, larvae accumulate osmoprotectants such as glycerol and trehalose, alter their cuticular hydrocarbon profile, and decrease their respiration rate as a means to conserve water and enhance dehydration tolerance (Benoit et al.,

Abbreviations: DM, dry mass; TEC, total energy content; CEC, carbohydrate energy content; TCC, total carbohydrate content; RD, repeated dehydration; PD, prolonged dehydration.

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doi:10.1016/j.jinsphys.2011.11.011

2007). Several other metabolic changes are elicited by dehydration, including elevation of specific polyols and amino acids, although the adaptive benefits of these changes remain unexplored (Michaud et al., 2008). Furthermore, a number of stress related genes, including several heat shock proteins and genes involved in oxidative stress, are upregulated in response to dehydration (Lopez-Martinez et al., 2009). At the protein level, dramatic changes in the abundance of cytoskeletal proteins occur during dehydration, presumably to counteract the change in body size accompanying dehydration (Li et al., 2009).

Despite obvious benefits to the larvae, many of these physiological changes during dehydration would appear to be energetically costly. Also, additional energy input is required during rehydration to repair the accrued damage. For example, the cellular machinery responsible for restoring perturbed ion gradients during desiccation consumes a large amount of energy (Harvey et al., 1998). In *Drosophila melanogaster*, the link between energy reserves and the ability to survive dehydration is well established; populations of *D. melanogaster* selected for desiccation resistance have increased carbohydrate stores, which provide additional energy to acclimate and recover from dehydration and bind bulk water to increase the pool of available water (Chippindale et al., 1998; Djawdan et al., 1998). However, the energetic consequences of dehydration in *B. antarctica*, or any other polar species, have not been examined.

Previous studies on dehydration in *B. antarctica* only assessed physiological changes in response to a single bout of dehydration, whereas in nature larvae are likely to be exposed to numerous rounds of dehydration and rehydration. While most studies of stress tolerance have only used a single bout of stress, several recent studies have demonstrated the importance of using repeated stress exposures that better reflect natural conditions. Previously, we demonstrated that repeated freeze/thaw cycles in larvae of *B. antarctica* cause significant mortality, damage to midgut tissue, expression of stress-related genes, and a severe reduction in energy reserves, and that these effects worsen with each freeze/thaw cycle (Teets et al., 2011). While several other studies have addressed the question of repeated cold exposure (Colinet et al., 2007; Marshall and Sinclair, 2010, 2011), few have addressed the consequences of repeated dehydration in insects. In *Drosophila*, prior exposure to dehydration can rapidly enhance dehydration-tolerance, primarily by altering cuticular water loss rates (Bazinet et al., 2010; Hoffmann, 1991). Meanwhile, only one study has addressed the consequences of repeated, cyclic bouts of dehydration; in the mosquito, *Culex pipiens*, repeated bouts of dehydration cause a significant reduction in energy reserves and fecundity, effects that are not measurable after a single bout of dehydration (Benoit et al., 2010). In all cases, the use of repeated stress exposures reveals novel physiological changes that cannot be predicted from a single exposure to stress.

Thus, our primary objectives in this study were to (1) measure the effects of repeated dehydration and rehydration on the water balance and osmolality of *B. antarctica* larvae, (2) determine the ability of larvae to survive multiple bouts of severe dehydration and (3) compare the energetic consequences of repeated and prolonged dehydration on the energy balance and osmoprotectant levels. Together, these experiments provide the first overview of the dynamics and energetic consequences of dehydration that confront a polar insect.

2. Materials and methods

2.1. Insects

Larvae of *B. antarctica* were collected from Humble, Cormorant, and Christine Islands near Palmer Station on the Antarctic Peninsula (64°46′S, 64°04′W) in January and February of 2011. Larvae were extracted from substrate (containing soil, rocks, and detritus) into ice water using a modified Berlese apparatus, and concentrated samples of larvae were placed back in their natural substrate and held at 2 °C until the time of experiments. On the day prior to beginning an experiment, larvae were hand-sorted from substrate in ice water and placed on moist filter paper overnight to standardize body water content. Only fourth instar larvae were used in this study.

2.2. Experimental conditions

Temperature was maintained at 2 °C for the duration of the experiment. Different relative humidities were generated in sealed desiccators using saturated salt solutions according to Winston and Bates (1960); 75% RH was obtained with a saturated solution of NaCl, while 99% humidity was obtained with a saturated solution of K_2SO_4. Larvae were placed in groups of 10 in microcentrifuge tubes with mesh tops for dehydration exposures, while control animals were held in groups of 10 in vials with moist paper towel for the duration of the experiment. For the repeated dehydration treatment, larvae were placed at 75% RH for 24 h, followed by 24 h of rehydration by transferring the larvae to a moist paper towel. This cycle was repeated for a total of five dehydration and rehydration cycles. Water content and osmolality were measured each day for 10 d, while samples for survival, cell survival, and metabolite analyses were taken every 2 d after the rehydration phase of the treatment. For the prolonged dehydration treatment, larvae were held for 10 d at 99% RH, followed by 24 h of rehydration on a moist paper towel. Water content and osmolality were determined after both the 10 d dehydration and rehydration, while larval survival, cell survival, and metabolite contents were assessed after rehydration.

2.3. Water content and osmolality

On each day of the experiment, water content was determined gravimetrically. From each group, 15 individuals were weighed to the nearest 0.2 μg on an electrobalance and immediately transferred to a drying oven at 65 °C. Individuals were weighed to constant dryness, and body water content, expressed as mg H_2O mg^{-1} dry mass (DM), was calculated for each individual.

