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ScienceDirect
Journal of Insect Physiology 53 (2007) 656–667
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

Mechanisms to reduce dehydration stress in larvae of the Antarctic midge, *Belgica antarctica*

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Received 26 January 2007; received in revised form 16 April 2007; accepted 17 April 2007

Abstract

The Antarctic midge, *Belgica antarctica*, is exposed to frequent periods of dehydration during its prolonged larval development in the cold and dry Antarctic environment. In this study, we determined the water requirements of the larvae and the mechanisms it exploits to reduce the stress of drying. Larvae lost water at an exceptionally high rate (>10%/h) and tolerated losing a high portion (>70%) of their water content. Larvae were unable to absorb water from subsaturated water vapor ($\leqslant 0.98\ a_v$) to replenish their water stores, thus this midge relies exclusively on the intake of liquid water to increase its pool of body water and maintain water balance. To reduce dehydration stress, the midge employed a variety of mechanisms. Behaviorally, the larvae suppressed water loss by clustering. In response to slow dehydration, glycerol concentration increased 2-fold and trehalose concentration increased 3-fold, responses that are known to decrease the rate of water loss and increase dehydration tolerance. No changes in the mass of cuticular lipids occurred in response to desiccation, but the observed shift to longer hydrocarbons likely contributes to reduced water loss as the larvae dehydrate. As the larvae dehydrated, their oxygen consumption rate dropped, resulting in a reduction of water loss by respiration. Lastly, one bout of slow dehydration also enhanced the larva's ability to survive subsequent dehydration, suggesting that the larvae have the capacity for drought acclimation. Thus, these hydrophilic midge larvae prevent dehydration by multiple mechanisms that collectively reduce the water loss rate and increase dehydration tolerance.

Keywords: Water balance; Chironomid; Dehydration resistance; Drought acclimation; Antarctica

1. Introduction

The habitat range of the Antarctic midge, *Belgica antarctica*, extends along the west coast of the Antarctic Peninsula with sporadic, but highly dense, populations in localized areas (Usher and Edwards, 1984; Sugg et al., 1983). During its 2-year life cycle, larvae feed on moss, terrestrial algae and other types of organic debris (Convey and Block, 1996; Sugg et al., 1983). All four larval instars are capable of overwintering. The adults emerge, mate, lay eggs and die with a 10–14 d period during the austral summer.

During most of the year, the larvae are frozen in their hibernacula, buffered at 0 to −5 °C by thick snow and ice cover (Baust and Lee, 1981; Lee et al., 2006). While frozen, the midge larvae are in vapor pressure equilibrium with the local environment, and no dehydration can occur (Holmstrup et al., 2002a, b; Lee et al., 2006). Even when not frozen, the highly permeable larvae likely are very close to vapor pressure equilibrium due to their relatively high osmolality (Lee et al., 2006; Hayward et al., 2007). Thus, with their high permeability, the midges will likely dehydrate and rehydrate in response to minor changes in the immediate habitat and continually maintain vapor pressure equilibrium, as described by Holmstrup et al. (2002a) for Collembola. Interestingly, the high permeability that allows larvae of *B. antarctica* to obtain vapor pressure equilibrium during most of the year is a severe

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0022-1910/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2007.04.006

problem during summer when periods of subfreezing temperatures, along with drought conditions, are common.

During our recent field seasons, we observed larvae of *B. antarctica* representing a range of hydration states. While most of the larvae we collected during the austral summer were fully hydrated, others were conspicuously less so. These field observations were backed by laboratory measurements showing that some of the larvae contained 30–40% less water than fully hydrated individuals. In an earlier study, we demonstrated that midge larvae tolerate a loss of up to 70% of their body water and that by slowly dehydrating they can dramatically enhance their freeze tolerance (Hayward et al., 2007). In contrast to the long-lived larvae, the short-lived adults of this species lose their body water slowly but are much less tolerant of water loss (Benoit et al., 2007).

In this study, we evaluate the water balance properties of the midge larvae and define the mechanisms it uses to prevent and reduce the stress of dehydration during the Antarctic summer. We report that as the larvae dehydrate they increase their ability to tolerate water loss and their capacity to retain their internal water pool. This is accomplished by increasing their internal concentrations of trehalose and glycerol, forming aggregations, altering the composition of their cuticular lipids, drought acclimation and reducing respiratory water loss.

2. Materials and methods

2.1. Insects

The third and fourth instars of *Belgica antarctica* Jacobs used in these experiments were collected in substrate on Cormorant Island, Torgersen Island and Bonaparte Point, near Palmer Station on Anvers Island (64°45′ S, 64°04′ W) on the Antarctic Peninsula in January 2006. In all cases larvae were held at 4 °C in their natural substrate until they were used for experimentation. Immediately before the experiments, the midges were sorted in ice-cold water. Water on the surface of the midges was gently removed by blotting individuals on a paper towel.

2.2. Experimental conditions

Midges were held at the ecologically relevant temperature of 4 °C or at 25 °C to permit comparison with previous studies on water balance (Wharton, 1985; Hadley, 1994), unless otherwise indicated. Specimens were weighed with an electrobalance (CAHN 25, Ventron Co.; Cerritos, CA; precision of 0.2 μg SD and accuracy of ±6 μg at 1 mg). Relative humidities (%RH) were generated using saturated salt solutions or a glycerol–water mixture in sealed desiccators (Winston and Bates, 1960). Dry calcium sulfate yielded near-complete dryness (1.4×10^{-4} RH, Benoit et al., 2005). Test atmospheres were measured with a hygrometer (±3.0% RH; Thomas Scientific, Philadelphia, PA).

2.3. Standard water balance characteristics

Water balance characteristics were determined according to Wharton (1985) with interpretations by Hadley (1994) and modifications from Benoit et al. (2005). To relate the water content within the larva to that of the atmosphere %RH was expressed as water vapor activity (a_v, $a_v = \%RH/100$). The water activity within the larva (a_w) is equal to 0.99 (Wharton, 1985). Thus, a water deficit occurs at all water vapor activities below saturation ($\leqslant 0.99$ a_v) unless a mechanism for water vapor uptake is present (Wharton, 1985; Hadley, 1994). Before experimentation, larvae were held at 0.98 a_v until a 4–6% loss in body mass occurred to negate the effects on water loss due to digestion, defecation and water adherence to the larval cuticle (Yoder and Denlinger, 1991). For all experiments, the mass of the larva was measured within 1 min to minimize time outside of the experimental conditions.

Dehydration tolerance was determined by placing the midges at 0.98 a_v, 0.93 a_v, 0.85 a_v, 0.75 a_v, 0.65 a_v and 0.33 a_v and weighing them hourly until the larvae could no longer recover when placed at 1.00 a_v. Midges were classified as alive if they moved in response to gentle probing after rehydration. The amount of water lost from initial mass to dehydrated mass was expressed as a percentage of the initial water mass (Benoit et al., 2005). The water mass was the difference between initial mass and the dry mass (mass after complete dehydration in 0.00 a_v at 65 °C).

