Polar Biology (2019) 42:1147–1156
https://doi.org/10.1007/s00300-019-02503-6

ORIGINAL PAPER

Characterization of drought-induced rapid cold-hardening in the Antarctic midge, *Belgica antarctica*

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Received: 1 October 2018 / Revised: 11 April 2019 / Accepted: 15 April 2019 / Published online: 20 April 2019

Abstract

Survival of the terrestrial midge, *Belgica antarctica*, on the Antarctic Peninsula is promoted, not only by their adaptations to prolonged exposures to seasonal stresses, but also by their ability to respond to unpredictable changes in their environments. Rapid cold-hardening (RCH) is an extremely swift acclimatory response of insects that occurs within minutes to hours. While the RCH response is most commonly induced by a brief exposure to mildly low temperatures, a similar rapid acclimatory response can also be elicited by exposure to drought. In this study, we characterized this drought-induced RCH in larvae of *B. antarctica.* Compared to fully hydrated larvae, those desiccated at various relative humidity (R.H.) conditions between 0 and 99% R.H. for 2 h had a significantly greater survival (~50%) to freezing at − 14 °C. The amount of water loss varied between 4 and 16% depending on R.H. conditions; however, all treatments were equally effective in eliciting the protective response against freezing stress, and its induction was evident within 30 min of desiccation. Lack of substantial changes in body-fluid osmolality or levels of major cryoprotectants suggest that accumulation of these protective solutes is not a primary mechanism of this response. Interestingly, the RCH protection induced by desiccation persisted after larvae were allowed to recover a significant portion of the lost water. Our results indicate that larval midges are highly sensitive to desiccation, capable of swiftly initiating physiological changes in response to a small reduction in their body water content.

Keywords Antarctic Peninsula · Cross tolerance · Drought · Freeze tolerance · Rapid cold-hardening

Introduction

Terrestrial environments of the Antarctic Peninsula impose a myriad of physiological challenges to the survival of insects and other arthropods (Lee and Denlinger 2015). Although these animals are protected from temperature extremes by thermal buffering from the ocean and accumulated snow cover (Baust and Lee 1981; Convey et al. 2018), their microhabitat temperatures remain below freezing for several months each winter (Elnitsky et al. 2008b; Kawarasaki et al. 2014a). Additionally, the risk of desiccation persists throughout this period because water is frozen and thus biologically unavailable as ice, and because the vapor pressure gradient between ice and unfrozen body fluids generally promotes water loss (Elnitsky et al. 2008a, b; Kawarasaki et al. 2014a). Even during the austral summer when conditions are relatively favorable, unpredictable fluctuations in temperatures and liquid water availability are inevitable, and these fluctuations are predicted to become more frequent in response to climate change (Bale and Hayward 2010). Thus, among terrestrial invertebrates of the Antarctic Peninsula, adaptations to rapid changes in environmental conditions that occur within the time scale of hours are arguably just as important as those for prolonged exposures to seasonal stresses (Lee and Denlinger 2015).

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Rapid cold-hardening (RCH) describes an extremely swift response of insects and related arthropods to changes in environmental conditions (review by Lee and Denlinger 2010). The RCH response is one of the fastest known acclimatory responses, and occurs within a time course of minutes to hours. For example, in the flesh fly, *Sarcophaga crassipalpis*, exposure to 0 °C for as little as 10 min dramatically improves survival of a subsequent exposure to − 10 °C (Lee et al. 1987). Since this original report in 1987, the RCH response has been documented in > 30 insect species from around the world, including polar and subpolar species of arthropods (*e.g.*, Worland and Convey 2001; Everatt et al. 2012). The ecological significance of RCH is well established, as this response is induced by cooling regimes that resemble diurnal fluctuations in temperatures (Kelty and Lee 1999, 2001) and is observed under field conditions in the olive fruit fly, *Bactrocera oleae* (Koveos 2001) and *Drosophila melanogaster* (Kelty 2007). Furthermore, not only does RCH improve insect survival in cold, but it also promotes preservation of critical ecological functions, such as flight, courtship behavior, longevity, egg production, and fertilization success, within a thermally variable environment (review by Lee and Denlinger 2010). Consequently, this response is now considered a general adaptation to fine-tune organismal and cellular functions to subtle changes in thermal conditions of their environment (Elnitsky and Lee 2009).

Rapid cold-hardening also occurs in response to a brief exposure to desiccating conditions (*i.e.*, drought) in some insects. In the goldenrod gall fly, *Eurosta solidaginis,* desiccation for 6 h significantly enhances larval tolerance of freezing stress (Levis et al. 2012), and this protective effect is evident at the cellular level within 1 h following the advent of desiccation (Gantz and Lee 2015). Similarly, in larvae of *Sarcophaga bullata,* mild desiccation for as little as 3 h enhances cell survival against non-freezing, chilling injury (Yi et al. 2017). The rapidity with which drought exposure triggers the RCH response closely resembles that induced by chilling. The capacity of these species to undergo this drought-induced RCH response indicates that some insects are highly sensitive to desiccation and initiate physiological changes in response to a relatively small disturbance to their hydration state.