Body fluid osmolality was determined using a vapor pressure depression technique according to Elnitsky et al. (2008). Briefly, 5 individuals from each treatment were crushed with a Teflon pestle, sealed in a C-52 sample chamber (Wescor Inc., Logan, UT), and allowed to equilibrate for 30 min at room temperature. The osmolality was then measured using a Wescor HR-33T Dew Point Microvoltmeter (Wescor Inc., Logan, UT), with values being compared to standard curves produced from solutions of known salt concentration (Opti-Mole, Wescor Inc., Logan, UT). For each treatment, osmolality was based on the mean of 5 biological replicates of 5 individuals.

2.4. Survival and cell survival

At the end of each dehydration/rehydration cycle, survival was assessed by checking for larval movement. Concurrently, survival was checked for control animals that were held on moist paper towel. Groups of 10 larvae were placed in a drop of water under a microscope, and those that either moved spontaneously or in response to gentle prodding were considered alive. For each treatment, survival was based on 5 replicates of 10 larvae each.

To test for sublethal tissue damage caused by dehydration, cell survival of midgut tissue was measured according to Yi and Lee (2003). The midgut was selected because it is highly susceptible

to damage from freezing (Teets et al., 2008) and because it is amenable to the staining procedures of this assay. After each rehydration cycle, the midgut was dissected in Coast's solution (Coast and Krasnoff, 1988) and stained with the LIVE/DEAD sperm viability kit (Invitrogen, Carlsbad, CA). Tissue samples were stained for 15 min in SYBR followed by an additional 15 min in propidium iodide. After staining, images were obtained using fluorescent microscopy at the Ohio State Campus Microscopy and Imaging Facility. The nuclei of living cells with intact cell membranes appear green, while dead cells with damaged membranes are red. Cell viability for each sample was determined by counting three groups of 100 cells and calculating the mean proportion of green cells from these three counts. For each treatment, viability was based on the mean of six biological replicates.

2.5. Metabolite assays

To assess the effects of dehydration on energy stores and osmoprotectant levels, we conducted assays for total lipid, triglycerides, glycogen, trehalose, glucose, and glycerol. After each dehydration/rehydration cycle, 10 groups of 5 individuals were weighed and quickly frozen at −70 °C for lipid and triglyceride assays (5 groups for lipid, 5 groups for triglycerides). For water-soluble metabolites, 5 groups of 20 individuals were weighed and frozen from each treatment. Samples were shipped frozen on dry ice from Palmer Station, Antarctica to Ohio State University, where the assays were conducted. Samples for the water-soluble metabolites were homogenized in 1 M perchloric acid, centrifuged at 14,000*g*, and the supernatant was neutralized with 1 M KOH. Neutralized extracts were stored at −70 °C until analysis.

Total lipid content was measured using vanillin-phosphoric acid reagent according to Sim and Denlinger (2009). Groups of 5 individuals were homogenized in 500 μl 1:1 chloroform:methanol and centrifuged, and the solvent was evaporated by heating at 90 °C. Samples were then re-suspended in H_2SO_4, followed by the addition of vanillin-phosphoric acid reagent. Absorbance was read at 490 nm in a NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA) and compared to absorbance of known standards containing canola oil dissolved in chloroform.

Triglycerides were measured according to Marshall and Sinclair (2010), with the following modifications: Samples of 5 larvae were homogenized in 1:1 chloroform:methanol and centrifuged to remove pigments. The supernatant was evaporated by heating at 90 °C, and the sample was re-suspended in 0.05% Tween-20. Triglyceride content was measured using triglyceride reagent (T2449, Sigma, St. Louis, MO) to liberate glycerol from triglyerides. Glycerol content was then measured using free glycerol reagent (F6428, Sigma, St. Louis, MO) by measuring absorbance at 540 nm and comparing to known glycerol standards.

Glycogen content was measured in perchloric acid extracts by first using amyloglucosidase from *Aspergillus niger* (A1602, Sigma, St. Louis, MO) to liberate free glucose (Kepler and Decker, 1984). Free glucose was then assayed using a glucose assay kit (GAGO20, Sigma, St. Louis, MO), and the absorbance at 540 nm was compared to standards of known glycogen concentration. Trehalose content was measured according to Chen et al. (2002), by using trehalase from porcine kidney (T8778, Sigma, St. Louis, MO) to hydrolyze trehalose into glucose monomers. The resulting glucose content was then measured as described previously. In each sample, glucose content was also measured in undigested perchloric acid extracts and used to correct the values of glycogen and trehalose. Finally, glycerol content in perchloric acid extracts was measured with free glycerol reagent according to Teets et al. (2011). For each metabolite, values were based on the mean of 5 replicates and are reported as μg metabolite mg^{-1} DM.

2.6. Total energy and carbohydrate energy content

To summarize the results of the energy reserve assays, the total energy content (TEC) and carbohydrate energy content (CEC) values were calculated according to Djawdan et al. (1998). Using thermodynamic values for lipid and carbohydrate, the formula for TEC is TEC = 0.0393(lipid content) + 0.0176TCC, where TEC is in J mg^{-1} DM, lipid content is in μg lipid mg^{-1}DM, and TCC is total carbohydrate content in μg carbohydrate mg^{-1} DM and is the sum of glycogen, trehalose, and glucose content. Because TEC predominantly reflects lipid content, we also calculated CEC, in J mg^{-1} DM, using the formula CEC = 0.0176CCC.