When insects are held at 0.00 a_v, no water can be absorbed (Wharton, 1985). Thus, under these conditions no water can be gained, and the loss can be analyzed without interference from water in the atmosphere (Eq. (1); Wharton, 1985)

$$m_t = m_0 e^{-k_t} \text{ or } \ln(m_t/m_0) = -k_t, \quad (1)$$

where m_0 is the initial water mass, m_t is the water mass at any time t, and k_t is the amount of water lost between the measurements m_0 and m_t (Wharton, 1985). Thus, the slope of the plot of $\ln(m_t/m_0)$ vs. time is the water loss rate expressed as %/h. Water mass was determined during 30 min intervals as the differences between initial and dry mass. It is important to note that no changes occurred in the dry mass throughout the experiment, indicating that metabolic water does not contribute significantly to the water pool for larvae of *B. antarctica*.

Activation energies (E_a) and the critical transition temperatures (CTT) were used to describe water flux through the cuticle over a broad range of temperature. A barrier that is robust to temperature changes is characterized by a low E_a, and individuals that lose water uncontrollably as temperature increases will yield a greater E_a (Yoder et al., 2005). Activation energies were derived by Arrhenius analysis (ln rate vs. reciprocal absolute temperature ($1/T$)) according to Eq. (2)

$$\ln k = -E_a/(RT) + A, \quad (2)$$

where k is the water loss rate, E_a is the energy of activation, R is the gas constant, T is absolute temperature, and A is the frequency factor. A CTT is present when changes in water loss rates deviate from a standard exponential increase, thus indicating the presence of a factor that suppresses or highly increases water loss with temperature. Only living larvae were used to allow for ecologically relevant comparisons (Benoit et al., 2007).

To assess whether there is a group (clustering) effect that suppresses water loss, individuals in groups of different sizes ($N = 5$, 10, and 20) were monitored to establish their water loss rates. A small spot of white paint (Pactra, Van Nuys, CA) was used to mark individual larvae. The paint had no discernable effect on the mass changes (Benoit et al., 2005). In all cases the midge larvae used in this portion of the study were similar in size, with nearly identical percent water content, thus differences could be attributed directly to differences in the number of larvae within a group (Benoit et al., 2007; Hayward et al., 2007).

Water vapor was tested as a source of water by placing pre-weighed larvae at 1.00 a_v, 0.98 a_v, 0.93 a_v, 0.85 a_v and 0.75 a_v and then reweighing the larvae every 12 h. Maintenance of the water mass levels below saturation (<0.99 a_v) indicates that loss is countered by gains from the air, thus indicating the presence of a water vapor uptake mechanism. Additionally, to determine if dehydrated larvae can uptake water, individual midges were held at 0.75 a_v for 12 and 24 h before exposure to 0.98 a_v. An increase of water mass after a period at 0.98 a_v would indicates that dehydration may trigger water vapor uptake. The lowest a_v where water gain occurred is designated as the critical equilibrium activity (CEA; Wharton, 1985), and water balance can be maintained at this point. Liquid water uptake was analyzed by allowing the larvae to move freely on a paper towel soaked with Evans blue (10%) dyed water. After 4 h of access, the midges were dissected and examined for blue coloration in their gut.

2.4. Drought acclimation

Drought acclimation was tested according to Holmstrup et al. (2002a, b) with modifications. Individuals were placed at the dehydrating water activities of 0.98 a_v, 0.93 a_v, or 0.75 a_v for 12, 24, 36 or 48 h. After the midge larvae had been held at these conditions, the larvae were returned to 1.00 a_v for 6 h, a recovery period that allowed complete rehydration. Only midges that were responsive to stimulation by probing were used to determine water loss rates and survival at 0.00 a_v.

2.5. Osmolality of dehydrated larvae

Hemolymph osmolality was determined using a vapor pressure depression technique (Holmstrup and Sømme, 1998). Briefly, groups of 5–6 larvae were placed in a sample chamber and crushed with a Teflon rod to expose body fluids. Samples were then allowed to equilibrate for ~30 min following placement within a C-52 sample chamber (Wescor Inc., Logan, UT, USA). The osmolality of the sample was measured using a Wescor HR-33T Dew Point Microvoltmeter (Wescor Inc., Logan, UT, USA) operated in the dew point mode. For this experiment, midges (10 groups of 5–6 larvae) were dehydrated until a loss of 50% of their water content at either 0.75 a_v or 0.98 a_v and compared to individuals held at saturation (1.00 a_v).

2.6. Oxygen consumption of dehydrated larvae

Oxygen consumption was determined according to Lee and Baust (1982). Respirometers were made by sealing a 50 µl micropipette onto the tip of a 1 cc plastic syringe. Rates of O_2 consumption were determined for groups of 5 larvae that were held at 5 °C and 1.00 a_v (fully hydrated) and at different times during dehydration at 0.75 a_v. A 10% KOH solution was introduced into the pipette to absorb the CO_2. The system was equilibrated for 1 h, and then O_2 consumption was measured during the following 2 h. A control syringe with no midge larvae served as a thermobarometer.

2.7. Glycerol and trehalose analysis

The effects of desiccation on whole-body glycerol and trehalose content were determined. Two types of drying were tested. Slow dehydration was accomplished by exposing larvae to 0.98 a_v and rapid dehydration was achieved at 0.75 a_v. For both cases, concentrations were measured for fully hydrated larvae and at multiple points throughout dehydration. An enzymatic assay (Sigma Chemical Co. #377-40A) was used to determine glycerol, as described by Yoder et al. (2006). Groups of five larvae were homogenized in 25 mM sodium phosphate (pH 7.4) and centrifuged at 25 °C for 10 min at 12,000g to collect the supernatant. To deproteinize the samples, 6% perchloric acid (w/v) was added, and the precipitate was removed by centrifugation at 12,000g for 5 min. The supernatant was collected and neutralized to pH 7.0 with 5 M potassium carbonate. Spectrophotometric absorbance was used to determine the glycerol levels of each sample by comparison to standards.

Trehalose concentrations were determined by a protocol similar to Van Handel (1985). First, five larvae were homogenized in a 200 µl solution of sodium sulfate (2% w/v). The samples were then mixed with 1 ml methanol and centrifuged at 3000g for 2 min. The supernatant was removed, and the previous step was repeated with 0.5 ml methanol to ensure that all the trehalose was extracted. The samples were concentrated to 0.5, and 0.1 ml of the supernatant was combined with 0.5 ml 1 M HCl and heated at 90 °C for 7 min. After heating, 0.5 ml 1 M NaOH was added and the solution was heated as before. Anthrone reagent (150 ml DI water, 380 ml conc. sulfuric acid, 750 mg anthrone) was subsequently added to the sample to yield a total of 5 ml. Once the samples cooled to room

temperature, the absorbance of each sample was measured at 625 nm, and concentrations were determined by comparison to standards.

2.8. Cuticular lipid analysis

Cuticular lipids were quantified according to the protocol of Yoder et al. (1992). The midges ($N = 30$) were washed with a 500 µl chloroform:methanol solution (2:1, v:v) twice for 5 min. The extracts were concentrated to dryness with N_2. The lipid samples were then reconstituted in 200 µl chloroform:methanol (2:1) and passed through a silica gel column (Millipore) with hydrocarbons and other nonpolar lipids eluted with hexane and polar components with chloroform. The eluted samples were dried under N_2 at 0.00 a_v on preweighed aluminum pans. After 5 d, the lipid mass on each pan was measured and subsequently reweighed the following day to ensure the complete dryness of the lipid samples.