The terrestrial midge, *Belgica antarctica* Jacobs (Diptera: Chironomidae), is the southernmost free-living insect species and is found exclusively along the west coast of the Antarctica Peninsula. Its two-year life cycle includes four larval stages, with pupation and synchronized emergence of apterous adults occurring early in the austral summer (Sugg et al. 1983). Larvae are freeze tolerant year-round (Baust and Lee 1987) but are capable of undergoing chilling-induced RCH to swiftly enhance their levels of freeze tolerance (Lee et al. 2006; Teets et al. 2008). Larvae of *B. antarctica* are the first freeze-tolerant species reported to have the capacity for RCH, and in our previous study, we extensively characterized conditions for its induction (Kawarasaki et al. 2013). Similarly, because these larvae are highly susceptible to desiccation, extensive efforts have focused on their adaptations to dehydration stresses that occur over a relatively long period (*i.e.,* days to weeks; see review by Lee and Denlinger 2015). Prolonged exposure to dehydrating conditions promotes cross tolerance to freezing stress in this species (Hayward et al. 2007; Benoit et al. 2009; Elnitsky et al. 2009); however, little is known about the rapidity by which this acclimatory response is elicited by desiccation.

Consequently, the objective of this study was to characterize drought-induced RCH in larvae of *B. antarctica.* We assessed the threshold of water loss required to elicit this protective response by exposing larvae to a range of relative humidities (R.H.). Increased survival to a subsequent freezing stress was used as evidence for induction of the RCH response. We also examined the amount of water loss, as well as changes in osmolality of body fluid and levels of several cryoprotectants. Rates of RCH induction and the effect of subsequent rehydration were also investigated.

Materials and methods

Source of insects

Substrates containing larvae of *B. antarctica* were collected on islands near Palmer Station, Antarctica (64°46′S, 64°04′W) in January 2018. Larvae were extracted using a modified Berlese apparatus, and concentrated samples were maintained in their natural substrate at 2 °C. In preparation for the experiment, larvae were hand-sorted in ice water and placed on wet filter paper for at least 12 h to ensure gut clearance and to standardize for body water content (Baust and Edwards 1979). All physiological experiments were completed at Palmer Station within three weeks of collection.

Experimental conditions for larval desiccation

Different R.H. conditions were obtained using saturated salt solutions in sealed containers at 2 °C. According to Winston and Bates (1960), the following chemicals were used: K_2SO_4 for 99% R.H., KCl for 85% R.H., NaCl for 75% R.H., and $MgCl_2$ for 35% R.H. Additionally, Drierite was used to generate near-complete dryness (~ 0%). Larvae were gently blotted dry on an absorbent tissue, confined in 1.6-ml microcentrifuge tubes with fine nylon mesh in place of the lids, and exposed to these desiccating conditions. None of these desiccation treatments *per se* resulted in mortality of larvae. Control larvae were maintained on a wet filter paper within a sealed container at 2 °C.

Assessment of larval freeze tolerance

To determine the effectiveness of desiccation in inducing the RCH response, groups of 5 individuals were first exposed to different humidity conditions at 2 °C for 2 h as described above. Subsequently, ~ 500 µl of water and a small piece of ice were quickly added to tubes containing larvae to promote ice formation and reduce variation in supercooling among treatment groups during exposure to the discriminating temperature. Because larvae are highly susceptible to inoculative freezing (Elnitsky et al. 2008b), internal ice formation likely occurred at the melting point of their body fluids (~ − 0.7 °C). We used the discriminating temperature of − 14 °C for 24 h because direct exposure to this condition caused ~ 80% mortality in untreated larvae in our preliminary experiment. Accordingly, increased survival at this temperature was used as evidence for the RCH induction. Refrigerated baths were used for freezing exposures, and their temperatures were maintained within ± 0.2 °C of the set temperature.

We also investigated the rate of RCH induction by exposing larvae to either 85% R.H. or 0% R.H. conditions for 30 min, 1 h, or 2 h at 2 °C; changes in levels of freeze tolerance were assessed by subsequent exposure to − 14 °C for 24 h. To determine whether protection conferred by the drought-induced RCH response was transient, the effect of rehydration was assessed by maintaining larvae on wet filter paper at 2 °C for 1, 2, 6, 12, or 24 h following the initial 2-h dehydration at either 85% or 0% R.H. conditions; changes in levels of freeze tolerance were subsequently examined at − 14 °C for 24 h.

Following exposure to the discriminating temperature, larvae were quickly thawed and allowed to recover on wet filter paper for 24 h at 2 °C. Survival was scored based on the larva's ability to move spontaneously or in response to gentle probing; only those exhibiting whole-body contractions were judged alive. Each treatment group consisted of ~ 50 individuals.

