The Journal of Experimental Biology 215, 3768-3773

doi:10.1242/jeb.076885

RESEARCH ARTICLE

Mild desiccation rapidly increases freeze tolerance of the goldenrod gall fly, *Eurosta solidaginis*: evidence for drought-induced rapid cold-hardening

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SUMMARY

Overwintering insects may experience extreme cold and desiccation stress. Both freezing and desiccation require cells to tolerate osmotic challenge as solutes become concentrated in the hemolymph. Not surprisingly, physiological responses to low temperature and desiccation share common features and may confer cross-tolerance against these stresses. Freeze-tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae), experience extremely dry and cold conditions in winter. To determine whether mild desiccation can improve freeze tolerance at organismal and cellular levels, we assessed survival, hemolymph osmolality and glycerol levels of control and desiccated larvae. Larvae that lost only 6–10% of their body mass, in as little as 6 h, had markedly higher levels of freeze tolerance. Mild, rapid desiccation increased freezing tolerance at –15°C in September-collected larvae (33.3±6.7 to 73.3±12%) and at –20°C in October-collected larvae (16.7±6.7 to 46.7±3.3%). Similarly, 6 h of desiccation improved *in vivo* survival by 17–43% in fat body, Malpighian tubule, salivary gland and tracheal cells at –20°C. Desiccation also enhanced intrinsic levels of cold tolerance in midgut cells frozen *ex vivo* (38.7±4.6 to 89.2±5.5%). Whereas hemolymph osmolality increased significantly with desiccation treatment from 544±16 to 720±26 mOsm, glycerol levels did not differ between control and desiccated groups. The rapidity with which a mild desiccation stress increased freeze tolerance closely resembles the rapid cold-hardening response, which occurs during brief sub-lethal chilling, and suggests that drought stress can induce rapid cold-hardening.

Key words: Dipteran, osmotic challenge, cross-tolerance, drought acclimation.

Received 29 June 2012; Accepted 7 August 2012

INTRODUCTION

During the winter, many temperate insects are threatened simultaneously by desiccation and low temperature. Desiccation and freezing of body water challenge cells by creating a hypertonic, extracellular environment that osmotically draws water from cells causing cryoinjury, or even death, due to solution effects and membrane damage (Mazur, 2004). Desiccation increases hemolymph osmolality by removing water (Williams and Lee, 2011), whereas extracellular ice formation removes water as pure ice, which results in the freeze concentration of remaining ions and other solutes (Lee, 1989). In addition, severely desiccating conditions or low temperature can directly compromise membrane fluidity and function (Holmstrup et al., 2002; Bennett et al., 1997) and denature proteins (Prestrelski et al., 1993; Hayward et al., 2004; Rinehart et al., 2007).

Not surprisingly, the physiological and molecular responses to these osmotic challenges are also similar. Accumulation of low-molecular-mass polyols and sugars (such as glycerol and trehalose) functions to colligatively reduce water loss, and maintain cellular and membrane integrity (Yancey, 2005; Williams and Lee, 2008). Desiccation and cold stress also increase the unsaturation of membrane lipids and upregulate chaperone stress proteins to maintain membrane fluidity and function and to stabilize proteins (Holmstrup et al., 2002; Bennett et al., 1997; Hayward et al., 2004; Rinehart et al., 2007). Recently, Michaud et al. (Michaud et al., 2008) used a metabolomics approach to identify similarities in the response of the Antarctic midge *Belgica antarctica* to desiccation and freezing stress; glycerol, erythritol, nonanoic acid and succinate levels increased, while serine levels decreased.

Although similarities between mechanisms conferring enhanced desiccation and low-temperature tolerance are known in various invertebrate taxa, relatively few studies have investigated the existence of cross-tolerance, in which physiological acclimation to one stress enhances tolerance to another (Bayley et al., 2001). In the soil collembolan *Folsomia candida*, sub-lethal desiccation for 7 days confers increased tolerance against cold shock (Bayley et al., 2001). Likewise, slow dehydration dramatically increased the freeze tolerance of larvae of *B. antarctica* (Hayward et al., 2007).

