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ORIGINAL PAPER

Wet hibernacula promote inoculative freezing and limit the potential for cryoprotective dehydration in the Antarctic midge, *Belgica antarctica*

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Abstract The terrestrial midge, *Belgica antarctica*, occupies a diverse range of microhabitats along the Antarctic Peninsula. Although overwintering larvae have the physiological potential to survive by freezing or cryoprotective dehydration, use of the latter strategy may be constrained by inoculative freezing within hibernacula. To investigate the influence of microhabitat type on larval overwintering, we selected four substrate types that differed markedly in their composition and hydric characteristics. Organic content of these substrates ranged from 14 to 89 %. High organic content was associated with higher values for saturation moisture content (up to 2.0 H_2O g^{-1} dry mass) as well as elevated levels of field moisture content. Seasonal values of field moisture content remained near or above the saturation moisture value for each microhabitat type, and when larvae were cooled in substrates rehydrated to field-based levels, they were unable to avoid inoculation by environmental ice, regardless of substrate type. Consequently, our data suggest that wet hibernacula would force most larvae to overwinter in a frozen state. Yet, dehydrated larvae were collected in April during the seasonal transition to winter suggesting that spatial and temporal variations in precipitation and microhabitat conditions may expose larvae to dehydration and promote larval overwintering in a cryoprotectively dehydrated state.

Keywords Antarctic Peninsula · Cryoprotective dehydration · Freeze tolerance · Inoculative freezing · Microenvironments · Overwintering

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Introduction

On a macroscale, the terrestrial environments of Antarctica are often described as deserts (Kennedy 1993). However, the maritime Antarctic receives a considerable amount of precipitation due to the oceanic influence and westerly airflow through the Drake Passage (Peck et al. 2006). Along the western coast of the Antarctic Peninsula, terrestrial invertebrates occupy a diverse range of habitats that vary in water availability depending on substrate type, exposure to wind and sun, slope, vegetation, drainage, and precipitation (Kennedy 1993). Owing to their small size, and correspondingly high surface area to volume ratio, arthropods and other invertebrates are particularly susceptible to dehydration (Hadley 1994). Thus, understanding the ecology and physiology of Antarctic soil invertebrates requires characterization of hydric conditions at the microscale level.

Hydric characteristics within microhabitats also influence the survival of terrestrial ectotherms during winter by affecting the likelihood of inoculative freezing due to contact with environmental ice (Lee and Costanzo 1998). Since most species are freeze intolerant, winter survival

depends on the avoidance of internal ice formation by supercooling. Among Antarctic arthropods, the mites, *Alaskozetes antarcticus* and *Halozetes belgicae*, are particularly susceptible to inoculative freezing, and their mortality rates greatly increase when cooled in contact with ice (Convey and Worland 2000a). Although some species are capable of resisting inoculative freezing (e.g., the springtail, *Cryptopygus antarcticus*; Convey and Worland 2000a), in general, dry microhabitats that are less likely to induce inoculation are better for the survival of freeze-intolerant organisms (e.g., Forge and MacGuidwin 1992).

In contrast, a few species are freeze tolerant, and their survival often depends on ice nucleation occurring at a relatively high subzero temperature (Lee and Costanzo 1998). Although some of these organisms physiologically increase their supercooling point (SCP; the temperature at which spontaneous ice formation occurs; e.g., Mugnano et al. 1996), others rely on inoculation from environmental ice within their hibernaculum. For example, extreme freeze tolerance of larval *Chymomyza costata* to temperatures as low as −80 °C requires inoculative freezing at high subzero temperatures (i.e., −2 °C; Shimada and Riihimaa 1988). Similarly, freezing by inoculation promotes survival of the wood frog, *Rana sylvatica* (Layne et al. 1990) and Antarctic soil nematodes (Convey and Worland 2000b).

The potential for hibernacula to induce inoculative freezing varies markedly depending on the type and composition of their substrate. The addition of clayey soil and/or peat to sandy soil decreased the proportion of individuals that froze inoculatively in adults of Colorado potato beetles, *Leptinotarsa decemlineata,* even when tested at the same moisture content (Costanzo et al. 1997). It was speculated that the experimental addition of clayey soil and/or peat to sandy soil reduced ice content, thereby decreasing the chance of direct contact between the organism and environmental ice. Similarly, in hatchlings of the painted turtle, *Chrysemys picta*, the likelihood of inoculative freezing was decreased by adding clay or peat to their native nest substrate (Costanzo et al. 1998). In fact, the hydric and substrate characteristics of the hibernaculum are the primary determinants of whether hatchlings overwinter in a frozen or a supercooled state (Costanzo et al. 1998, 2001).