Determination of body water content and body-fluid osmolality

To examine changes in body water content, larvae were individually exposed to various R.H. conditions for 2 h, and their body mass was compared before and after exposure, using a gravimetrical balance capable of measuring to the nearest 0.002 mg (Mettler Toledo, Columbus, OH, USA). Subsequently, larvae were dried to constant mass at 65 °C to determine dry mass (DM), and their initial water content was calculated by subtracting DM from the initial mass. Given the duration of the exposure to desiccating conditions, any changes in the body mass were assumed to be caused by loss of body water, and percent water loss was calculated based on the initial water content. To assess the ability of larvae to rehydrate, larvae were individually exposed to 0% R.H. for 2 h and subsequently maintained on wet filter paper for 24 h at 2 °C. Body mass of each individual was tracked throughout the experiment.

Changes in body-fluid osmolality in response to desiccation were measured using a vapor pressure depression technique (Holmstrup and Sømme 1998; Elnitsky et al. 2008a, b). Larvae were exposed to different humidity conditions in groups of five and subsequently crushed in a sample holder to release the body fluid. Following at least 30 min of equilibration within a C-52 sample chamber, osmolality was measured using a Wescor HR-33T Dew Point Microvoltmeter (Wescor Inc., Logan, UT, USA).

Cryoprotectant analysis

To determine whether desiccation elicited accumulation of low-molecular-mass cryoprotectants, groups of five larvae were exposed to 75% R.H. at 2 °C for 2 h. Control larvae were maintained on wet filter paper in groups of five for 2 h. Immediately after exposure, 20 larvae were grouped and quickly weighed before freezing at − 80 °C for later analysis. Frozen samples were shipped on dry ice from Palmer Station, Antarctica to Gustavus Adolphus College, where biochemical assays were conducted.

We chose to measure levels of glucose, trehalose, and glycerol because they are major cryoprotectants in larvae of *B. antarctica* (Baust and Lee 1983; Teets et al. 2011). Briefly, frozen samples were homogenized in 7% PCA using a bead homogenizer (Benchmark Scientific Inc., Edison, NJ, USA), and neutralized with KOH. Trehalose levels were determined after enzymatic digestion by trehalase from porcine kidney (T8778, Sigma Aldrich, St. Louis, MO, USA). Glucose concentrations were measured using the liquid glucose (oxidase) reagent set (G7521, Pointe Scientific, Canton, MI, USA); changes in absorbance were measured at 450 nm. Finally, glycerol levels were determined using the free glycerol reagent (F6428, Sigma Aldrich). The suggested ratio of sample to reagent was increased by ~ tenfold to get a signal according to Teets et al. (2011), and absorbance was measured at 570 nm; serial dilutions of glycerol standards (G7793, Sigma Aldrich) generated a linear relationship for concentrations ranging between 3.25 and 26 µg ml^{-1}. Values for each cryoprotectant were expressed as µg mg^{-1} DM.

Statistical analysis

All statistical analyses were completed using R Version 3.5.1 (R Core Team 2018) on R Studio (RStudio Team 2015). Survival data were analyzed using a generalized linear model with the logistic link and binomial error distribution (Hosmer et al. 2013). Water loss and body-fluid osmolality

at various R.H. conditions were analyzed using one-way ANOVA after the assumptions of parametric tests were checked. Data for glucose levels were compared using t test, while those for trehalose and glycerol were analyzed with the permutation test using independent_test() function in the coin package for R because the assumption of normality was violated in these datasets. Finally, changes in body mass during rehydration following the initial dehydration were analyzed with the linear mixed model using lmer() function in the lmerTest package for R; variations among time points were considered the fixed effect, while those among individuals were considered the random effect. Statistical significance was judged at $p < 0.05$ and Tukey's post hoc test was applied to multiple comparison tests where overall significance was detected.

Results

Dehydration-induced RCH

Two-hour exposure to sub-saturated air dramatically enhanced freeze tolerance in larvae of *B. antarctica* (Fig. 1). Compared to control larvae that were maintained on wet filter paper, those exposed to a range of R.H. for 2 h had significantly greater survival rates at − 14 °C for 24 h ($\chi^2_5 = 55.16$, $p < 0.0001$). The amount of larval water loss varied according to the relative humidity conditions (Table 1). While exposure to 99% R.H. resulted in body water loss of < 4%, larvae that were exposed to 0% R.H. lost ~ 16% of the initial water content. Yet, regardless of the severity of desiccation, all treatments were equally effective in eliciting a protective response against freezing stress in *B. antarctica* (Fig. 1). This protective effect induced by desiccation developed rapidly; as little as 30 min at either 85% or 0% R.H. was sufficient to enhance larval freeze tolerance (85% R.H.: $\chi^2_3 = 26.00$, $p < 0.0001$; 0% R.H.: $\chi^2_3 = 17.68$, $p = 0.0005$; Fig. 2).

Body-fluid osmolality and cryoprotectant analyses

In response to body water loss, numerically greater values for body-fluid osmolality were observed among larvae that had been exposed to sub-saturated air (Table 1). Although the result of ANOVA was significant ($F_{5, 30} = 3.74$, $p = 0.0095$), Tukey's post hoc test generally failed to detect significant differences among these values. Similarly, levels for glucose did not vary between control larvae that had been maintained on wet filter paper and those exposed to 75% R.H. for 2 h ($t_{10} = 0.95$, $p = 0.3627$). Significant, albeit small, changes in trehalose and glycerol levels occurred in response to desiccation at 75% R.H. (trehalose: $z = -2.4$, $p = 0.0184$; glycerol: $z = -2.0$, $p = 0.0422$).