Larvae of the goldenrod gall fly, *Eurosta solidaginis* (Fitch 1855), inhabit stem galls on goldenrod plants (*Solidago* spp.) across North America from Florida and Texas to New Brunswick and British Columbia (Uhler, 1951; Miller, 1959). During the summer, the moist gall tissue provides nutrients for larval growth and development. In late summer, as the plant senesces and gall tissues dry, third-instar larvae cease feeding and prepare for winter by acquiring freeze tolerance and increasing their resistance to desiccation (Williams et al., 2004). Because the gall provides little buffering against the extreme cold and desiccating conditions of winter (Layne, 1991), this species provides a useful model for cross-tolerance research.

To investigate the effect of drought acclimation on freeze tolerance in this species, we used larvae collected in September and October before they had acquired fully developed levels of desiccation and freeze tolerance found in winter larvae. Unlike some previous studies, we focused on the effects of mild desiccation

(<10% body mass) occurring over only a few hours to several days of exposure. The effects of drought acclimation were assessed based on organismal and cellular levels of freeze tolerance and possible mechanistic underpinnings of the differences we observed.

MATERIALS AND METHODS

Gall collection, body mass and desiccation resistance

Spherical galls of *E. solidaginis* were collected from goldenrod plants at the Miami University Ecology Research Center in August and September 2003 and in October 2011 prior to acquisition of the well-developed levels of freeze tolerance found in mid-winter larvae. Body mass and rates of water loss of the third-instar larvae, modified from Ramløv and Lee (Ramløv and Lee, 2000) and Williams et al. (Williams et al., 2004), were measured in late August and early September 2003, and early October 2011. Rates of water loss were based on start and end values. Larvae were either used immediately or stored outdoors for up to a week before use.

Whole-organism viability

Three replicate groups of 30 larvae were placed in a desiccator at 15°C, dried at 33 or 4% relative humidity (over saturated $CaCl_2$ solution or Drierite) until they lost ~7% of body mass (Table 1) and then frozen in microcentrifuge tubes for 24 h at –15 or –20°C for September- and October-collected larvae, respectively. Because larvae collected in October are more cold tolerant than those collected in September, a lower test temperature was required to discriminate between levels of freezing tolerance in the treatment groups. Control larvae were not desiccated prior to freezing. Using the freezing regimen of Philip et al. (Philip et al., 2008), larvae were cooled at a rate of 0.2°C min^{-1} (from 4°C to –15 or –20°C) and inoculatively frozen at –4°C by spraying microcentrifuge tubes with Super Friendly Freeze'It (Fisher Scientific, Hanover Park, IL, USA). Viability was assessed by responsiveness to tactile stimuli after thawing for 2 h at 22°C.

In vivo tissue viability

For assessment of cellular freeze tolerance *in vivo*, two groups of four larvae from the September 2003 collection were used: the treated group was desiccated for 6 h at 33% relative humidity and 15°C before freezing for 24 h at –20°C, and the control group was frozen without the desiccation treatment. After a 2-h recovery from freezing at 22°C, fat body, Malpighian tubule, salivary gland and tracheal tissue were dissected and survival was assessed with a LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA) according to Yi and Lee (Yi and Lee, 2003; Yi and Lee, 2004). This assay involves incubating cells in SYBR-14 stain and propidium iodide solution; whereas the SYBR-14 stain penetrates the nuclei of all cells, propidium iodide can only enter damaged cells that have lost plasma membrane integrity. Live cells fluoresced green and dead cells fluoresced bright red or orange-red. Survival rates were based on the mean of three counts of 100 cells except in Malpighian tubules, where all cells (~65) were counted.

Ex vivo tissue viability

Larvae collected in October 2011 (N=4) were treated as described above, except that tissues (fat body, Malpighian tubule and midgut) were dissected and then placed into 100 µl of Coast's solution (Coast, 1988) in microcentrifuge tubes prior to freezing. After freezing at –20°C for 2 h and a 2-h thaw at 22°C, survival was assessed using fluorescent vital dyes as described above.

Hemolymph osmolality and glycerol content

Hemolymph osmolality was measured for desiccated and control larvae (N=10) by drawing 7–10 µl of hemolymph into a microcapillary tube through an incision in the larval cuticle. The hemolymph was then analyzed using a Wescor Vapro 5520 Osmometer (Advanced Instrument Inc., Norwood, MA, USA).

Glycerol content for desiccated and control larvae was determined according to Philip and Lee (Philip and Lee, 2010). Whole larvae (N=10) were homogenized using a Teflon pestle in 200 µl of 0.6 mol l^{-1} perchloric acid (PCA) and incubated on ice for 5 min. The supernatant was retained after centrifugation for 2 min at 16,000 ***g***. To neutralize the PCA, an equivalent amount of 1 mol l^{-1} potassium bicarbonate was added to the supernatant and incubated on ice with a vented lid for 15 min. Following brief centrifugation, the supernatant was retained and immediately analyzed.