2.7. Statistical analyses

All statistical analyses were conducted with JMP 9 (SAS Institute Inc., Cary, NC). Means were compared using ANOVA with a post hoc pooled *T*-test. A false discovery rate correction (Benjamini and Hochberg, 1995) with $\alpha = 0.05$ was applied to each hypothesis test to control for the probability of a type I error. Based on the number of hypothesis tests conducted, the approximate false discovery rate for the entire study was 0.025. Whole-animal and cell survival data were arcsin-square-root-transformed prior to analyses. Statistical significance was set at $P < 0.05$, and all data are reported as mean ± SE.

3. Results

3.1. Water content and osmolality

To facilitate discussing the results, odd numbered days (i.e. 1, 3, 5, 7 and 9) represent the dehydration phase of the dehydration/rehydration cycle, while even numbered days (i.e. 2, 4, 6, 8 and 10) represent the rehydration phase. Also, for convenience, larvae subjected to repeated dehydration/rehydration will be referred to RD larvae, while those subjected to prolonged dehydration (i.e. 99% for 10 d) will be referred to as PD larvae. After each dehydration cycle, the water content of RD larvae was between 26% and 48% less than control larvae, differences that were statistically significant in all cases ($P < 0.05$). After the first rehydration event, the water content of RD larvae (2.41 ± 0.11 μg H_2O mg^{-1} DM) remained significantly lower than the water content of control larvae (2.92 ± 0.09 μg H_2O mg^{-1} DM; $P < 0.05$). However, after each subsequent rehydration cycle the water content of RD larvae was statistically indistinguishable from that of control larvae ($P > 0.05$). Over the course of the experiment, the water content of RD animals after both dehydration and rehydration increased slightly (Fig. 1A). The water content of RD larvae on days 7 and 9 (1.88 ± 0.12 and 2.15 ± 0.06 mg H_2O mg^{-1} DM, respectively) was significantly greater than in RD larvae on days 3 and 5 (1.46 ± 0.07 and 1.47 ± 0.14 mg H_2O mg^{-1} DM, respectively; $P < 0.05$). Likewise, the water content of RD larvae on days 6, 8, and 10 was significantly higher than that of RD larvae on day 2 ($P < 0.05$). In the prolonged dehydration experiment, water content of PD larvae was 26% less than that of control larvae after day 10 ($P < 0.05$) but returned to control levels following rehydration ($P > 0.05$; Table 1).

In the repeated dehydration experiment, changes in body fluid osmolality tracked the observed changes in water content (Fig. 1B). Following each of the five dehydration events, body fluid osmolality of RD larvae was significantly higher than that of control larvae ($P < 0.05$), while there was no difference between RD and control larvae after each cycle of rehydration ($P > 0.05$). The greatest difference in osmolality was observed on day 7, when the osmolality of control larvae was 466.0 ± 45.4 mOsm kg^{-1} while that of RD larvae was 1519.4 ± 290.9 mOsm kg^{-1} ($P < 0.05$). Follow-

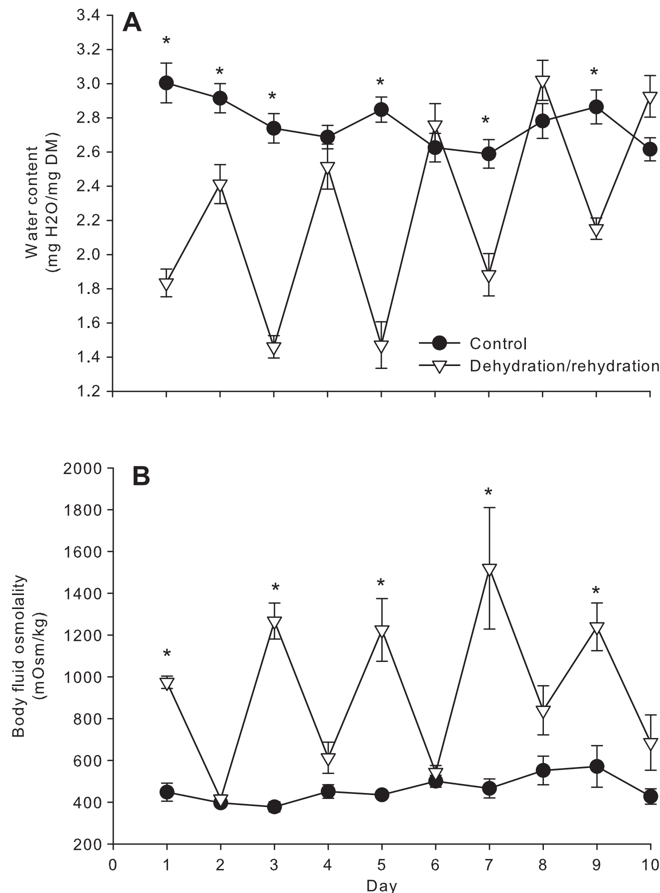

Fig. 1. Water content (A) and osmolality (B) of larvae of *B. antarctica* during repeated bouts of dehydration and rehydration. Each bout of dehydration and rehydration consisted of dehydration for 24 h at 75% RH followed by rehydration for 24 h on a moist paper towel. Odd numbered days represent days following dehydration while even numbered days represent days following rehydration. Each data point represents the mean ± SE of 15 individuals for water content and for five samples for osmolality. An "*"indicates a significant difference between control and repeated dehydration larvae at a particular time point (ANOVA, FDR, $P < 0.05$). DM, dry mass.

ing prolonged dehydration, the osmolality of PD larvae was 889.9 ± 83.0 mOsm kg^{-1}, compared to 616.9 ± 59.9 mOsm kg^{-1} in control larvae, but this difference was not statistically significant ($P > 0.05$; Table 1). Likewise, following rehydration, there was no difference in osmolality between control and PD larvae ($P > 0.05$).