Effect of rehydration following drought-induced RCH

The protective effect of drought-induced RCH persisted even after larvae were allowed to rehydrate (Fig. 3). Initial desiccation at either 85% or 0% R.H. for 2 h significantly enhanced larval freeze tolerance, amounting in ~ 40% difference in survival rates compared to control larvae (85%

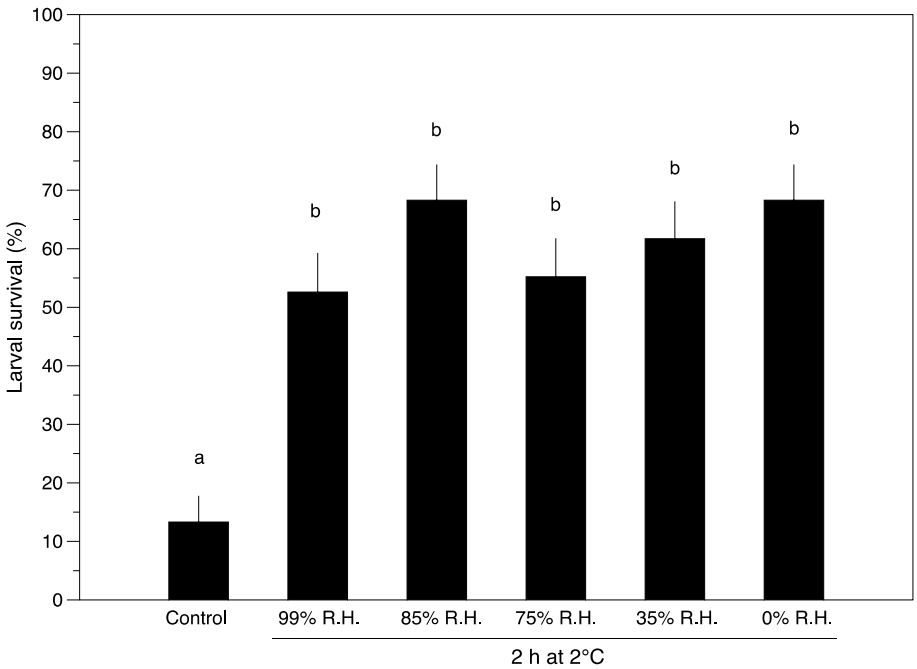

Fig. 1 Effects of desiccation at various relative humidity (R.H.) conditions on freeze tolerance in larvae of *B. antarctica.* Larvae were exposed to one of the R.H. conditions for 2 h at 2 °C prior to testing their freeze tolerance at − 14 °C for 24 h. The control group was exposed to the discriminating temperature after being maintained on wet filter paper for 2 h at 2 °C. Survival for each treatment group was based on 57–60 individuals (± standard error of proportion). Values with different letters are significantly different from each other (Logistic regression, Tukey family-wise $p < 0.05$)

Table 1 Effects of exposures to various relative humidity (R.H.) conditions on the hydration state, body-fluid osmolality, and cryoprotectant levels in *B. antarctica*

	Initial body mass (mg; $N=18$)	Body mass after desiccation (mg; $N=18$)	Body water loss (% initial water content.; $N=15$–18)	Body-fluid osmolality (mOsm kg^{-1}; $N=6$)	Glucose (µg mg^{-1} DM; $N=6$)	Trehalose (µg mg^{-1} DM; $N=6$)	Glycerol (µg mg^{-1} DM; $N=6$)
Control	0.89 ± 0.03	0.87 ± 0.03	2.46 ± 0.21^{a}	423 ± 16^{a}	0.57 ± 0.05	46.13 ± 1.03	0.35 ± 0.04
99% R.H.	0.90 ± 0.03	0.88 ± 0.03	3.72 ± 0.41$^{a, b}$	467 ± 24$^{a, b}$	–	–	–
85% R.H.	0.91 ± 0.04	0.87 ± 0.04	5.60 ± 0.32$^{b, c}$	541 ± 21$^{a, b}$	–	–	–
75% R.H.	1.00 ± 0.06	0.96 ± 0.06	6.43 ± 0.47^{c}	533 ± 28$^{a, b}$	0.64 ± 0.04	42.55 ± 0.45*	0.50 ± 0.05*
35% R.H.	0.99 ± 0.04	0.91 ± 0.04	11.30 ± 0.51^{d}	577 ± 46^{b}	–	–	–
0% R.H.	1.05 ± 0.05	0.93 ± 0.05	16.12 ± 0.70^{e}	535 ± 30$^{a, b}$	–	–	–

Larvae were exposed to one of R.H. conditions for 2 h at 2 °C prior to analysis of water loss (measured individually), body-fluid osmolality (in groups of five), or cryoprotectant levels (in groups of 20). Control larvae were maintained over wet filter paper for 2 h at 2 °C. Values are means ± S.E.M