As the southernmost, free-living insect, the terrestrial midge, *Belgica antarctica* Jacobs (Diptera: Chironomidae), is locally abundant along the west coast of the Antarctic Peninsula. During its 2-year life cycle, this species undergoes four larval stages, with pupation and synchronized emergence of apterous adults occurring in early summer (Sugg et al. 1983). Overwintering larvae are encased in a frozen matrix of substrate for 8–9 months. Physiologically, winter-acclimatized larvae can survive prolonged subzero exposure in a frozen state (Baust and Lee 1987). Alternatively, larvae may avoid freezing by supercooling because their SCPs are seasonally depressed to ∼−15 °C, a value below the reported minima for their microhabitat temperatures (∼−10 °C; Kawarasaki et al. 2014). Prolonged maintenance of the supercooled state in the presence of environmental ice inevitably leads to loss of body water, resulting in cryoprotective dehydration (Holmstrup et al. 2010). Therefore, larvae may overwinter either by freezing or by cryoprotective dehydration if inoculative freezing is avoided. A previous study demonstrated that a relatively dry substrate allowed many larvae to avoid inoculative freezing and become cryoprotectively dehydrated (Elnitsky et al. 2008). However, that study used summer-acclimated larvae and tested only one type of substrate.

Consequently, the present study investigated the likelihood of larval inoculative freezing in different types of hibernacula to infer whether winter-acclimatized larvae overwinter in a cryoprotectively dehydrated or a frozen state in the field. Larvae occupy a wide range of microhabitats comprised of various types of substrates, including moss detritus and sandy soil. The heterogeneity in physicochemical characteristics among larval microhabitat sites is further diversified by their sporadic associations with the terrestrial alga *Prasiola crispa*, the grass *Deschampsia antarctica*, and/or penguin and seal rockeries. We selected four substrate types representing the diverse range of larval microhabitats. Changes in field moisture content were monitored to determine natural hydration levels for each type during the seasonal transition to winter conditions. Based on these field measurements, we determined hydric characteristics for each substrate type and its potential to induce inoculative freezing of winter-acclimatized larvae. Additionally, we conducted field-based surveys of larval body water content to determine whether larvae dehydrate under natural conditions.

Materials and methods

Source of insects for inoculation trials

Substrate containing larvae of *B. antarctica* was collected on Humble, Christine, and Cormorant Islands near Palmer Station on the Antarctic Peninsula (64°46′S, 64°04′W) in early February 2011. After collection, larvae in their native substrates were combined and placed in an open container in a site behind Palmer Station until May 10, 2011 (duration of ∼3 months). The site was carefully chosen to avoid artificial lights from the station that could potentially influence larval activity. This field acclimatization allowed larvae to experience seasonal changes in the environmental conditions that likely promoted seasonal cold-hardening.

Table 1 Substrate characteristics of different microhabitats of *B. antarctica*

Variable	Site			
	A	B	C	D
General description	Moss detritus	Gravelly soil with decomposing algae	Sandy soil	Sandy soil with organic material
Saturation moisture content (g H_2O g^{-1} DM)	N/A*	1.2 ± 0.1^a	0.6 ± 0.1^b	2.0 ± 0.1^c
Organic content (%)†	88.8 ± 0.6^a	44.4 ± 1.8^b	14.0 ± 1.0^c	41.5 ± 0.9^b
Water potential (kPa)				
1.4 g H_2O g^{-1} DM	N/A*	-63 ± 28	–	>− 60
0.6 g H_2O g^{-1} DM	N/A*	-267 ± 60	~0	-117 ± 70
0.3 g H_2O g^{-1} DM	N/A*	–	-231 ± 57	–
0.15 g H_2O g^{-1} DM	N/A*	–	-817 ± 322	–

Different letters indicate significant differences between microhabitat sites (Bonferroni, family-wise $P < 0.05$)

*An attempt to rehydrate this substrate to ecologically relevant levels after complete drying at 65 °C was not successful, and thus, this substrate was excluded from these analyses

† Percent of DM Values are mean ± SEM

Following acclimatization, the container was brought into the laboratory and quickly thawed before extracting larvae into ice-cold water via a modified Berlese method. Subsequently, larvae were handpicked and held on moist filter paper at 0 °C for 12–24 h to ensure clearance of the gut (mean gut clearance ~6 h; Baust and Edwards 1979).