3.2. Whole-animal and cell survival

Throughout much of the repeated dehydration experiment, there were no significant differences in survival between control and RD larvae (Table 2). For the first four dehydration/rehydration cycles, survival ranged from 94.4 ± 4.0 to 100.0 ± 0.0% for control larvae and from 92.8 ± 4.9% to 98.0 ± 2.0% for RD larvae. However, after the fifth cycle, survival of RD larvae (63.9 ± 12.3%) was significantly lower than that of corresponding control larvae (96.2 ± 2.3%; $P < 0.05$). In the prolonged dehydration experiment, there was no difference in survival between PD larvae (100.0 ± 0.0%) and control larvae (98.0 ± 2.0%; $P > 0.05$; Table 1).

In the repeated dehydration experiment, cell survival of midgut tissue in RD larvae was consistently lower than that of control larvae (Table 2). After each of the five dehydration/rehydration cycles, cell survival of RD larvae was significantly lower than that of control larvae, ranging between 9.4% and 18.5% lower than their control counterparts ($P < 0.05$). In the prolonged dehydration experiment, there was no difference in cell survival between control and PD larvae ($P > 0.05$; Table 1).

3.3. Energy reserves and osmoprotectant levels

For the entire repeated dehydration experiment, total lipid levels did not vary across treatments, ranging from 171.0 ± 10.2 to 201.8 ± 11.2 μg lipid mg^{-1} DM (Fig. 2A; $P > 0.9827$). Likewise, triglyceride levels did not significantly differ between control and RD larvae after any of the dehydration/rehydration cycles (Fig. 2B; $P > 0.05$), ranging from 79.2 ± 6.2 to 128.8 ± 10.3 μg triglyceride mg^{-1} DM. Following prolonged dehydration and rehydration, PD larvae had 27% less lipid than their control counterparts, a difference that was significant ($P < 0.05$; Fig. 3A). However, the observed difference in triglyceride content between control (88.5 ± 11.8 μg triglyceride mg^{-1} DM) and PD (73.8 ± 7.1 μg triglyceride mg^{-1} DM) larvae was not significant ($P > 0.05$).

While repeated dehydration had little effect on lipid energy reserves, carbohydrate energy reserves were dramatically altered by repeated dehydration. After each of the five cycles of dehydration/rehydration, the glycogen content of RD larvae was significantly lower than that of control larvae ($P < 0.05$; Fig. 2C). The glycogen content of RD larvae continued to decrease with each successive cycle, decreasing from 29.5 ± 2.4 μg glycogen mg^{-1} DM after one dehydration/rehydration cycle to 4.9 ± 0.9 μg glycogen mg^{-1} DM after five dehydration/rehydration cycles. By the fifth cycle of dehydration/rehydration, the glycogen content of RD larvae was 89% lower than their control counterparts. Prolonged dehydration likewise caused a significant reduction in glycogen, although not to the levels seen in the RD larvae. After rehydration, the glycogen content of PD larvae (31.9 ± 3.9 μg glycogen mg^{-1} DM) was 43% less than that of control larvae (55.9 ± 1.6 μg glycogen mg^{-1} DM; $P < 0.05$; Fig. 3A).

The other major carbohydrate energy store, trehalose, was also significantly depleted by repeated bouts of dehydration/rehydration (Fig. 2D). After one dehydration/rehydration cycle, the trehalose content of RD larvae (42.8 ± 0.8 μg trehalose mg^{-1} DM) did not differ from the control value (48.3 ± 1.5 μg trehalose mg^{-1} DM). However, trehalose levels of RD larvae were gradually diminished with each cycle and were significantly lower than control larvae for cycles 2–5 ($P < 0.05$). After the fifth cycle of dehydration/rehydration, trehalose levels were 48% lower in RD larvae compared to controls. In the prolonged dehydration experiment, there was no difference in trehalose content between control

Table 1
Effect of prolonged dehydration (10 d at 99% RH) and rehydration (1 d at 100% RH) on water content, osmolality, survival, and midgut cell survival in larvae of *B. antarctica*. Values are presented as mean ± SE, $N = 15$ for water content, $N = 5$ for osmolality and survival, $N = 6$ for cell survival. Different letters within the same row represent significant differences between treatment groups (ANOVA, FDR, $P < 0.05$). DM, dry mass.

	Control, 10 d	Prolonged dehydration	Control, 11 d	Prolonged dehydration + rehydration
Water content (mg H_2O/mg DM)	2.48 ± 0.06^a	1.83 ± 0.07^b	2.54 ± 0.07^a	2.75 ± 0.12^a
Body fluid osmolality (mOsm/kg)	616.9 ± 59.9^a	889.9 ± 83.0^a	552.0 ± 21.3^a	673.9 ± 15.6^a
Survival (%)	NA	NA	98.0 ± 2.0^a	100.0 ± 0.0^a
Cell survival (%)	NA	NA	92.0 ± 2.8^a	90.5 ± 2.7^a

Table 2
Effects of repeated bouts of dehydration and rehydration on whole-animal and midgut cell survival in larvae of *B. antarctica*. Each cycle of dehydration and rehydration consisted of 24 h at 75% RH followed by 24 h on a moist paper towel. Values are given as mean ± SE, $N = 5$ for organismal survival and $N = 6$ for cell survival. An "*"indicates a significant difference between control and repeated dehydration larvae at a particular time point (ANOVA, FDR, $P < 0.05$).