Different letters denote significant differences (ANOVA, Tukey family-wise $P<0.05$)

Asterisks (*) denote significant differences compared to the control group (Permutation test, $P<0.05$)

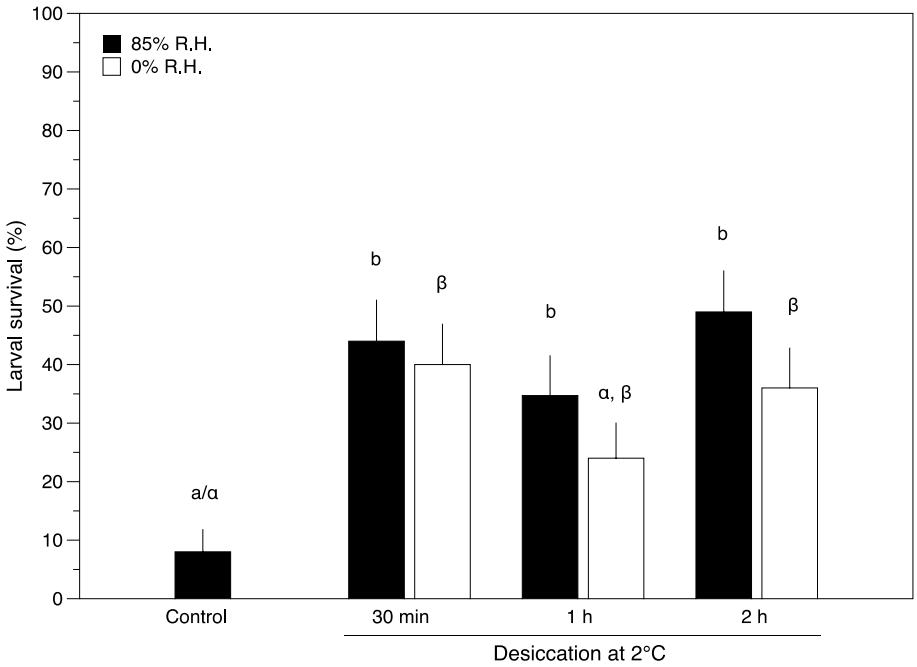

Fig. 2 Effects of varying duration of exposures to desiccating conditions (85% or 0% R.H.) on freeze tolerance of larvae of *B. antarctica.* Larvae were exposed to one of the R.H. conditions for 30 min, 1 h, or 2 h at 2 °C prior to testing freeze tolerance at − 14 °C for 24 h. The control group was exposed to the discriminating temperature after maintained on wet filter paper for 2 h at 2 °C. Survival was based on 49–51 individuals (± standard error of proportion). Different English letters denote significant differences among groups exposed to 85% R.H., while Greek letters are used for those exposed to 0% R.H. (Logistic regression, Tukey family-wise $p<0.05$)

R.H.: $\chi^2=102.08$, $p<0.0001$; 0% R.H.: $\chi^2_6=50.47$, $p<0.0001$). Among larvae initially desiccated at 0% R.H., freeze tolerance remained at an enhanced level through the 24-h period of rehydration. Among larvae initially desiccated at 85% R.H., protection against freezing stress continued to increase through the rehydration period; the greatest level of freeze tolerance was achieved after 24 h with nearly 90% of larvae surviving exposure to − 14 °C for 24 h (Fig. 3).

We investigated the ability of larvae to rehydrate by monitoring changes in body mass after an initial desiccation at 0% R.H. (Fig. 4). Exposure to 0% R.H. for 2 h resulted in a significant loss of body water (~ 12% of initial body water content; $F_{6,84}=23.48$, $p<0.0001$). Within the first hour of rehydration on wet filter paper, larvae recovered a significant portion of their lost water. However, body mass remained at a slightly, but significantly lower level than the initial mass throughout the 24-h period.