Free glycerol reagent (Sigma-Aldrich, St Louis, MO, USA) was reconstituted and 800 µl was added to glycerol standards prepared from a stock solution or experimental samples (200 µl). Following a 15-min incubation, the absorbance was read at 540 nm on a spectrophotometer; values are reported as micromoles of glycerol per gram of wet mass.

Statistical analysis

Organismal- and tissue-level rates of survival, hemolymph osmolality and glycerol concentration were evaluated using unpaired *t*-tests to compare control and desiccated treatment groups. Additionally, for tissue survival, a two-way ANOVA was performed with treatment and tissues as the factors and the proportion of tissue surviving as the response. The response variable was arcsine square-root transformed to stabilize the variance. We used a test of interaction to determine whether the effect of a treatment differed between tissue types. If this test was significant, then we examined contrasts between the tissues to identify differences. Otherwise, we

Table 1. Mass loss of *Eurosta solidaginis* larvae due to desiccation treatment at 15°C before they are fully winter-acclimatized

Date	Relative humidity (%)	Duration (h)	Initial mass (mg; mean ± s.e.m.)	Percent mass loss (mean ± s.e.m.)	Rate of water loss (µg mm^{-2} h^{-1})
2003					
26 August	33	3	20.7±2.4	8.3±0.8	29.9
5 September	33	6	28.2±2.9	6.5±0.8	12.7
11 September	33	6	34.6±4.0	4.3±0.3	9.1
2011					
7 October	33	29	56.3±2.2	6.3±0.7	3.2
8 October	33	168	54.5±1.5	8.4±1.1	0.7
12 October	4	144	56.0±2.2	7.6±1.3	0.7

Date refers to the starting date of the desiccation treatment. Treatment duration was increased and/or relative humidity was decreased to maintain 6–10% mass loss during the seasonal acquisition of extreme desiccation resistance.

tested the main effects of tissue and treatment. Analyses were performed using SAS (SAS Institute, Cary, NC, USA) with significance set at α=0.05.

RESULTS

Seasonal changes in body mass and desiccation resistance

The body mass of third-instar larvae increased from 20.7±2.4 to 34.6±4.0 mg from 26 August to 11 September 2003 (Table 1). For experiments conducted in October 2011, larval body mass remained relatively constant at ~54–56 mg. Consistent with previous reports (e.g. Williams et al., 2004), desiccation resistance increased seasonally through the autumn. August-collected larvae exposed to 33% relative humidity lost 8.3% of their initial body mass after only 3 h, whereas October-collected larvae required 168 h to lose a similar proportion of their body mass (Table 1).

Mild desiccation enhanced larval freeze tolerance

Mild desiccation, in which larvae lost 6–10% of their initial body mass (Table 1), enhanced freeze tolerance at both organismal (Fig. 1) and tissue levels (Figs 2, 3). A loss of 6–10% of initial mass corresponds to a loss of 10–17% initial water and results in a final water content of 1.35–1.25 mg water mg^{-1} dry mass (Williams and Lee, 2005). Organismal survival of freezing in September-collected larvae was significantly improved (33.3±6.7 *versus* 73.3±12.0%) by exposure to desiccating conditions for as little as 6 h (P<0.05; Fig. 1). Because October-collected larvae were more resistant to water loss, larvae were exposed to the desiccation treatment longer in order to achieve the same level of desiccation (~7% reduction) as larvae collected a month earlier (Table 1). Nevertheless, this desiccation treatment markedly increased larval freeze tolerance by 30% (16.7±6.7 to 46.7±3.3%).

Mild desiccation enhanced freeze tolerance of tissues

Desiccation-induced increases in larval freezing tolerance were also evident at the tissue level. Following larval desiccation and freezing at –20°C for 24 h, fat body, Malpighian tubule, salivary gland and tracheal cells were immediately dissected and cell survival was assessed (Yi and Lee, 2003). For each tissue, cell survival was significantly higher for larvae that were desiccated prior to freezing (P< 0.05; Figs 2, 3); compared with controls, relative levels of freeze tolerance increased by 17–43% for each type of tissue. There was no interaction between tissue type and treatment ($F_{3,88}$=1.25, P=0.215), indicating that the effect of treatment did not differ among tissue types. However, treatment and tissue type both had significant effects on survival ($F_{1,88}$=61.62, P<0.0001 and $F_{3,88}$=7.57, P=0.0001, respectively).