	Number of cycles of dehydration/rehydration				
	1	2	3	4	5
Control survival (%)	100.0 ± 0.0	100.0 ± 0.0	94.0 ± 4.0	98.0 ± 2.0	96.2 ± 2.3
Dehydration/rehydration survival (%)	98.0 ± 2.0	94.4 ± 3.7	92.8 ± 4.9	94.0 ± 4.0	63.9 ± 12.3*
Control cell survival (%)	93.7 ± 2.2	96.5 ± 1.6	90.9 ± 3.0	96.3 ± 1.1	92.0 ± 2.8
Dehydration/rehydration cell survival	84.3 ± 1.2*	78.0 ± 1.0*	78.3 ± 2.8*	79.5 ± 3.8*	79.5 ± 4.7*

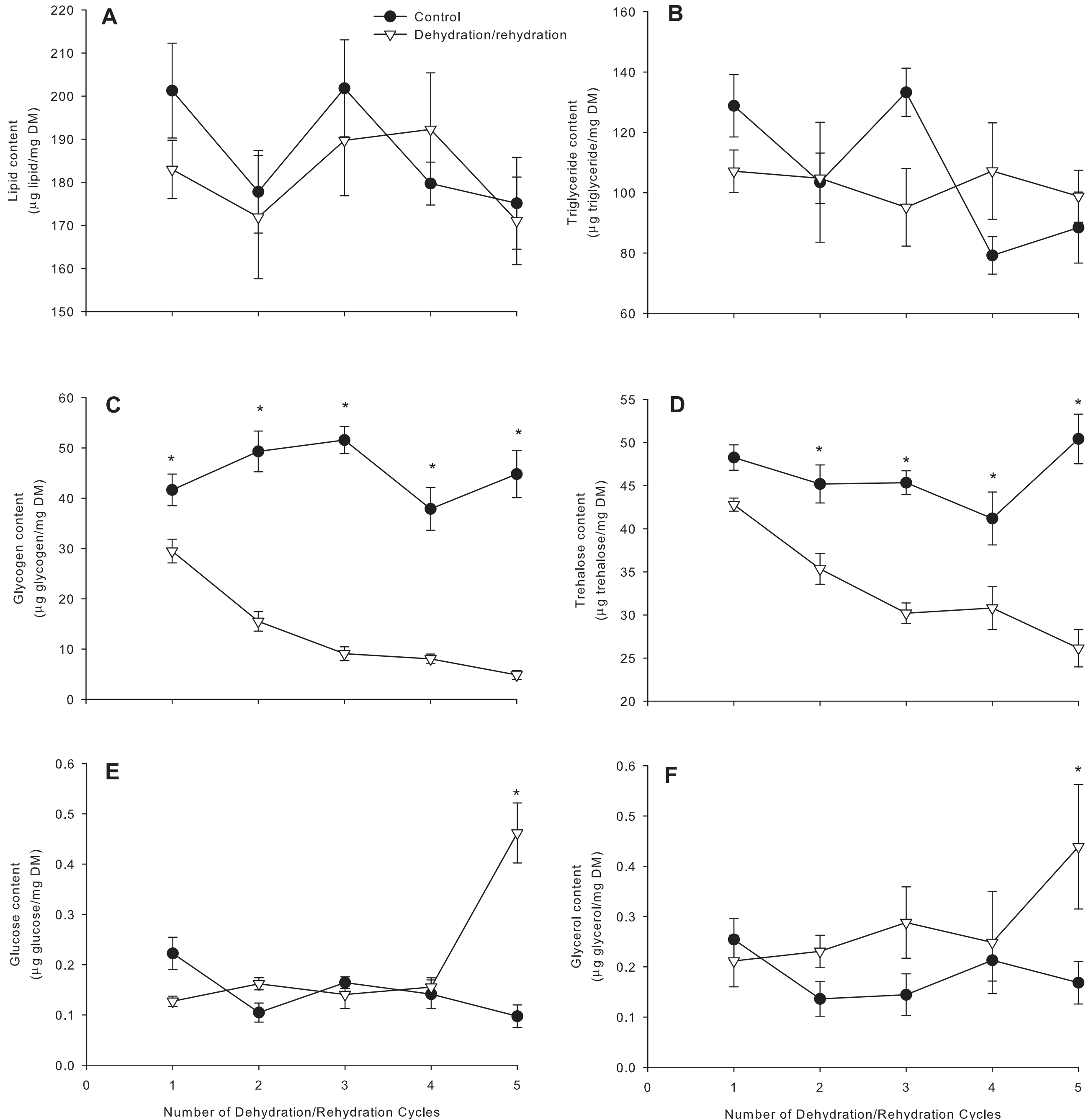

Fig. 2. Lipid (A), triglyceride (B), glycogen (C), trehalose (D), glucose (E), and glycerol (F) content in larvae of *B. antarctica* during repeated dehydration/rehydration cycles. Metabolites were sampled at the end of each of five dehydration/rehydration cycles, consisting of 24 h dehydration at 75% RH followed by 24 h rehydration on a moist paper towel. For lipid and triglyceride, values are the mean ± SE of 5 replicates of 5 individuals each, while for the other metabolites values are mean ± SE of 5 replicates of 20 individuals. An "*"indicates a significant difference between control and repeated dehydration larvae at a particular time point (ANOVA, FDR, $P < 0.05$). DM, dry mass.

(52.6 ± 0.5 μg trehalose mg^{-1} DM) and RD (46.4 ± 1.4 μg trehalose mg^{-1} DM) larvae (Fig. 3A).