Seasonal changes in field moisture content of different microhabitat substrates

During the sampling period from February 3 to April 11, 2011, substrates from four different microhabitat sites were collected semi-weekly to determine changes in field moisture content. Substrate samples (~50 ml each) were collected in watertight 50-ml conical tubes and kept at 2 °C until analyzed. Any materials >~3 mm were quickly removed from the sample, and the moisture content ($N = 3$) was determined gravimetrically as the difference between fresh mass (FM; to the nearest 1 mg) and dry mass (DM) after drying to constant mass at 65 °C. Values were expressed as g H_2O g^{-1} DM.

Hydric characterization of substrates

All measurements were conducted on pooled, field-collected samples dried to constant mass at 65 °C. Saturation moisture content (i.e., water-holding capacity) of each substrate ($N = 4$ per substrate) was determined as the amount of deionized water absorbed and held against gravity. Organic content ($N = 4$) was determined gravimetrically as the difference between DM and the mass of residue remaining after incinerating the samples at 550 °C for 21 h. The water potential of each substrate was determined after dried samples were rehydrated to ecologically relevant hydration levels. The ranges of hydration levels were determined based on the field moisture content and were specific to each substrate type (Table 1). Measurements ($N = 4$) were made using C-52 sample chambers and a Wescor HR-33T Dew Point Microvoltmeter (Wescor Inc., Logan, UT, USA) operated in the dew point depression mode.

Protocol for inoculation trials

After drying to constant mass at 65 °C, each type of field-collected substrates was pooled and subsequently rehydrated to ecologically relevant hydration levels. High and low levels of moisture content were chosen for each substrate type based on the range of values from field measurements. Larvae were placed individually ($N = 17$–20 for each treatment) in caps of 15-ml conical tubes with ~2 ml of the appropriate substrate. Caps were then covered with parafilm to prevent the evaporative loss of water. Following overnight equilibration at 0 °C, parafilm was quickly removed, and a crushed piece of ice was added to the substrate. After a new piece of parafilm was applied, each sample was transferred to a cold bath at −1.5 °C. This procedure was performed as quickly as possible in an environmental chamber at 2 °C to minimize melting of the ice. The addition of ice ensured that the substrate froze near its melting point. Following ~12-h equilibration at −1.5 °C, temperatures of refrigerated baths were lowered incrementally (~0.5 °C day^{-1}) to −3 °C and held for 5 days.

Evaluation of larval inoculative freezing

Since we could not determine directly whether larvae froze by inoculation, we used larval water content as an indirect measure (Elnitsky et al. 2008). Cryoprotective dehydration occurs due to the vapor pressure gradient between supercooled body fluids and the environmental ice; this gradient is terminated once internal ice formation is induced by inoculative freezing (Holmstrup et al. 2010). Consequently, larvae with a lower water content than control values likely avoided inoculative freezing and became cryoprotectively dehydrated. In contrast, larvae that remained fully hydrated were assumed to have frozen. The determination of larval body water content was performed within 10 min of thawing the substrate. Control larvae were held at 0 °C on moist filter paper for 24 h before their water content was measured. Water content of individual larvae was determined gravimetrically from measurements of FM (to the nearest 0.002 mg) and DM after drying to constant mass at 65 °C.

Statistical analysis

Differences in mean saturation moisture content and organic content for each substrate type were analyzed using the nonparametric, permutation test (Edgington 1995); Bonferroni's post hoc test distinguished means between each type. Differences in mean larval body water content, which were used as an indirect measure of whether larvae froze inoculatively, were compared between control versus experimental groups using the Kruskal–Wallis test. Lastly, mean body water content of field-collected larvae was compared to that of fully hydrated, control larvae collected on the same day using the Wilcoxon's rank-sum test. All statistical analyses were performed using R (R Development Core Group 2013) with the lmPerm package (Wheeler 2010); statistical significance was set at $\alpha < 0.05$.

Results

Physical description of larval microhabitats

Larvae of *B. antarctica* were found in a diverse range of microhabitats, from which we selected four substrate types representing this spectrum (Table 1). Site A on Humble Island was composed of a layer (depth of 3–5 cm) of decaying mass. Generally, larvae were most abundant at the surface of this decaying moss layer, which was covered by a carpet of living moss or large rocks (diameter >20 cm). On Cormorant Island (Site B), larvae were found beneath a thin layer of decomposing terrestrial algae and within a thin layer (depth of <2 cm) of sandy soil containing larger gravel (diameter 3–10 mm). Relatively large (diameter ~10 cm) rocks overlaid this site, providing insulation from extreme cold and evaporative water loss.