2.9. Gas chromatography–mass spectrometry (GC–MS)

Surface cuticular hydrocarbons from *B. antarctica* larvae were also analyzed using GC–MS . Three groups of 25 individual larvae (fully hydrated or 50% reduction in water content) were placed in a 7 ml test tube along with 200 µl hexane containing a 1 ng/µl heptadecanoic acid methyl ester (17:0, not found in *B. antarctica* surface hydrocarbon samples, as determined by preliminary results) as an internal standard. After 30 min, the hexane was removed, capped under a nitrogen stream, and stored at −20 °C until GC–MS analysis.

Hexane samples containing fatty acid methyl esters were loaded into a Finnigan Trace GC/MS and 1 µl of each sample was subjected to chromatographic analysis. The oven temperature ranged from 70 to 300 °C and the temperature increment was 8 °C/min, which produced a chromatograph of sufficient resolution to separate the peaks of all hydrocarbons observed. The column was a Restek 30 m fused silica column (I.D. 25 mm, 95% dimethyl siloxane, 5% diphenyl) with helium gas used as a carrier at a rate of 50 ml/min. After each sample was run, the oven remained at 300 °C for 7 min to clean impurities from the column.

Identification of each hydrocarbon by GC–MS was attained by comparing the spectrum of each peak with multiple chemical libraries (Xcalibur software, Thermo). Hydrocarbons identified with an SI value greater than 800 were considered identified with a reasonable degree of confidence. Peak intensities were measured by integrating the area under each peak, and these measurements were normalized to the internal standard and converted to molar percentage ratios ($\text{signal}_1/\text{MW}_1 = \text{signal}_2/\text{MW}_2$, where MW is the molecular weight) for statistical testing.

2.10. Statistics

Analysis of variance was used to compare data with multiple comparisons accomplished with Tukey's HSD. Percentages were arcsin-transformed prior to analysis, and regression lines were analyzed by testing for the equality of slopes (Sokal and Rohlf, 1981). Each experiment was replicated 5 times with 10 individuals for a total of 50 tested specimens unless noted elsewhere. Data were reported as mean ± SE unless otherwise noted.

3. Results

3.1. Water pool

The total water pool for 3rd and 4th instar larvae collected on three islands near Palmer Station is presented in Table 1. Water mass, dry mass and water content did not vary significantly for larvae of the same stage collected at

Table 1
Water balance characteristics of 3rd and 4th instar larvae of the Antarctic midge, *Belgica antarctica* collected on three different islands near Palmer Station
E_a, activation energy; CEA, critical equilibrium activity; +, does occur

Characteristic	Cormorant Island		Togersen Island		Bonapart Point	
Internal water pool	3rd instar	4th instar	3rd instar	4th instar	3rd instar	4th instar
Initial mass (mg)	0.761 ± 0.011	1.163 ± 0.082	0.782 ± 0.045	1.106 ± 0.056	0.732 ± 0.046	1.082 ± 0.063
Dry mass (mg)	0.168 ± 0.019	0.339 ± 0.072	0.194 ± 0.031	0.311 ± 0.042	0.176 ± 0.053	0.299 ± 0.045
Water mass (mg)	0.580 ± 0.021	0.844 ± 0.071	0.588 ± 0.054	0.795 ± 0.081	0.556 ± 0.041	0.783 ± 0.062
Water content (%)	76.2 ± 1.2	72.6 ± 2.5	75.3 ± 2.1	71.9 ± 2.2	75.9 ± 2.3	72.4 ± 2.7
Water loss						
Rate (%/h)	14.2 ± 0.4	12.1 ± 0.8	14.1 ± 1.3	12.3 ± 1.7	13.6 ± 1.7	12.2 ± 1.4
Loss tolerance (%)	74.5 ± 1.6	72.5 ± 2.1	75.7 ± 1.5	71.9 ± 2.2	74.6 ± 2.3	72.2 ± 2.1
E_a (kJ)	4.91 ± 0.11	4.92 ± 0.31	4.81 ± 0.21	4.96 ± 0.24	4.94 ± 0.13	4.99 ± 0.33
Water gain						
Free water uptake	+	+	+	+	+	+
CEA (a_v)	1.00	1.00	1.00	1.00	1.00	1.00

Each value represents the mean ± SE of 30 individuals. Data was compared with one-way ANOVA.

the three sites (ANOVA, $P>0.05$, $F=2.01$, d.f. $=4, 45$) but the 3rd and 4th instars were significantly different from each other (ANOVA, $P<0.05$, $F=8.64$, d.f. $=1, 98$). From the 3rd to 4th instars, the dry and water masses increased by $2\times$ and $1.5\times$, respectively, which caused percent water content to decline by 2–4% since the water mass did not increase proportionally (Table 1). Identical water mass to dry mass ratios were observed among the islands for the 3rd and 4th instar larvae, indicating that the observed differences were likely the result of overall size differences (Table 1). In all cases, dry mass correlated positively with water mass, indicating that water loss is standardized according to size (Wharton, 1985; Benoit et al., 2005).

3.2. Water uptake

The overall water content of the midges decreased when exposed to subsaturated water vapor (Fig. 1). At no point could sufficient water be absorbed from the atmosphere to counter water loss, thus the water content could not equilibrate. The only point at which the midge larvae maintained their water balance without drinking was at saturation (1.00 a_v), indicating that the CEA was greater than that within the insect (0.99 a_v). To further verify that water could not be absorbed from the atmosphere, a subset of midges were first desiccated at 0.75 a_v for 12 or 24 h and then exposed to 0.98 a_v. As before, the midges failed to increase their water content (Fig. 2). Thus, pre-desiccation did not trigger a mechanism for water vapor uptake from subsaturated air. When allowed access to a paper towel soaked in water containing Evans blue dye, the midges readily drank, as verified by the presence of blue coloration observed in dissections of the digestive tract. Thus, free water is the major source of hydration for *B. antarctica* larvae.

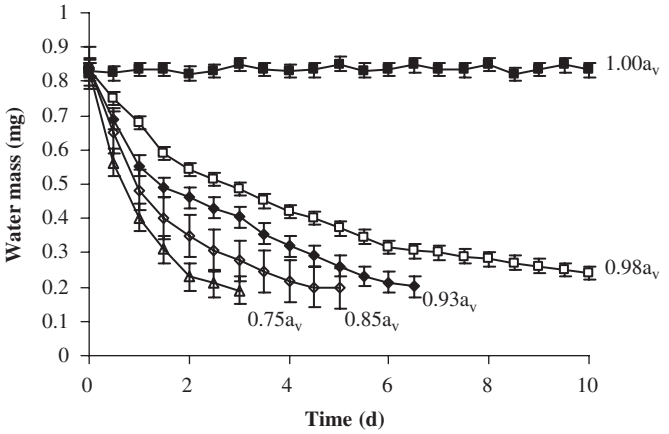

Fig. 1. Changes of water mass in 4th instar larvae of *Belgica antarctica* exposed to different relative humidities. The fact that none of the larvae other than those held in a water saturated environment (1.00 a_v) gained mass indicates that the larvae cannot absorb atmospheric water vapor. Each point represents the mean ± SE mass of 30 individuals.