In addition to the major energy reserves, we also measured levels of two osmoprotectants in *B. antarctica*, glucose and glycerol. Glucose levels did not change appreciably throughout the first four cycles of repeated dehydration/rehydration (Fig. 2D); however, after the fifth cycle, the glucose content of RD larvae sharply increased to 0.46 ± 0.06 μg glucose mg^{-1} DM, which was significantly higher than control larvae (0.10 ± 0.02 μg glucose mg^{-1} DM; $P < 0.05$). In the prolonged dehydration experiment, there was no difference in glucose content between PD and control larvae ($P > 0.05$; Fig. 3A). Meanwhile, glycerol levels followed a similar pattern to glucose (Fig. 2E). There were no observed differences in glycerol content until after the fifth dehydration/rehydration cycle, where glycerol levels were 2.6-fold greater in RD larvae ($P < 0.05$). As with glucose, there were no differences in glycerol between control and PD larvae ($P > 0.05$; Fig. 3A).

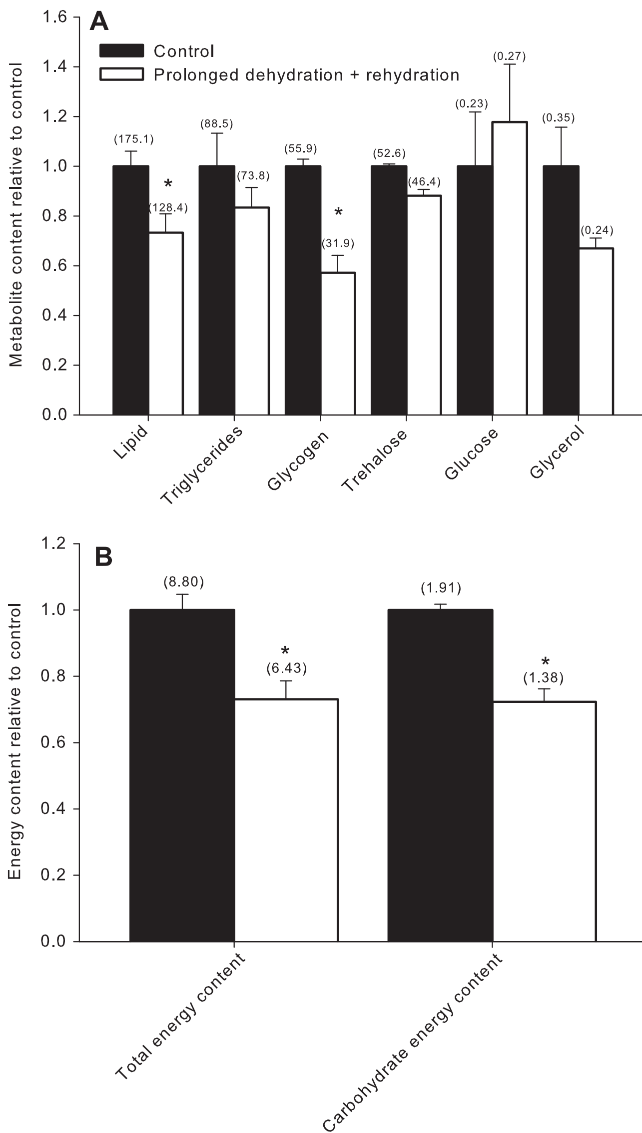

Fig. 3. Effect of prolonged dehydration on metabolite (A) and energy content (B) of larvae of *B. antarctica*. Prolonged dehydration consisted of 10 d at 99% RH followed by rehydration for 24 h on a moist paper towel. The bars represent the relative value of each parameter relative to the control; i.e. the control level is set at 1 for each metabolite or energy content. The actual measured values (mean) for each bar are included in parentheses. Units are μg mg^{-1} DM for metabolites and J mg^{-1} DM for energy content. Error bars represent the SE. For lipid and triglyceride, values are the mean of five replicates of five individuals, while for the other metabolites values are the mean of 5 replicates of 20 individuals. An "*"indicates a significant difference between control and prolonged dehydration larvae for a particular pair of values (ANOVA, FDR, $P < 0.05$).

3.4. Total energy and carbohydrate energy content

Using thermodynamic values for lipid and carbohydrate energy content, we calculated the change in energy content of larvae in response to dehydration and rehydration. In the repeated dehydration experiment, TEC was nominally lower in RD larvae than in control larvae after each cycle (Fig. 4A), ranging from 8.5 ± 0.3 to 9.6 ± 0.5 J mg^{-1} DM in control larvae and 7.3 ± 0.4 and 8.5 ± 0.3 J mg^{-1} DM in RD larvae. However, these differences were not statistically significant ($P > 0.05$). Because lipid levels, which did not change significantly, predominantly influence TEC we also calculated the CEC. Unlike TEC, there were dramatic changes in CEC (Fig. 4B); after each of the five cycles of dehydration and rehydration, the CEC was significantly lower in RD larvae ($P < 0.05$). By the fifth cycle, the CEC was 67% lower in RD larvae than in their control counterparts. In contrast to the repeated dehydration treatment, prolonged dehydration caused a significant decrease in both TEC and CEC (Fig. 3B). Following prolonged dehydration and rehydration, the TEC was 27% lower in PD larvae, and the CEC was 28% lower.