Sites C and D were located within a few meters of each other on Christine Island. These sites had a thick layer (depth >5 cm) of sandy soil with relatively little gravel. Larvae were most abundant at the surface of the sandy soil beneath large rocks (diameter > 20 cm). However, on some occasions, larvae were found at a depth of 1–3 cm, whereas the substrate of Site C was composed primarily of sandy soil, at Site D organic material, including shed seal hair and/or plant material, strongly augmented the substrate.

Seasonal changes in moisture content and characterization of microhabitat substrates

During the sampling period from February 3 to April 11, 2011, precipitation as rain or snow occurred on 33 days (total of 11.8 cm (melted): University of Wisconsin-Madison Antarctic Meteorological Research Center 2013). Mean daily air temperatures gradually decreased during this period from ~+5 to −4 °C (minimum = −6.8 °C on April 10, 2011; University of Wisconsin-Madison Antarctic Meteorological Research Center 2013), and the ground began to freeze in late March. In general, the substrate under large rocks, where larvae were most abundant, was initially protected from extreme cold and froze later in the autumn compared with exposed surface areas.

Field moisture contents of each substrate type exhibited distinctly different patterns of fluctuation (Fig. 1). The moisture content of moss detritus at Site A was relatively constant at ~3.5 g H_2O g^{-1} DM (range 2.5 ± 0.3 to 4.1 ± 0.1 g H_2O g^{-1} DM). By contrast, moisture levels were highly variable in gravelly soil with decomposing algae (Site B), ranging from 1.4 ± 0.2 to 7.4 ± 0.1 g H_2O g^{-1} DM. Field moisture content differed considerably within different microhabitats on Christine Island (Fig. 1). Whereas Site C was generally characterized by relatively low moisture content (range 0.5 ± 0.1–2.1 ± 0.1 g H_2O g^{-1} DM), Site D values fluctuated widely, ranging from 1.1 ± 0.1–4.2 ± 0.2 g H_2O g^{-1} DM.

At Sites B, C, and D, hydration levels observed in the field often exceeded the saturation moisture content of the substrate (Fig. 1; Table 1). Notably, although the substrate at Site B was fully saturated at 1.2 ± 0.1 g H_2O g^{-1} DM, it had field moisture contents that were consistently higher than this value, reaching as much as 7.4 g H_2O g^{-1} DM. When this highest hydration level was recorded on March 10, 2011, the microhabitat site was inundated by water, and larvae were found in a stagnant pool. We attempted to measure the saturation moisture content of substrate from