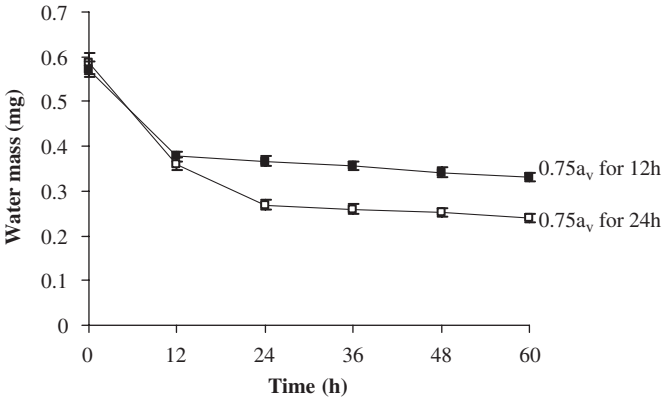

Fig. 2. Changes in water mass of 3rd instar larvae at 0.98 a_v, preceded by prior exposure to 0.75 a_v for 12 or 24 h. Each value represents the mean ± SE mass of 30 individuals.

3.3. Dehydration tolerance

Midge larvae became noticeably dehydrated after only 1 h at 0.00 a_v due to their excessively high rate of water loss (Table 1). The midges first lost their normal coloration (metallic to dull black) due to the reduction of water associated with the cuticle (at approx. 10–20% reduction in their water mass), and with further loss (20–30%) the larvae were conspicuously smaller yet they continued to move. Finally, the larvae ceased moving (40–50% water mass reduction) and continued to dehydrate until reaching a mortality point when their loss in water content exceeded 70%. To define their limit of dehydration tolerance, larvae were rehydrated using saturated water vapor (1.00 a_v), not liquid immersion, because saturated water vapor is less stressful to the larvae than liquid water (Hayward et al., 2007).

Desiccation tolerance varied when different rates of dehydration were employed to dry the larvae (Fig. 3). Between 0.00 a_v and 0.65 a_v dehydration tolerance increased by only 2% (from 72% to 74%), but the change in dehydration tolerance was more substantial between 0.65 a_v and 0.98 a_v (~5%). The difference between the most rapid rate of dehydration at 0.00 a_v and the slowest rate (0.98 a_v) represents a >6% change in their dehydration tolerance, a change that is significantly different (ANOVA, $P<0.05$, $F=1.18$, d.f. $=1, 98$). Thus, midge larvae are capable of tolerating greater water loss if they are dehydrated more slowly.

3.4. Water loss

The hourly water loss rates of the midge larvae at 0.00 a_v were 14%/h and 12%/h for 3rd and 4th instar larvae, respectively. This implies that the larvae would likely survive for only 5–6 h before succumbing to dehydration in dry (0.00 a_v) air at 4 °C (Fig. 4). This indeed was the case, with 3rd instar larvae surviving 5.3 ± 0.6 h and 4th instar larvae for 6.1 ± 0.8 h. Rapid dehydration is further accelerated with rising temperature (Fig. 5a). At 15 °C,

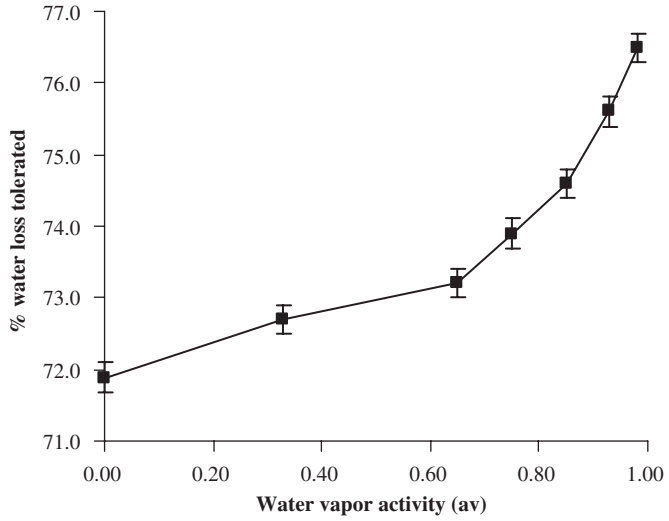

Fig. 3. Dehydration tolerance of midge larvae held at different water vapor activities. After exposure to the subsaturated water vapor activities, individuals were allowed to recover for 6 h at 1.00 a_v. Dehydration tolerance was designated as the greatest amount of water loss tolerated by the larvae. Each point represents the mean ± SE of 30 individuals compared with two-way ANOVA.

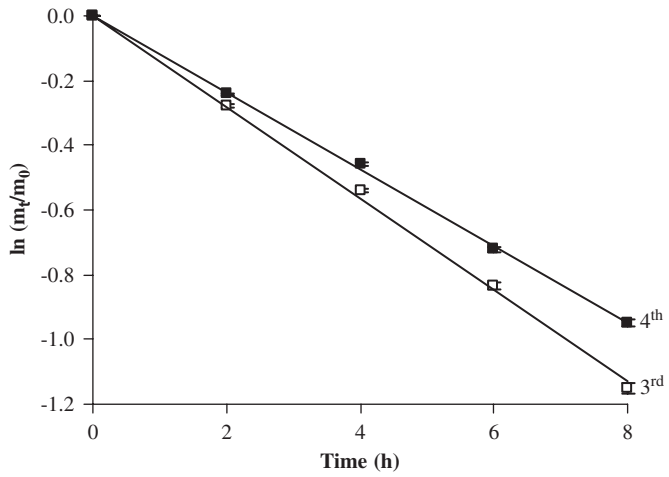

Fig. 4. Water loss rates for 3rd and 4th instar larvae at 4 °C and 0.00 a_v. The slope for the regression line through the points represents the water loss in %/h. m_t represents the mass at any time t and m_0 is the initial mass. Each point is the mean ± SE of 45 larvae compared with one-way ANOVA.

larvae survived only 2–3 h, and exposure to the same dry conditions at 25 °C prompted midge death in 1–2 h. No CTT was present and the activation energies did not change (Fig. 5b), indicating that water was lost exponentially across the temperature range, and there was no point at which cuticular permeability abruptly changed, resulting in a more rapid rate of water loss.

3.5. Group effect

When *B. antarctica* larvae were held in groups, individuals were able to retain water more effectively than