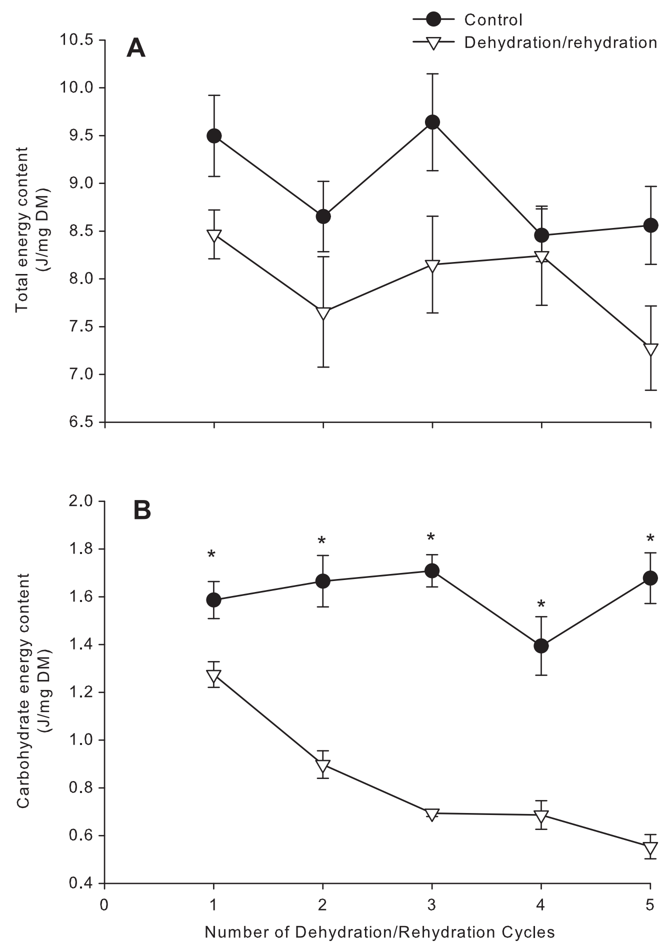

Fig. 4. Effect of repeated dehydration on the total energy content (TEC) (A) and carbohydrate energy content (CEC) (B) of larvae of *B. antarctica*. Larvae were exposed to five cycles of 24 h dehydration at 75% RH followed by 24 h rehydration on a moist paper towel. TEC and CEC were calculated according to Djawdan et al. (1998) using the following equation: TEC = 0.0393(lipid content) + 0.0176 (carbohydrate content), CEC = 0.0176(carbohydrate content), where lipid and carbohydrate content are in μg mg^{-1} DM. Values are the mean ± SE of 5 replicates. An "*"indicates a significant difference between control and repeated dehydration larvae at a particular time point (ANOVA, FDR, $P < 0.05$).

4. Discussion

While previous studies have elucidated a number of physiological responses to dehydration in larvae of *B. antarctica*, the present study is the first to address the physiological consequences of repeated dehydration in a polar insect. During the repeated dehydration trials, larvae lost 30–40% of their total body water during each 24 h exposure to 75% RH (Fig. 1A). However, the dynamics of dehydration and rehydration changed noticeably over the course of the experiment. After the first bout of dehydration/rehydration, larvae failed to fully rehydrate within 24 h, with water levels being 17% lower in RD larvae on day 2 of the experiment. With each successive cycle, larvae took in more water during rehydration, with water content in RD larvae peaking on day 8 at 3.02 ± 0.12 mg H_2O mg^{-1} DM. Also, over time, there was a trend towards lower rates of water loss; for example, between days 8 and 9, larvae lost 29% of their water, compared to a 39% loss of body water between days 2 and 3. While we did not probe the mechanisms of this reduced water loss, we suspect that changes in cuticular hydrocarbons may be responsible (Benoit et al., 2007). Ultimately, these changes resulted in larvae having significantly higher water content following dehydration on days 7 and 9 compared to days 3 and 5, when the lowest water levels were observed.

Thus, it appears these changes are adaptive as they allow larvae to maintain higher water content during each successive bout of dehydration. In general, insects can increase their desiccation resistance by either (1) increasing water content, (2) reducing water loss rates, or (3) increasing the amount of water that can be lost before death (Gibbs et al., 1997). Here, we observed evidence for both 1 and 2 in response to repeated dehydration, although a thorough study of water balance parameters is needed to substantiate these claims. Because these results were somewhat unexpected, our study was not designed to fully test the mechanisms by which the dynamics of dehydration/rehydration change in RD larvae. Also, it is worth noting that because water content is expressed as μg H_2O/mg DM, a decrease in DM over the course of the experiment due to respiration could skew the results slightly. However, because of the large variation in size in field-collected larvae, expressing water content per individual was not practical.

To test how repeated dehydration affected the osmotic balance of larvae, we measured changes in body fluid osmolality during repeated dehydration and rehydration. On the whole, osmolality of larvae closely tracked observed changes in water content (Fig 1B). After each dehydration cycle, there was a significant increase in osmolality, which is not surprising due to the concentration of solutes following evaporation of water. However, the reduction in water content alone cannot account entirely for the observed changes in osmolality. For example, between days 2 and 3, the osmolality increased by a factor of 3, while the change in water content could account for only a 1.7-fold increase in osmolality. Thus, it appears additional osmolytes are produced during dehydration, although the identity of these osmolytes is unknown. Elnitsky et al. (2008) observed similar results in response to cryoprotective dehydration, with some of the increased osmolality coming from glucose and trehalose synthesis. In the present study, we did not observe any accumulation of osmoprotectants (i.e. trehalose, glucose, and glycerol), although metabolite composition was only measured after recovery. Thus, it's possible that additional osmolytes were accumulated during dehydration but recycled into insoluble energy stores during recovery.