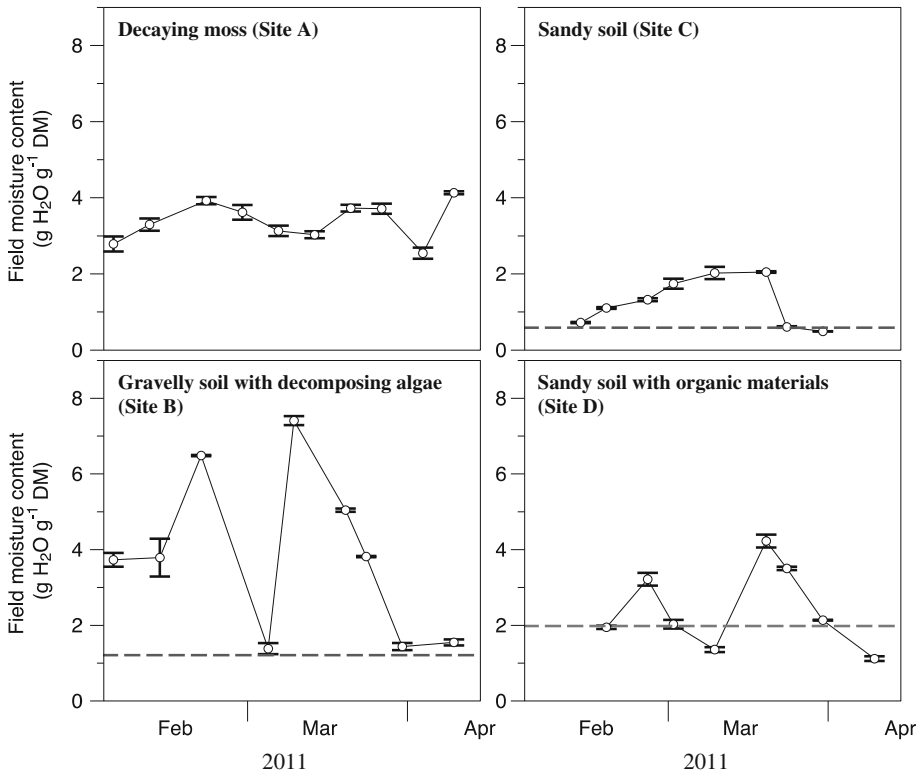

Fig. 1 Seasonal changes in moisture content of different larval microhabitats. Values are mean ± SEM. ($N = 3$). *Dashed lines* indicate the moisture content of saturated substrate from each site. Because an attempt to rehydrate the substrate from Site A after drying at 65 °C was not successful, the saturation content could not be determined

Site A. This substrate appeared relatively dry when the field moisture content of 2.5 ± 0.3 g H_2O g^{-1} DM was observed on April 4; however, after complete drying at 65 °C and then being rehydrated in the laboratory, it became fully saturated at only ~1.0 g H_2O g^{-1}. Thus, we concluded that rehydration of this substrate did not simulate natural conditions and excluded it from subsequent analyses.

Substrates differed markedly in their organic content (Table 1). Site A, composed primarily of decaying moss, had the highest organic content (88.8 ± 0.6 % of DM). Intermediate values of 44.4 ± 1.8 and 41.5 ± 0.9 % were observed in substrates from Sites B and D, respectively. Despite being only a few meters away from Site D, the organic content in substrates from Site C was only 14.0 ± 1.0 %.

To determine whether substrates hydrated to ecologically relevant levels would create dehydrating conditions for larvae, we determined the water potential of each substrate. Water potential describes the energy state of water in a given system, and the difference in these values dictates the net direction of water movement between the microhabitat and permeable organisms (Shoemaker et al. 1992). Water potential is strongly influenced by substrate type and composition; thus, even at the same hydration level, different substrates may produce distinctly different values (Saxton and Rawls 2006). Accordingly, when microhabitat substrates of *B. antarctica* were rehydrated to 0.6 g H_2O g^{-1} DM, Sites B and D produced lower values for water potential (~−60 kPa) compared with Site C (Table 1). Nonetheless, it should be noted that the water potential for all substrates rehydrated to ecologically relevant levels was relatively high (0 kPa produced by pure water is the highest possible value for this measurement), producing relatively moist condition within these substrates.

Effect of hydration level and substrate type on inoculative freezing of larvae

To determine the effect of substrate type on the potential for inoculative freezing of larvae, we exposed larvae to subzero temperatures in substrates from Sites B, C, and D that were rehydrated to ecologically relevant levels (Fig. 2). Because prolonged larval supercooling in the presence of environmental ice results in the loss of body water (i.e., cryoprotective dehydration), a decrease in larval body water content to ~1.80 mg H_2O mg^{-1} DM after equilibration at −3 °C would indicate avoidance of inoculative freezing (Elnitsky et al. 2008). Irrespective of low or high moisture content or substrate type, larval body water content after cold exposure did not differ from control larvae that were fully hydrated (mean of control: 2.40 ± 0.03 mg H_2O mg^{-1} DM; Kruskal–Wallis, $H = 4.158$, $P = 0.655$; Fig. 2). These data suggest that larvae could not avoid inoculation from environmental ice under