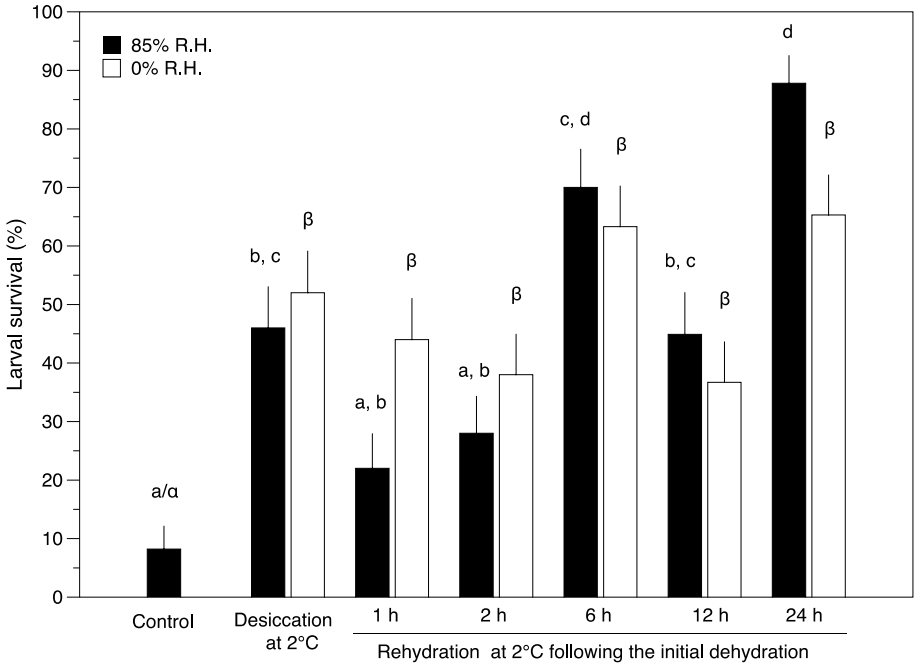

Fig. 3 Effects of rehydration on freeze tolerance of *B. antarctica.* Following desiccation at either 85% or 0% R.H. for 2 h at 2 °C, larvae were allowed to rehydrate on wet filter paper for varying durations at 2 °C before testing of their freeze tolerance at − 14 °C for 24 h. The control group was exposed to the discriminating temperature after being maintained on wet filter paper for 2 h at 2 °C. Survival was based on 49–50 individuals (± standard error of proportion). Different English letters denote significant differences among groups exposed to 85% R.H., while Greek letters are used for those exposed to 0% R.H. (Logistic regression, Tukey family-wise $p < 0.05$)

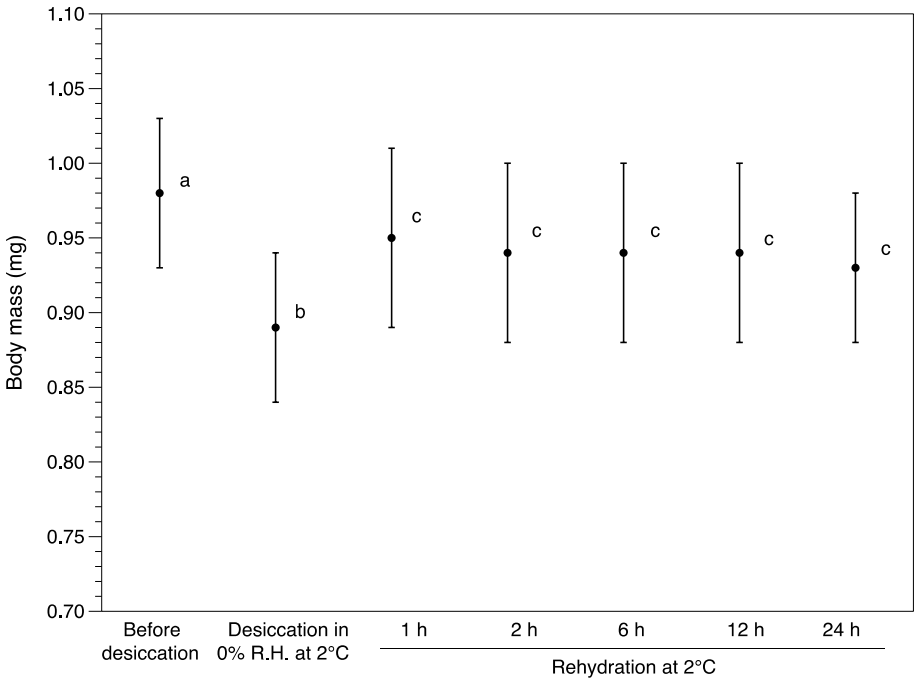

Fig. 4 Changes in larval water content during rehydration. Following exposure to 0% R.H. for 2 h, larvae were allowed to rehydrate on wet filter paper, and fresh weight of each larva was monitored for 24 h. Values are means ± S.E.M. ($N = 15$). Different letters denote significant differences (Linear mixed model, Tukey family-wise $p < 0.05$)

Discussion

Most commonly, RCH in insects is induced by a brief exposure to mildly low temperatures (reviews by Lee and Denlinger 2010; Teets and Denlinger 2014). In. *B. antarctica,* chilling-induced RCH occurs at the subzero range, and an exposure to temperatures as low as − 12 °C is effective in eliciting this protective response (Kawarasaki et al. 2013). Recently, induction of RCH by exposure to desiccating conditions (*i.e.,* drought) was reported in both freeze-tolerant (Sinclair and Chown 2003; Levis et al. 2012; Gantz and Lee 2015) and freeze-intolerant (Yi et al. 2017) species. Cross tolerance to cold elicited by desiccation is well documented among insect and other invertebrates in the context of drought-acclimation that takes place over a time course of days to weeks (reviews by Holmstrup et al. 2010; Everatt et al. 2015). In contrast,

the drought-induced RCH response can occur within a time frame of minutes to hours, strikingly resembling the chilling-induced response. In the present study, we characterized the capacity of *B. antarctica* larvae to undergo a drought-induced RCH response. Even in the absence of chilling, mild desiccation resulting in ~ 4–16% loss of body water enhanced survival of larvae to a subsequent exposure to freezing stress, and this protective response developed within 30 min of desiccation (Figs. 1, 2; Table 1).