Because mild desiccation significantly improved rates of cell survival *in vivo*, we investigated whether tissues that were removed from desiccated larvae (6–10% loss of initial body mass) and then frozen *ex vivo* in Coast's solution were also more tolerant of freezing. Although there was a trend in which all three tissues from desiccated larvae had higher rates of cell survival compared with controls, only in the midgut 38.7±4.6 *versus* 89.2±5.5% was the difference significant (Fig. 4). Fat body cells showed relatively low rates of survival in control and desiccated groups, while Malpighian tubule cells had survival rates >50% in both treatments (Fig. 4). Similar to the results of the *in vivo* experiment, there was no interaction between tissue type and treatment ($F_{2,18}$=3.49, P=0.0525), but treatment and tissue type both affected survival ($F_{1,18}$=19.90, P=0.0003 and $F_{2,18}$=19.80, P<0.0001, respectively). These data indicate that, at least in cells from the midgut, larval desiccation increases the intrinsic level of cellular cold tolerance.

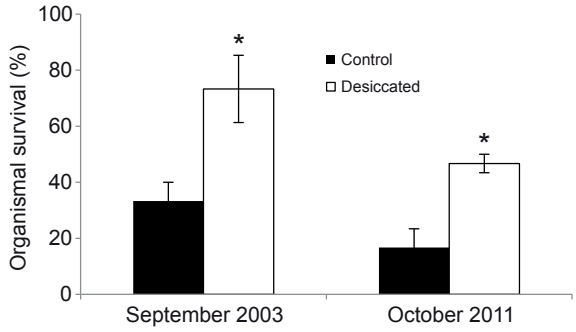

Fig. 1. Desiccation treatment significantly increased organismal survival of *Eurosta solidaginis* larvae after freezing at –15°C (15 September) or –20°C (7–8 October) for 24 h (P<0.05). Means (±s.e.m.) marked with an asterisk were significantly higher than the control of the same year.

Hemolymph osmolality and glycerol content

To investigate a possible mechanism by which desiccation enhanced larval freeze tolerance, we measured hemolymph osmolality and organismal levels of glycerol. In October-collected larvae that were desiccated for 168 h at 33% relative humidity, the hemolymph osmolality was significantly higher (~175 mOsm) than that of controls (720±26 *versus* 544±16 mOsm; P<0.05; Fig. 5A). However, glycerol levels in control (100.0±7.6 μmol g^{-1} fresh mass) and desiccated (95.8±8.8 μmol g^{-1} fresh mass) larvae did not differ (P>0.05; Fig. 5B), and thus could not explain the observed increase in hemolymph osmolality. Likewise, the molar concentration of glycerol did not differ between treatments (control, 0.17±0.01 mol glycerol l^{-1}; desiccated, 0.18±0.02 mol glycerol l^{-1}; P>0.05).

DISCUSSION

For insects, winter survival is essentially a complex problem of water management that includes cuticular permeability to water loss, regulation of ice content, hemolymph freezing point and supercooling capacity, and production of antifreeze and ice-nucleating proteins and low-molecular-mass cryoprotectants (for a review, see Denlinger and Lee, 2010). Increases in cold tolerance are frequently linked to desiccation and a reduction in body water content (Holmstrup et al., 2010). In the freeze- and desiccation-tolerant Antarctic nematode *Plectus murrayi*, desiccation significantly improved freeze survival (Adhikari et al., 2010). Drought acclimation increased survival by 60% at –2°C and 30% at –4°C during a 48-h exposure of the freeze-intolerant collembolan *F. candida* (Bayley et al., 2001). In desiccated larvae of *B. antarctica*, a 20–50% reduction in body mass improved survival at –10°C by >90% (Hayward et al., 2007). Similarly, when this species was exposed to hyperosmotic (1000 mOsm) seawater, larval water content decreased and survival at –12 and –15°C increased by 30 and 40%, respectively (Elnitsky et al., 2009).