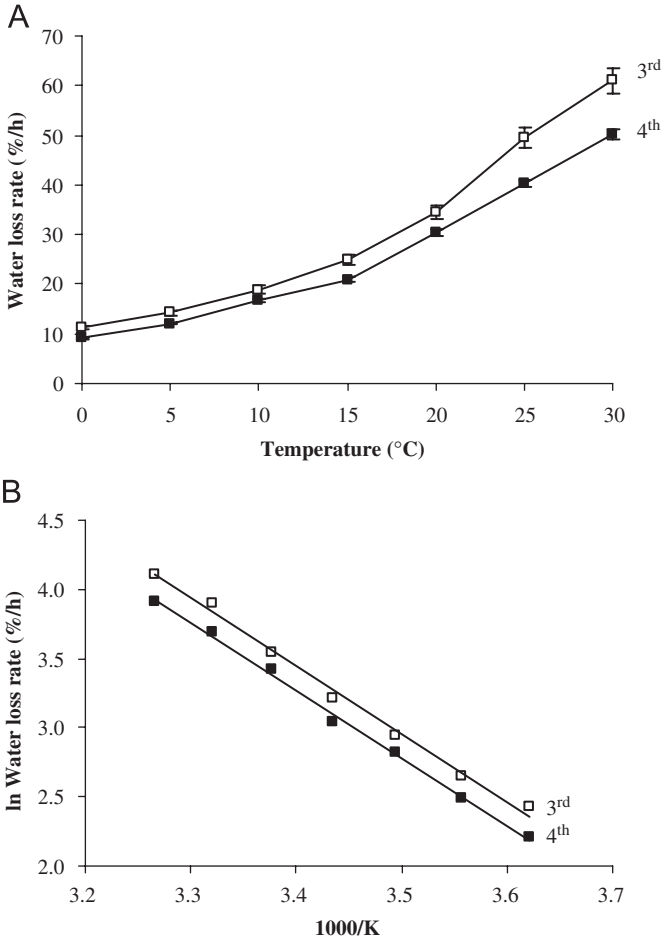

Fig. 5. Influence of temperature on the water loss rates (%/h) of 3rd and 4th instar larvae. The water loss rates were determined as in Fig. 4. (A) Water loss rates across a temperature (°C) range and (B) ln water loss rate across a temperature range (1000/°K). No critical transition temperature was observed, i.e. two statistically different regression lines (one-way ANOVA; $P < 0.05$) were not observed for either 3rd or 4th instar larvae. Each point represents the mean ± SE of 30 individuals.

solitary larvae (Table 2). The ability to reduce water loss increased with group size, as indicated by lower rates in groups of 20 than for groups of 5 individuals. Larvae formed tightly packed groups; in most cases individuals were coiled around neighboring larvae (Fig. 6). Similar effects in reducing water loss occurred at 10 and 20 °C, indicating that the clustering effect occurs at both high and low temperatures (data not shown). To diminish the effect of surface area to volume properties, larvae in groups had similar percent water content (Table 2) to those used in experiments on individuals (Table 1; ANOVA; $P > 0.05$, $F = 1.21$, d.f. = 3, 196), implying that the difference in water conservation can be attributed to clustering.

3.6. Drought acclimation

In an experiment designed to evaluate the potential of drought acclimation to enhance dehydration tolerance, 4th instar larvae were first exposed to a range of water vapor

Table 2
The effects of clustering on the percent body water content and water loss rates of 3rd and 4th instar larvae of *Belgica antarctica* exposed to 0.00 a_v

Group size	Body water (%)		Water loss rate (%/h)	
	3rd instar	4th instar	3rd instar	4th instar
1	75.9 ± 1.1^a	72.5 ± 1.2^a	14.3 ± 0.8^a	12.3 ± 1.1^a
5	75.6 ± 1.3^a	72.3 ± 1.6^a	11.4 ± 0.7^b	9.9 ± 1.0^b
10	76.2 ± 2.2^a	72.4 ± 1.7^a	9.1 ± 0.6^c	7.5 ± 0.9^c
20	75.7 ± 2.4^a	72.1 ± 1.5^a	8.7 ± 0.9^c	7.2 ± 1.2^c

Values are the mean ± SE of 15 individuals. Data within a column followed by the same letter are not statistically different (one-way ANOVA, $P > 0.05$; percentage data were arcsin-transformed prior to analysis).

Fig. 6. Larval cluster of *Belgica antarctica* observed during field collection.

activities for different times, then were fully rehydrated at 1.00 a_v before being subjected to a second bout of dehydration at 0.00 a_v (Table 3). Midges that were dehydrated slowly (0.98 a_v), moderately (0.93 a_v) or rapidly (0.75 a_v) were compared to control individuals that were not dehydrated (1.00 a_v) but were removed from the substrate. In all cases, larvae used in this experiment were allowed to recover their water pool, and they showed no other signs of dehydration. Larvae held at 1.00 a_v remained fully hydrated throughout the experiments and had water loss rates similar to those of larvae removed directly from their soil substrate (Table 3). When larvae were dehydrated slowly (both 0.98 a_v and 0.93 a_v) and then rehydrated, water loss rates decreased and survival time increased when compared to larvae held at 1.00 a_v (Table 3; ANOVA; $P < 0.05$, $F = 12.21$, d.f. = 8, 441). Dehydration of midge larvae at 0.75 a_v did not prompt a reduction in the water loss rate nor an increase in survival time in comparison to larvae held at 1.00 a_v (Table 3; ANOVA, $P > 0.05$, $F = 1.23$, d.f. = 8, 441). Thus, slow, but not fast, changes in the water pool enhanced the ability of the midge larvae to resist subsequent dehydration.

3.7. Osmolality dependent on dehydration rate

Midges examined immediately after removal from the substrate had an osmolality of 442 ± 4 m Osm Kg^{-1}. After losing nearly 50% of their water content at 0.98a_v, the osmolality nearly doubled to 872 ± 27 m Osm Kg^{-1} (ANOVA, $P < 0.05$, $F = 26.34$, d.f. = 1, 18). The rate of dehydration had a significant effect on the osmolality of midge larvae: larvae dehydrated to 50% of their water mass at 0.75 a_v, a level of water loss attained more rapidly at 0.75 a_v than at 0.98 a_v, yielded an osmolality of 700 ± 34 m Osm Kg^{-1} (ANOVA, $P < 0.05$, $F = 22.34$, d.f. = 1, 18). Thus, the rate of dehydration had an impact on osmolality: slower dehydration resulted in higher osmolality.

3.8. Effect of dehydration on polyol and sugar concentration

Concentrations of glycerol and trehalose increased when larvae were dehydrated at both 0.75 a_v and 0.98 a_v, but the increases were more pronounced in larvae dehydrated at the slower rate (0.98 a_v) (Fig. 7). Overall, glycerol concentrations increased 2-fold while trehalose increased 3-fold when larvae lost 70% of their water content by exposure to 0.98 a_v (ANOVA, $P < 0.05$, $F = 17.8$, d.f. = 7, 72). Thus, midges altered their internal polyol and sugar content in response to dehydration, and this was enhanced by slow dehydration.

3.9. Changes in cuticular lipids

During dehydration, no differences were noted in the overall quantity of cuticular lipids (data not shown), but differences in the amounts of specific components were observed. The masses of nonpolar hydrocarbons and the polar components of the cuticular lipids were not significantly different (ANOVA, $P > 0.05$, $F = 1.32$, d.f. = 5, 55) at any point during dehydration, including immediately before the midges succumbed to dehydration (data not shown). When the proportional amount of each lipid was determined, slight changes in the amounts of specific lipids were noted. In response to desiccation (0.75 a_v and 0.98 a_v for 24 h), the hydrocarbons with fewer carbons decreased while larger hydrocarbons consistently increased (Fig. 8). Thus, dehydration elicited changes in the types of lipids, favoring larger hydrocarbons, but did not affect the overall lipid mass.