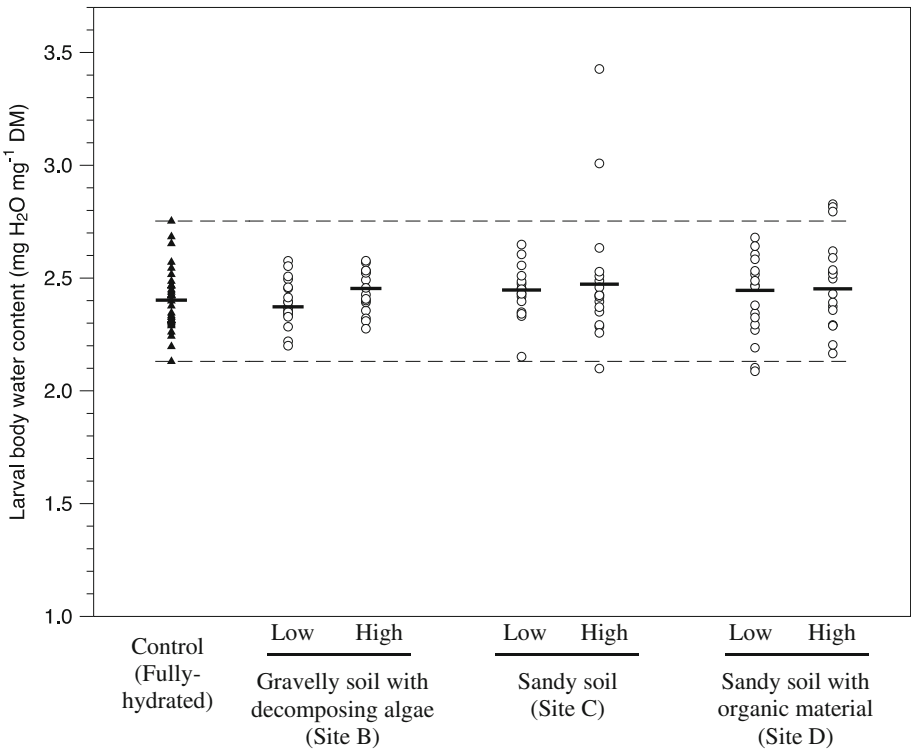

Fig. 2 Effect of exposure to subzero temperature in substrates rehydrated to ecologically relevant levels on body water content of *B. antarctica* larvae. Each *dot* represents the water content of an individual larva. *Dashed lines* indicate the range of control values for fully hydrated larvae ($N = 30$) held for 24 h on moist filter paper at 0 °C collected on the same day and held for 24 h on moist filter paper at 0 °C prior to water content determination. *Solid lines* represent the mean. Body water content of larvae exposed to −3 °C for 5 days was used as an indirect measure of whether larvae froze inoculatively. Larvae that maintained full hydration were assumed to have frozen, whereas those with a lower water content likely avoided inoculative freezing and became cryoprotectively dehydrated. The potential of each substrate type to induce inoculative freezing was tested at "Low" and "High" moisture contents based on the range of values from field measurements. Specifically, the substrate from Site B was rehydrated to 1.4 and 2.5 g H_2O g^{-1} DM; Site C to 0.6 and 1.4 g H_2O g^{-1} DM; Site D to 1.4 and 1.8 g H_2O g^{-1} DM

these conditions and froze soon after temperatures dropped below their hemolymph melting points.

Field observation of dehydrated larvae

Although field-collected larvae generally appeared to be fully hydrated, we observed dehydrated larvae on Christine Island on April 4, 2012 (Fig. 3). During the preceding month, the ground had begun to freeze. However, on this particular day, larval microhabitats under large rocks (diameter > 20 cm) were only partially frozen; a thin (<1 cm), unfrozen layer of sandy soil augmented with shed seal hair/plant material covered the underlying frozen layers. Within the general area (3 m × 3 m), most microhabitats were wet. However, substrates under some rocks appeared exceptionally dry, and larvae found in these sites were actively wiggling in a loose, flaky layer of substrate. After rapid transfer to the laboratory, we determined the body water content of each of larva ($N = 17$). Compared to control larvae that were collected on the same day and held for 24 h on moist filter paper at 0 °C (mean body water content 2.66 ± 0.05 mg H_2O mg^{-1} DM; range 2.36–3.15 mg H_2O mg^{-1} DM; $N = 14$), hydration states of field-collected larvae were significantly lower (mean 2.16 ± 0.07 mg H_2O mg^{-1} DM; Wilcoxon, $W = 222$, $P < 0.0001$; Fig. 3), with some individuals having a body water content as low as 1.52 mg H_2O mg^{-1} DM.

Discussion

Each year, larvae of *B. antarctica* are encased in a matrix of frozen substrate for 8–9 months during the winter (Baust and Lee 1981; Elnitsky et al. 2008). Although ambient air temperature reaches winter lows of −40 °C, thermal environments within larval hibernacula are buffered by the oceanic influence and accumulated snow and remain relatively stable between −2 and −10 °C (Elnitsky et al. 2008; Kawarasaki et al. 2014). Under idealized laboratory conditions, we demonstrated previously that larvae had the