As expected, the experimental perturbations in water content had substantial costs. While larvae survived the first four cycles of dehydration/rehydration very well, by the fifth cycle there was significant mortality in RD larvae (Table 2). The exact cause of mortality is unclear, but it could be related to damage to midgut tissue (Table 2). Compared to control larvae, midgut cell mortality was between 2.5 and 6 times greater in RD larvae. However, midgut mortality did not increase with each successive cycle, suggesting there is some repair of dead cells during rehydration. Because of the substantial volume changes that accompany dehydration stress in *B. antarctica* (Li et al., 2009), the midgut may be particularly sensitive to dehydration since it occupies such a large proportion of the animal's total volume. On the whole, the observed differences in midgut cell survival were very similar to our previous study of repeated cold exposure in *B. antarctica* (Teets et al., 2011), suggesting that both repeated freezing and repeated dehydration cause considerable damage to the midgut.

In addition to the above effects on larval mortality and tissue damage, repeated dehydration had severe energetic consequences for larvae. While total lipid and triglyceride levels were unaffected by repeated dehydration (Fig. 2A and B), we observed a severe depletion of carbohydrate energy reserves in response to dehydration. After five cycles of dehydration/rehydration, RD larvae had 89% less glycogen and 48% less trehalose than their control counterparts (Fig. 2C and D), corresponding to a 67% loss of carbohydrate energy content (Fig. 4B). These results were analogous to our previous study on repeated cold exposure, where frozen larvae lost ~40% of their glycogen and ~25% of their trehalose (Teets et al., 2011).

The depletion of glycogen during repeated dehydration is in line with hypotheses of dehydration adaptations in other species. Glycogen is often accumulated in desiccation-resistant *Drosophila* strains, both to serve as an energy source and as a means of sequestering additional water, since glycogen can bind 3–5× its weight in water (Gibbs et al., 1997). Using these estimates, the observed breakdown of glycogen would provide an additional 120–200 μg H_2O mg^{-1} DM over the course of the study, which is roughly 4–7% of the larvae's total body water after five cycles of dehydration/rehydration. Also, since glycogen is the primary source of glucose for osmoprotectant production in insects (Storey, 1997), glycogen depletion may be in part due to osmoprotectant production during dehydration. The observed increase in glucose and glycerol after cycle 5 is one possible fate of glycogen, although these modest increases only accounted for <1% of the total glycogen depletion.

One surprising result of this study was the observed depletion of trehalose, the other major carbohydrate energy store. Trehalose is accumulated during dehydration in *B. antarctica* (Benoit et al., 2007; Elnitsky et al., 2008), and is perhaps the most commonly used solute to protect against dehydration in insects (Danks, 2000). Thus, using trehalose as an energy source for recovery would seem to be maladaptive if it generates a deficit of osmoprotectants in the next dehydration cycle. Also, as with glycogen, the accumulation of glucose and glycerol after cycle 5 represented only a fraction of the amount of trehalose lost, so conversion of trehalose to other carbohydrates does not appear to be a major factor here. Perhaps, the inability to maintain adequate trehalose content is partially responsible for mortality during the last cycle of repeated dehydration.

In addition to quantifying the energetic consequences of repeated dehydration, a secondary goal of this study was to measure the energetic costs of gradual, prolonged dehydration on larvae. Thus, the prolonged dehydration experiment was designed to assess how the duration and severity of dehydration exposure affected the energy balance of larvae. The 10 d prolonged dehydration treatment encompassed the same amount of time as five cycles of repeated dehydration (i.e. 2 d per cycle), allowing us to make energetic comparisons between the two treatments. We anticipated that prolonged, gradual dehydration would be less energetically costly than rapid dehydration, because during

gradual dehydration larvae (1) become drought-acclimated, thereby reducing the damage caused by dehydration (Hayward et al., 2007), and (2) decrease their respiration rates, presumably to conserve energy while they are dehydrated and unable to feed (Benoit et al., 2007). However, somewhat surprisingly, while prolonged dehydration at 99% RH did not deplete carbohydrate energy to the extent of repeated dehydration, prolonged dehydration did cause a significant reduction in lipid content. As a result, PD larvae had 27% less TEC than their control counterparts, while after five cycles RD larvae only had a 15% deficit in TEC, a difference that was not statistically significant. This suggests that larvae at 99% RH maintain a higher average meatabolic rate than larvae at both 75% and 100% RH. However, further experiments using identical relative humidities are needed to directly compare the energetic consequences of repeated and prolonged dehydration.

All told, the energy depletion observed in response to dehydration caused by both repeated and prolonged dehydration likely has fitness consequences for *B. antarctica.* In the mosquito *C. pipiens*, repeated bouts of dehydration cause a similar reduction in energy stores that lead to reduced egg production by adult females (Benoit et al., 2010). Similarly, in *Drosophila*, repeated bouts of cold cause a concurrent reduction in both glycogen stores and fecundity (Marshall and Sinclair, 2010). While we are unable to raise larvae to adulthood and directly measure fitness, the depletion of energy stores likely reflects a diversion of energy from growth and reproduction. This may be especially problematic for polar insects, which have an extremely short growing season during which to replenish energy stores. Also, our results have implications related to climate change on the Antarctic Peninsula. While total precipitation has increased in response to climate warming, climate change has caused localized changes in snow melt and evaporation that could result in greater fluctuations in moisture regimes in the *B. antarctica* habitat (Fox and Cooper, 1998). Thus, even though *B. antarctica* is the most dehydration-tolerant polar insect known (Hayward et al., 2007), there are dramatic energetic costs to dehydration and rehydration that likely restrict the potential habitat of *B. antarctica* in nature.

Acknowledgements

This work was supported by NSF grants ANT-0837559 and ANT-0837613. We are especially grateful for the hard work and assistance of the Palmer Station support staff during our field season. We also thank Mary Hahn for assistance in collecting data during a pilot study for these experiments. Microscopic images were generated using the instruments and services at the Campus Microscopy and Imaging Facility, The Ohio State University.

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