Table 3
Changes in dehydration tolerance and water loss following drought acclimation at different water vapor activities for different durations in 4th instar *Belgica antarctica*

Group	Water mass (mg)			Dehydration tolerance	
	Initial	Dehydrated	Recovery	Water loss rate (%/h)	Survival (h) at 0.00a_v
1.00 a_v					
12 h	0.834±0.012[a]	0.837±0.021[a]	0.831±0.061[a]	12.5±0.7[a]	6.4±0.4[a]
24 h	0.829±0.022[a]	0.827±0.019[a]	0.825±0.051[a]	11.9±0.6[a]	6.6±0.3[a]
36 h	0.832±0.020[a]	0.833±0.021[a]	0.831±0.041[a]	12.3±0.4[a]	6.9±0.5[a]
48 h	0.827±0.031[a]	0.828±0.023[a]	0.827±0.023[a]	12.4±0.4[a]	6.7±0.4[a]
	0.833±0.029[a]	0.837±0.031[a]	0.833±0.031[a]	12.5±0.5[a]	6.8±0.6[a]
0.98 a_v					
12 h	0.843±0.025[a]	0.752±0.021[b]	0.839±0.021[a]	10.4±0.3[b]	7.2±0.3[a]
24 h	0.850±0.023[a]	0.693±0.029[c]	0.846±0.031[a]	9.1±0.4[c]	8.4±0.3[b]
36 h	0.849±0.022[a]	0.612±0.019[d]	0.843±0.033[a]	8.2±0.6[c]	9.2±0.6[b]
48 h	0.842±0.022[a]	0.561±0.023[e]	0.845±0.045[a]	9.2±0.5[c]	8.3± 0.5[b]
0.93 a_v					
12 h	0.853±0.023[a]	0.691±0.024[c]	0.849±0.021[a]	9.2±0.6[c]	8.4±0.5[b]
24 h	0.861±0.041[a]	0.579±0.031[e]	0.857±0.023[a]	9.6± 0.5[c]	7.2±0.3[a]
36 h	0.853±0.061[a]	0.496±0.025[f]	0.854±0.025[a]	9.7±0.7[c]	7.6±0.4[a]
48 h	0.849±0.031[a]	0.453±0.022[g]	0.852±0.021[a]	8.7±0.8[c]	7.6±0.3[a]
0.75 a_v					
12 h	0.859±0.041[a]	0.543±0.031[e]	0.869±0.034[a]	12.1±0.7[a]	6.2±0.4[a]
24 h	0.863±0.031[a]	0.393±0.021[g]	0.860±0.023[a]	12.4±0.6[a]	6.3±0.6[a]
36 h	0.859±0.029[a]	0.311±0.031[h]	0.858±0.024[a]	13.4±0.5[a]	6.2±0.4[a]
48 h	0.831±0.043[a]	0.253±0.025[i]	0.839±0.022[a]	13.6±0.9[a]	6.1±0.3[a]

For rehydration (recovery), the larvae were held at 1.00 a_v for 12 h. Values are the mean±SE of 50 individuals. Data within a column followed by the same letter are not statistically different (two-way ANOVA, $P>0.05$; percentage data were arcsin-transformed prior to analysis).

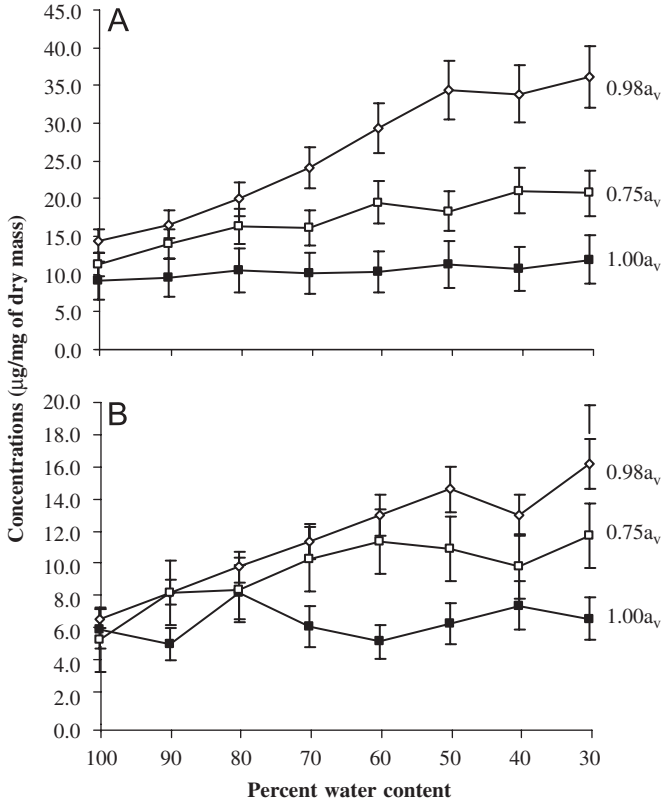

Fig. 7. Concentration of (A) trehalose and (B) glycerol in midge larvae dehydrated at 0.75 a_v and 0.98 a_v and in larvae held continually at 1.00 a_v. Mean±SE of 10 measurements compared with two-way ANOVA.

3.10. Depression of oxygen consumption

As the water content within the midges declined, the rate of O_2 consumption decreased (Fig. 9). A 25% loss in water mass corresponded to an ≈20% reduction in O_2 consumption, calculated on the basis of dry weight, which was significantly different (ANOVA, $P<0.05$, $F=10.24$, d.f. = 1, 18). The decline of oxygen consumption with water content did not occur linearly, but rather O_2 consumption declined exponentially. Thus, desiccation depressed the metabolic rate.

4. Discussion

To counter dehydration, terrestrial arthropods have evolved a number of adaptations to suppress water loss, to tolerate reductions in their internal water pool, and to uptake water (Wharton, 1985; Hadley, 1994). Methods to reduce water loss include the deposition of extra cuticular lipids (Yoder and Denlinger, 1991; Hadley, 1994; Benoit and Denlinger, 2007), modifications in cuticle structure (Hadley, 1994; Benoit et al., 2005), membrane changes (Hadley, 1994), reduction in metabolism (Hadley, 1994), elevation of osmotically active chemicals, i.e. polyols (Yoder et al., 2006), altering surface area to volume ratios (Benoit and Denlinger, 2007), and behavioral changes such as clustering (Hadley, 1994; Benoit et al., 2007). A number of insect larvae can tolerate high losses of water (Yoder

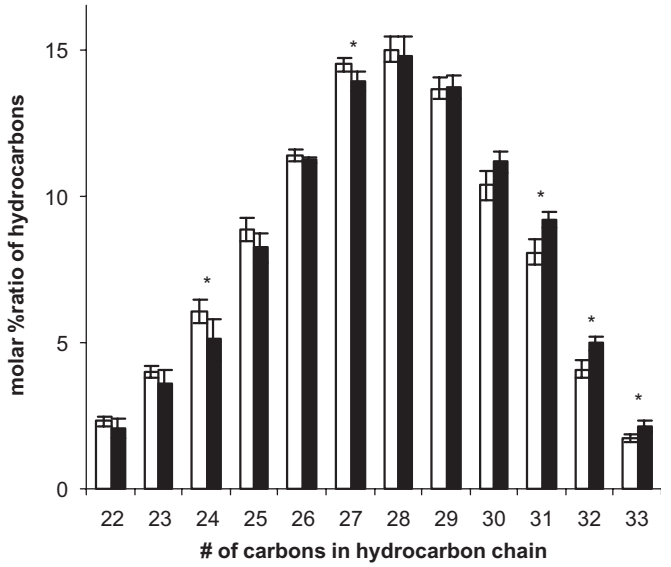

Fig. 8. Shift in the pool of surface hydrocarbons in larvae exposed to desiccation. Surface hydrocarbons removed from larvae desiccated for 6 days at 0.98 a_v (50% water loss) and untreated larvae (1.00 a_v) were examined using GC–MS. Desiccated larvae increased proportions of longer hydrocarbons (31, 32, 33 carbons) and decreased proportions of shorter hydrocarbons (27 carbons) when compared to untreated larvae (ANOVA, df = 8, $P<0.05$). Each bar represents the mean ± SD of 5 groups of 25 larvae.