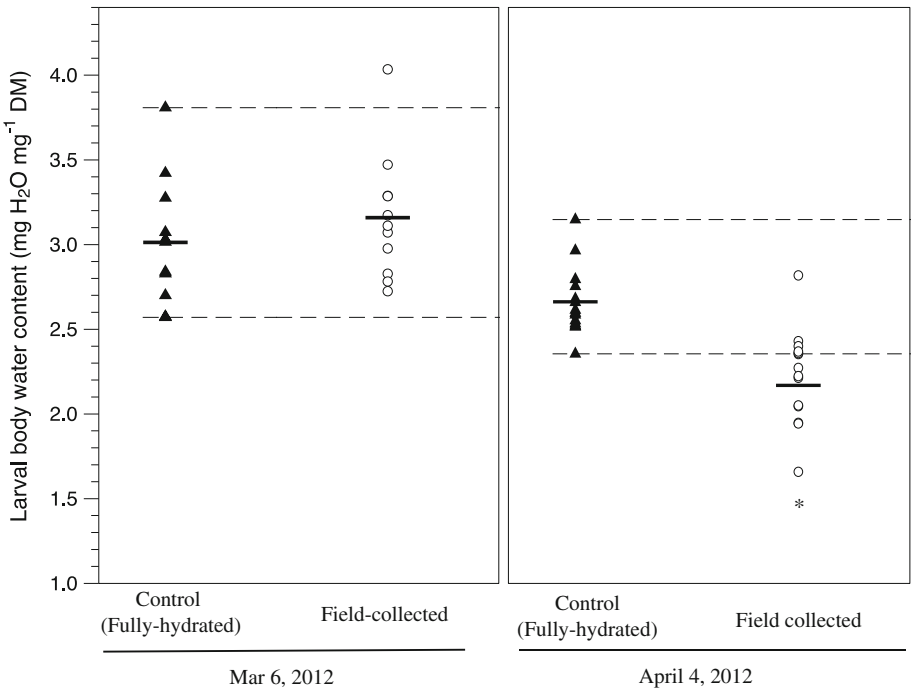

Fig. 3 Body water content of larvae field collected from a microhabitat site on Christine Island. Each *dot* represents the water content of an individual larva. *Dashed lines* indicate the range of control values for fully hydrated larvae ($N = 10$–14) collected on the same day and held for 24 h on moist filter paper at 0 °C prior to water content determination. *Solid lines* represent the mean. Whereas all larvae ($N = 11$) field collected on March 6, 2012 were fully hydrated, 11 out of 17 larvae field collected on April 6, 2012 had body water content lower than the range of fully hydrated, control larvae. *Asterisk* denotes the significant difference by the Wilcoxon's rank-sum test ($P < 0.0001$)

physiological potential to overwinter either in a frozen or a cryoprotectively dehydrated state (Kawarasaki et al. 2014). The present study examined hydric conditions within various microhabitat types to determine whether winter-acclimatized larvae could avoid inoculative freezing under natural conditions.

Although terrestrial habitats in Antarctica are generally characterized by a lack of free water, the maritime Antarctic Peninsula receives considerable precipitation owing to the oceanic influence and westerly winds through the Drake Passage (Peck et al. 2006). From February 3 to April 11, 2011, precipitation either as rain or snow occurred on 33 days for a total of 11.8 cm (University of Wisconsin-Madison Antarctic Meteorological Research Center 2013). Since ambient temperatures in this period generally remained above freezing, this precipitation together with the melting of accumulated snow from the previous winter created "moist" microenvironmental conditions for *B. antarctica*.

Hydration levels within microhabitat sites fluctuated seasonally, and the pattern of fluctuation varied among sites (Fig. 1). This difference in seasonal hydration was at least partly accounted for by substrate type and composition. In general, higher organic content promotes a greater retention of water by a substrate (Saxton and Rawls 2006). Experimental addition of as little as 10 % (w:w) horticultural peat increased by nearly twofold the amount of water that sandy soil could hold against gravity (i.e., saturation moisture content; Costanzo et al. 1997). In the present study, this effect was most evident when hydric characteristics of Sites C and D were compared (Table 1). Located within a few meters from each other on the same island, both sites were primarily composed of sandy soil. However, Site D had shed seal hair and plant material augmenting the substrate, increasing its organic content nearly threefold (from 14.0 to 41.5 %; Table 1). This difference in organic content likely contributed to the higher saturation content of the substrate from Site D compared to Site C (2.0 vs. 0.6 g H_2O g^{-1} DM), as well as higher levels of field moisture content (Fig. 1).