Induction of RCH by drought exposure requires only a small change in an insect's body water content. In the freeze-tolerant caterpillar, *Pringleophaga marioni*, a body mass reduction of ~ 12% over 6 h is effective in inducing physiological changes to improve survival to a subsequent freezing exposure (Sinclair and Chown 2003). Remarkably, in larvae of *Eurosta solidaginis,* a tiny loss of body water equating to less than 1% of fresh mass by 2 h of desiccation at 75% R.H. is sufficient to enhance freeze tolerance (Gantz and Lee 2015). Similarly, in *Sarcophaga bullata,* a brief (3–4.5 h) desiccation resulting in 2–3% loss of body mass dramatically increases survival of freeze-intolerant larvae against cold shock at the cellular level (Yi et al. 2017). Given these small changes in body mass, the protective effect of drought-induced RCH appears greater than what could be explained simply by the concentrating effects of body fluids by desiccation. In the present study, we observed that the effectiveness of the RCH protection in *B. antarctica* was independent of the severity of desiccation caused by exposures to a varying range of humidity conditions (Fig. 1; Table 1). Therefore, our results also provide evidence that drought-induced RCH is a coordinated physiological response elicited by a small reduction in body water content.

Generally, cross tolerance to cold that develops in response to desiccation is at least partly attributed to the accumulation of low-molecular-mass sugars and polyols (Holmstrup et al. 2010). At high concentrations, these molecules confer protection against both osmotic and low-temperature stresses through colligative effects (*e.g.*, depression of freezing and supercooling points and/or minimizing cellular dehydration). In *B. antarctica*, slow dehydration caused by acclimation over several days to either 98% R.H. (Hayward et al. 2007; Benoit et al. 2009) or high-osmolality seawater (Elnitsky et al. 2009) causes severalfold accumulation of glucose, trehalose, and/or glycerol and enhances larval tolerance to freezing stress. While cryoprotectant synthesis is the hallmark of seasonal cold-hardening in insect (review by Lee 2010), chilling-induced RCH generally occurs without de novo synthesis of these molecules (Teets and Denlinger 2013). In *B. antarctica,* drastic changes in levels of the major cryoprotectants appear absent when the RCH response is induced by an exposure to mildly low temperatures (Lee et al. 2006; Kawarasaki et al. 2013). Similarly, in the present study, we did not observe substantial changes in levels of glucose, trehalose, or glycerol in larvae rapidly cold-hardened by mild desiccation at 75% R.H. condition for 2 h (Table 1).

The extent to which cryoprotectant accumulation contributes to drought-induced RCH remains to be elucidated. In other species, this hardening response is accompanied by an increase in body-fluid osmolality to a level that is greater (by < ~ 50 mOsm kg^{-1}) than what could be explained by the loss of body water (Levis et al. 2012; Gantz and Lee 2015; Yi et al. 2017). However, levels of the major cryoprotectant systems (*e.g.*, glycerol, trehalose, glucose, and/or sorbitol) remain relatively unchanged in the species examined. Thus, accumulation of other molecules, such as free amino acids, is speculated. Recently, a strong cryoprotective effect of proline accumulation was reported in some insects (Koštál et al. 2011, 2012). Also, a twofold increase in abundance of alanine and glutamine occurs during the chilling-induced RCH response in *Sarcophaga crassipalpis* (Michaud and Denlinger 2007). When accumulated, even at a relatively low concentration, these molecules may provide protective effects against cold and/or freezing through their non-colligative functions (*e.g.*, by stabilizing the membranes and/or proteins).

One of the notable characteristics of chilling-induced RCH in larvae of *B. antarctica* is its ability to undergo this protective response in both frozen and supercooled states (Teets et al. 2008; Kawarasaki et al. 2013). When exposed to − 5 °C, a temperature above the supercooling point (~ − 7 °C in summer-acclimatized larvae; Hayward et al. 2007; Kawarasaki et al. 2014a), larvae freeze or remain supercooled depending on the occurrence of inoculative freezing. Interestingly, even at the same temperature, the protective effect of RCH develops more rapidly in frozen larvae (Kawarasaki et al. 2013). As ice forms extracellularly, solutes are excluded from the frozen portion of the solution and results in a freeze concentration that induces dehydration stress at the cellular level. We speculated that this cellular dehydration imposed by freeze concentration might play a synergetic role in eliciting the RCH response in the frozen state (Kawarasaki et al. 2013). In larvae of *S. bullata,* the protective effect of RCH is greater when induced by successive exposure to desiccation and cold than either treatment alone, thereby suggesting synergetic interactions between two abiotic cues (Yi et al. 2017). Occurrence of the drought-induced RCH response in larvae of *B. antarctica* without chilling also provides further support for this hypothesis, and future study should systematically investigate interactions between chilling and desiccation that elicit the RCH response in this species.