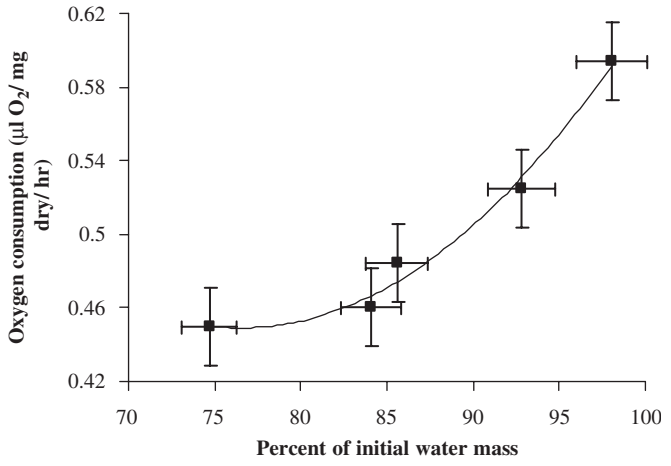

Fig. 9. Rate of oxygen consumption during dehydration of midge larvae at 0.98 a_v. Each point is the mean ± SE of oxygen consumed by groups of five midges. Each measurement was replicated five times at each level of dehydration and compared with two-way ANOVA.

and Denlinger, 1991; Hadley, 1994), a trait that is especially pronounced in chironomid larvae (Suemoto et al., 2004). Replenishing water stores is predominantly accomplished by drinking free water or from the ingestion of food, but in some cases an active water vapor uptake mechanism is present (Hadley, 1994; Bayley and Holmstrup, 1999). The exploitation of these diverse mechanisms permits terrestrial arthropods to survive a range of potentially desiccating environments.

Like many other chironomid larvae, *B. antarctica* displays a high level of dehydration tolerance. For most insects, only a 20–30% reduction in body water is tolerated before succumbing to dehydration (Hadley, 1994), but with respect to other chironomid larvae the dehydration tolerance of *B. antarctica* (70–75%) is not particularly unusual (Hadley, 1994; Suemoto et al., 2004). Like dehydration tolerance, the water content (75%) of *B. antarctica* is high when compared to most other insects, but is lower than that observed in some other chironomid larvae (Suemoto et al., 2004). Increased water content is usually associated with individuals that have high water requirements; insects that are resistant to dehydration commonly have higher fat content or a heavily waterproofed cuticle that lowers the overall water content (Wharton, 1985; Hadley, 1994). Attempts to link the amount of water loss that can be tolerated to the insect's overall water requirements has been rather unsuccessful because the amount of water required varies considerably among insects (Hadley, 1994; Benoit et al., 2005). In the case of *B. antarctica*, high water content and high dehydration tolerance permit the larvae to tolerate the loss of a considerable portion of their internal water reserves.

The water loss rates for *B. antarctica* in dry air (0.00 a_v) were quite high, but with these midges spending much of their larval stages within moist substrate (Lee et al., 2006), we would not anticipate that this species would exhibit mechanisms employed by xeric species to suppress water loss (Hadley, 1994). When the water loss rates of 3rd and 4th instar larvae were compared, it was evident that younger larvae lost water at a faster rate (nearly 2%/h faster). This decrease in water loss as a function of developmental stage is known in other insects (Hadley, 1994) and presumably is due to the decrease in surface area to volume ratio as the insect grows. No differences in water loss rates or other water balance characteristics were observed between larvae from different islands; likewise no such differences were noted for adults collected from different islands (Benoit et al., 2007). In relation to temperature, the water loss for midge larvae was greatly accelerated at 15–20 °C, relatively warm conditions for the Antarctic Peninsula, but considered relatively low in comparison to most tropical and temperate insects (Hadley, 1994). A number of Antarctic arthropods lose water rapidly at high temperatures (Worland and Block, 2003; Benoit et al., 2007), suggesting that many species in the Antarctic, including *B. antarctica*, are extremely vulnerable to increased dehydration that could occur in response to even mild temperature increases.

Larvae of *B. antarctica* use several mechanisms to counter dehydration. If dehydration occurs at a slow rate, the midge larvae accumulate trehalose and glycerol, as demonstrated in this study (Fig. 6) and as reflected in a previous study showing an overall increase in polysaccharide levels (Hayward et al., 2007). An African chironomid, *Polypedilum vanderplanki*, a species that can become anhydrobiotic, increases its internal concentration of trehalose nearly 5 ×, an elevation that allows it to tolerate extremely high levels of dehydration (Sano et al., 1999;

Kikawada et al., 2005; Goyal et al., 2005). This increase in dehydration tolerance is likely due to the replacement of water with trehalose, thus preventing dehydration-induced cellular damage (Sano et al., 1999; Goyal et al., 2005). The increase of trehalose for both *P. vanderplanki* (Kikawada et al., 2005) and *B. antarctica* larvae depends on slow dehydration, and if the dehydration occurs rapidly, the midges apparently do not have sufficient time to accumulate adequate levels of trehalose. An increase in glycerol content has been demonstrated to reduce rates of water loss (Yoder et al., 2006) and to protect membranes and proteins during dehydration (Tsvetkova and Quinn, 1994; Tang and Pikal, 2005). As with trehalose, slow dehydration of the midge larvae allowed the attainment of more glycerol than when dehydrated quickly (Fig. 7). We thus anticipate that increases in both glycerol and trehalose contribute to the desiccation tolerance of *B. antarctica*.

Along with the changes in glycerol and trehalose, Michaud et al. (2007) recently identified a number of other metabolites that changed in concentration during dehydration in larvae of *B. antarctica*. As in this study, the concentration of glycerol increased by nearly $2\times$ during dehydration, while sorbitol decreased by approximately 50%. Asparagine was the only amino acid that increased in dehydrated larvae (Michaud et al., 2007), suggesting that this molecule may serve as an osmolyte (Cohen et al., 1986). Free fatty acids also increased; this has been linked previously to desiccation stress in *Aedes aegyptii* (Sawabe and Mogi, 1999). Other changes noted by Michaud et al. (2007) during dehydration were associated with alterations in metabolic pathways. It is apparent from this study and the study by Michaud et al. (2007) that there are significant shifts in metabolites during larval dehydration.

The body fluid osmolality was altered significantly during dehydration and was further influenced by the rate of drying that the larvae encountered. Likely, a considerable portion of the osmolality change during dehydration is due to an increase in the concentration of solutes as the proportion of water within the midge decreases. In particular, the differences of $>$ 100 m Osm that occurred when the larvae were dehydrated to 50% of their original water content at 0.98 a_v, compared to achieving the same water loss at 0.75 a_v suggests that differences occur. The osmolality difference observed between 0.98 a_v and 0.75 a_v, i.e. the reduction from 872 to 700 m Osm, possibly is the result of different amounts of osmolyte sequestration under the two dehydration regimes (Baust and Edwards, 1979; Baust and Lee, 1983; Michaud et al., 2007). The net effect is that slow dehydration yields a higher osmolality than attained by fast dehydration.