Because substrates interact differently with water depending on their type and composition, the hydric relationship between an organism and its immediate surroundings cannot be inferred simply by its moisture content, and requires measurement of its water potential (Shoemaker et al. 1992; Hillel 1998). The net direction of water movement between systems (i.e., microhabitat vs. organism) follows the gradient in water potential from higher to lower. The water potential of an organism is primarily determined by the osmolality of its body fluid (Shoemaker et al. 1992). Based on the lowest value of hemolymph osmolality reported for *B. antarctica* (373 mOsm kg^{-1}; Lee et al. 2006), we calculated the water potential of larvae to be ~ -846 kPa. Seasonal acclimatization results in a higher osmolality (e.g., Kawarasaki et al. 2014; 620 mOsm kg^{-1}), further decreasing the water potential ($\sim -1{,}451$ kPa). However, compared to these calculated water potential values for larvae, all substrates rehydrated to ecologically relevant levels had higher potential values regardless of their type and composition

(Table 1). Consequently, although larvae are particularly vulnerable to rapid water loss in dry air (Benoit et al. 2007b), they would be expected to remain hydrated under field conditions when temperatures are above 0 °C. Wet environments are also favorable for adults, whose reproductive activities are likely affected by their hydration status (Benoit et al. 2007a).

What is the likelihood larvae can avoid inoculative freezing in these wet microhabitats? The potential for a hibernaculum to induce inoculative freezing is decreased, not only by a lower hydration level (e.g., Forge and MacGuidwin 1992), but also by the addition of organic materials to the substrate (Costanzo et al. 1997, 1998). Accordingly, we expected that hibernaculum substrates with a high organic content would allow a greater proportion of larvae to avoid inoculative freezing and become cryoprotectively dehydrated. However, when larvae were cooled in substrates rehydrated to ecologically relevant levels, they were not able to avoid inoculation from environmental ice, regardless of substrate type (Fig. 2). The moisture content of microhabitat substrates in February and March often exceeded the saturation moisture content of each substrate (Fig. 1), and by early winter, many larval hibernacula were covered by a thick layer of ice (Y. Kawarasaki personal observation). Our data suggest that inoculation from environmental ice is generally unavoidable in these wet hibernacula, and, thus, the potential of larvae to overwinter in a cryoprotectively dehydrated state is limited.

Although our assessment of hydric conditions in microhabitat substrates and inoculation trials suggests that *B. antarctica* larvae would be expected to remain hydrated throughout the year, spatial heterogeneity in microenvironmental conditions still exposed some individuals to dehydration in the field (Fig. 3). Additionally, the likelihood of dehydrating conditions in some microhabitat sites may be exacerbated by year-to-year variations in the amount of precipitation and snowmelt. Furthermore, under natural conditions, the pattern of ice formation within substrates is heterogeneous due to freezing-induced redistribution of water molecules (Holmstrup and Zachariassen 1996). Through this process, water molecules in fine pores diffuse to join ice in larger pores, thereby causing the formation of segregated ice. Thus, spatial and temporal variations in hydric levels, as well as the pattern of ice distribution, within a microhabitat may allow larvae to avoid inoculation and promote larval overwintering in a cryoprotectively dehydrated state.

The origin of contemporary Antarctica traces to the ancient super-continent Gondwana with its temperate environments and rich flora and fauna (Elliot 1985). At least 28 Mya with the opening of the Drake Passage, Antarctica separated from South America and began its transition to an ice-dominated continent (Allegrucci et al. 2012). Recent phylogenetic analyses comparing *B. antarctica* with other representative species of Chironomidae indicate its current distribution on the Antarctic Peninsula was through the process of vicariance, rather than by recent colonization by dispersal (Allegrucci et al. 2006). Ancestors of *B. antarctica* must have survived in situ through successive cycles of glaciation during the Pleistocene, as well as the dramatic changes in climatic conditions associated with the southward continental drift (Allegrucci et al. 2006). These changes include loss of freshwater environments occupied by ancestral chironomid larvae, changes that forced *B. antarctica* to adapt to life on land where it was exposed to cold and dry conditions. Thus, it is not surprising that larvae exhibit remarkable levels of dehydration tolerance, surviving 70–75 % loss of their body water (Baust and Lee 1987; Hayward et al. 2007). Furthermore, the molecular and physiological mechanisms underlying larval tolerance to dehydration stress likely served as a chief preadaptation for the evolution of freeze tolerance (Lee and Costanzo 1998; Elnitsky et al. 2009). Consequently, larvae developed the physiological flexibility to occupy a diverse range of microhabitats that vary temporally and spatially in their hydric conditions, and to overwinter in a frozen or a cryoprotectively dehydrated state. This flexibility likely contributed to its geographic distribution that extends further south than any other free-living insect.

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