For chilling-induced RCH, the protective effect is generally transient, and is quickly lost upon return to favorable conditions (Lee and Denlinger 2010). In the case of *B. antarctica,* the RCH protection elicited by freezing at − 5 °C

begins to diminish within 2 h of thawing at 4 °C (Kawarasaki et al. 2013). In contrast, not only did the protective effect of the drought-induced RCH response persist, but it also continued to develop after larvae were allowed to rehydrate (Fig. 3). Because larvae that were initially desiccated at 0% R.H. did not fully return to their initial mass upon rehydration (Fig. 4), it is plausible that they continued to experience desiccation stress through the rehydration period and thus maintained an enhanced level of freeze tolerance. Alternatively, disturbance of water balance may initiate changes that have relatively long-term effects on the physiology of the larvae. Some acclimatory responses to slow dehydration (*i.e.,* days of exposure) also persist after rehydration in this species. For example, larval cross tolerance to freezing induced by an exposure to hyperosmotic seawater for 3 days remains at an enhanced level even after 24 h of rehydration (Elnitsky et al. 2009).

Mild desiccation may trigger changes at the transcriptomic level in larvae of *B. antarctica.* When desiccated at 93% R.H. for 5 days, larvae lose ~ 40% of their body water, and this level of dehydration causes more than 3000 genes to change their expression levels (Teets et al. 2012). Some of the genes upregulated in response to slow dehydration include those that are closely linked to stress tolerance, such as those that encode heat shock proteins and enzymes involved in mobilization of cryoprotectants (Teets et al. 2012, 2013; review by Teets and Denlinger 2014). However, little is known about the threshold of water loss required to initiate such drastic changes in gene expression. Chilling-induced RCH proceeds with little-to-no changes in gene expression (Teets and Denlinger 2013), and it remains to be determined whether drought-induced RCH promotes changes at the transcriptomic level. Even without the synthesis of new gene products, changes in the activity or localization of existing proteins likely play a role in promoting the RCH protection. For example, the functional importance of aquaporins underlying cellular freeze tolerance is reported in *E. solidaginis* (Philip et al. 2008) and *B. antarctica* (Yi et al. 2011), and insertion of these proteins into apical membranes from storage vesicles may occur rapidly, within the time frame of RCH.

During rehydration following the initial dehydration, larvae were maintained on wet filter paper without access to their natural substrate. Thus, the larvae may have also experienced starvation stress during this period. Together with the overnight clearance of the gut that preceded these experiments, larvae had been deprived of food for a few days. In some insects, mild starvation promotes physiological changes that confer cross tolerance to cold. For example, in *D. melanogaster,* young adult females starved for 24 h exhibit a greater level of tolerance to 0 °C for 32 h, compared to fully fed individuals (Le Bourg 2013). Also, in *Drosophila immigrans,* starvation for 3 days causes a 1.5-fold increase in proline (Pathak et al. 2018) that may exert cryoprotective effects (Koštál et al. 2011, 2012). Although we reported previously that the maintenance of larval *B. antarctica* on wet filter paper at 2 °C for 2 or 5 days did not alter tolerance to freezing stress (Kawarasaki et al. 2013), future study should incorporate the use of a parallel control to differentiate the potentially confounding effect of starvation from that of initial dehydration.

Endemic to the Antarctic Peninsula, *B. antarctica* is the southernmost free-living insect species (Sugg et al. 1983). While maritime Antarctica receives considerable precipitation, which due to the climate change occurs more increasingly as rain during the summer months, the availability of liquid water is still considered one of the most critical factors determining the distribution of insect and other arthropods in the terrestrial environment (Kennedy 1993; Convey et al. 2014). Because larvae of *B. antarctica* are highly susceptible to water loss (Benoit et al. 2007), they typically occupy wet microhabitats (Kawarasaki et al. 2014b). However, during the austral summer, fluctuations in water availability occur both spatially and temporally according to weather conditions and likely impose dehydration stress in an unpredictable manner. The RCH response was originally proposed as a mechanism to instantaneously protect insects from a sudden cold-snap (Lee et al. 1987), and we used the enhanced level of larval freeze tolerance as evidence for RCH induction in this study. However, more recently, the ecological function of RCH has been expanded as a more general adaptation to promote fine-tuning of homeostatic performance to subtle changes in environmental conditions (reviews by Elnitsky and Lee 2009; Lee and Denlinger 2010). Thus, in this manner, the drought-induced RCH response might function to promote physiological adjustments in response to declines in liquid water availability within larval microhabitats. Additionally, we report that this RCH response elicited by mild desiccation may have a relatively long-lasting effect on larval physiology. Given that a decrease in liquid water availability is known to trigger seasonal cold-hardening in some insects (*e.g.,* Rojas et al. 1986; Williams et al. 2004; Williams and Lee 2005), the ability of *B. antarctica* to respond to subtle changes in this abiotic variable may have a role in promoting the process of winter-acclimatization in this species.

Acknowledgements We are grateful for the hard work and assistance of the support staff at Palmer Station. We also thank Peter Convey and two anonymous reviewers who provided comments and suggestions on this manuscript.

Funding This research was supported by the National Science Foundation Grants (PLP-1341385 and PLP-1341393). This work was also supported by the National Institute of Food and Agriculture, U.S.

Department of Agriculture, and Hatch Project 1010996 awarded to NMT.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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