Our results also show that one bout of dehydration followed by rehydration better equips the larvae to tolerate a subsequent exposure to dehydration. Although similar periods of acclimation are well known to provide protection against subsequent high and low temperature, drought acclimation is less well documented. Sjursen et al. (2001) reported such a finding in a collembolan, where exposure to dry conditions allowed enhanced protection against subsequent exposure to drying. Similar effects have also been noted in reptiles and other vertebrates (Perry et al., 1999; Lillywhite, 2004). Possibly the first bout of dehydration allows the midge to generate, accumulate or sequester protective molecules that persist during the second dehydration challenge. In conjunction, as suggested by Holmstrup et al. (2002a, b), as the amount of water declines within the larvae, membrane phosopholipid fatty acids may become more tightly packed, which will decrease membrane fluidity and thus retard water movement. Drought acclimation may be particularly beneficial to *B. antarctica* during the frequent flux between the moisture-rich and dehydrating environments that are common on the Antarctic Peninsula.

Changes in cuticular lipids likely play only a minor role in the ability of midge larvae to retain water. A fairly common mechanism used by many insects to reduce water loss is the accumulation of cuticular hydrocarbons (Yoder and Denlinger, 1991; Gibbs et al., 1998; Benoit and Denlinger, 2007), but this was not the case in *B. antarctica*. No changes were noted in the total amount of lipids nor in the polar or nonpolar portions of these samples. But, we did note a shift in the abundance of specific hydrocarbons: dehydration prompted a shift toward hydrocarbons with a higher carbon number. A shift to longer carbon chains is frequently associated with enhanced desiccation tolerance in terrestrial arthropods (Hadley, 1994), and this may be the case for the larvae of *B. antarctica*. In most cases, the change occurs in response to an increase in temperature, a change that decreases cuticular permeability (Toolson, 1982; Hadley, 1985). In our study, the hydrocarbon alteration was due to dehydration, but without testing the effects of temperature we cannot rule out the possibility that a temperature-induced hydrocarbon shift also occurs in *B. antarctica*. Likely, the increase in hydrocarbon length allows for tighter packing of the cuticular wax layer, thus reducing the rate of water loss (Hadley, 1994).

When midges were placed in groups, individuals quickly formed tightly packed aggregations. We observed this aggregation behavior in both the laboratory and, particularly in dry microhabitats, in the field. Thus, the reduced water loss rates observed in groups are unlikely to be a laboratory artifact. Two ways that the midge reduces water loss by clustering is that individuals in direct contact have reduced their overall surface area to volume ratios and/or clustering generates a protective boundary layer which acts to increase the water vapor activity (Hadley, 1994; Benoit et al., 2005). Regardless of the mechanism, aggregation effectively reduces larval rates of water loss.

As midge larvae dehydrate their rate of oxygen consumption decreases, a response that is expected to decrease respiratory water loss (Hadley, 1994). The reduction in oxygen consumption could possibly represent a shift from aerobic to anaerobic metabolism, a shift known to generate glycerol and trehalose as by-products of glycolysis (Storey, 2004). Although such a shift could

indeed account for the elevation of glycerol and trehalose noted in the midge, this possibility has not been experimentally tested. A reduction in metabolism associated with dehydration has also been demonstrated in another chironomid larva, *Chironomus dorsalis* (Buck, 1965). The only contrast between the response of *B. antarctica* in this study and *C. dorsalis* is that the reduction in respiration for *B. antarctica* occurs with minimal dehydration, but for *C. dorsalis* a loss of nearly 50% of its water content is required before eliciting a significant change in metabolism (Buck, 1965). Thus, a dehydration-induced reduction in metabolism may be a common mechanism used by midge larvae to reduce respiratory water loss.

Heat shock proteins (Hsps) have been implicated in response to both dehydration (Tammariello et al., 1999) and rehydration (Hayward et al., 2004), but in *B. antarctica* the larvae continually express the genes encoding Hsps (Rinehart et al., 2006). Though Hsps may contribute to enhanced stress resistance overall, there is no evidence that the expression levels of these Hsps are altered by desiccation (Hayward et al., 2007). However, the fact that they are continually present in abundance suggests that they are available to maintain or protect cellular function when the midge is in the dehydrated state.

From this study, it is evident that *B. antarctica* employs a variety of mechanisms to retard and tolerate dehydration. This is especially important due to the frequent, rapid and extreme, environmental changes the midge confronts in Antarctica. Also, it is clear that desiccation can generate tolerance to other environmental stresses. This is particularly evident for freeze tolerance, as desiccation dramatically increases freeze tolerance in *B. antarctica* (Hayward et al., 2007). Whereas fully hydrated larvae can survive only briefly (hours) at −10 °C, dehydrated larvae readily survive for several days at the same low temperature. Drought acclimation may also increase cold tolerance of fully hydrated midge larvae, as it does in Collembola (Bayley et al., 2001), but this has not yet been tested.

Recently, we described the water balance characteristics of both female and male adults of *B. antarctica* (Benoit et al., 2007). Comparatively, there are significant differences in the water balance between adults and larvae: adults have a 10–15% reduction in water content, a significant reduction in body size, a 2–4× reduction in water loss rate, and a lower dehydration tolerance (Table 4). Such dramatic changes are commonly seen during metamorphosis (Hadley, 1994; Yoder and Denlinger, 1991): larvae, although usually more permeable to water, can tolerate a significantly higher loss of their water pool than adults. Interestingly, in most cases, the adults have significantly lower water requirements than the larvae, as indicated by their survival time at 0.00 a_v (Yoder and Denlinger, 1991; Hadley, 1994; Benoit and Denlinger, 2007). However, larvae of *B. antarctica* are more resistant than adult males and nearly as resistant as females to dehydration due to the larva's capacity to lose a high proportion of their water mass. This difference likely reflects the fact that these larvae survive >2 years and during this time may be exposed to prolonged periods of dehydration, while the adults live for only 1–2 weeks. Thus, the long life of the larvae may necessitate a suite of responses to counter the persistent and wide range of hydric states they encounter throughout the austral seasons.

Table 4
Comparison between the water balance characteristics for larvae and adults of *Belgica antarctica*. WLR, water loss rate at 0.00 a_v

Characteristics	Larvae		Adults	
	3rd instar	4th instar	Male	Female
WLR (%/h)	14.2±0.4	12.1±0.8	7.09±0.08	3.42±0.10
Loss tolerance (%)	74.5±1.6	72.5±2.1	34.4±1.0	32.4±1.0
Survival at 0.00 a_v (h)	5.34±0.61	6.09±0.81	4.78±0.71	9.21±1.23
Body size (mg)	0.76±0.01	1.16±0.08	0.31±0.01	0.69±0.01
Water content (%)	76.2±1.2	72.6±2.5	64.5±1.1	60.9±1.0

Results for the water requirements of the adults are from Benoit et al. (2007).

Acknowledgments

Research was funded by NSF grants OPP-0337656 and OPP-0413786. The support of the Palmer Station staff is greatly appreciated.

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