During autumn, larvae of *E. solidaginis* undergo a major transition from being freeze intolerant and desiccation susceptible to having well-developed freezing tolerance and extreme resistance to water loss (Ramløv and Lee, 2000). The acquisition of desiccation resistance can occur quite rapidly. Williams et al. (Williams et al., 2004) found a sixfold decrease in the rate of water loss within a 13-day period in October. The extent of acclimatization can also vary markedly among individuals in a single population. From October to November, larval exposure to desiccating conditions varies depending on when the host plant senesces and gall tissues

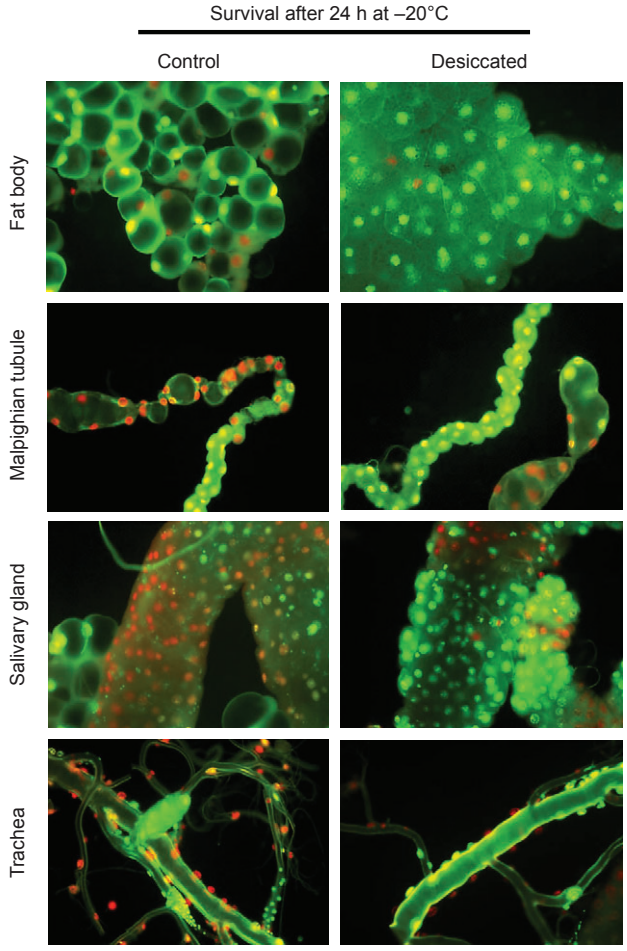

Fig. 2. Desiccation treatment improved *in vivo* cellular freeze tolerance in fat body, Malpighian tubule, salivary gland and trachea tissues of *Eurosta solidaginis* larvae collected on 15 September 2003. Larvae were either non-desiccated controls (left panels) or desiccated at 33% relative humidity and 15°C, 6 h (right panels) followed by freezing at –20°C for 24 h. Cell viability of each tissue was assessed using the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA).

Fig. 3. Cell survival (mean ± s.e.m.; N=4) after freezing *in vivo* at –20°C for 24 h was significantly higher for desiccated *Eurosta solidaginis* larvae than controls collected on 15 September 2003. Values marked with asterisks were significantly higher than controls of the same tissue (P<0.05).

dry. Rojas et al. (Rojas et al., 1986) reported that larvae from dry, brown galls accumulated more cryoprotectants than those in green, moist galls collected from the same field. Therefore, it is not surprising that larvae collected during a 6-day period in October had markedly different rates of water loss (Table 1).

Few studies have examined the effects of mild desiccation on cold tolerance. The desiccation treatment we used caused only a 6–10% loss of body mass (Table 1), which is the smallest reduction in water content reported to enhance cold tolerance. Despite the small reduction in body water, freeze tolerance increased by 30–40% at the organismal level (Fig. 1) and 20–40% at the tissue level (Figs 2, 3). Interestingly, this increase in freeze tolerance occurred in September-collected larvae that were still actively feeding and growing, and had not yet developed significant levels of freeze tolerance typical of winter larvae. Our results suggest that larvae are highly sensitive to slight losses in body water and undergo rapid physiological changes, including enhanced cold tolerance, when loss is detected.

Desiccation-induced increases in cold tolerance occur more slowly in other species than we observed in *E. solidaginis* larvae. In a study of cross-tolerance between cold-hardening and desiccation tolerance, *F. candida* was dehydrated for 7 days prior to cold exposure (Bayley et al., 2001). Elnitsky et al. (Elnitsky et al., 2009) and Hayward et al. (Hayward et al., 2007) dehydrated *B. antarctica* larvae for shorter periods, 3 and 2 days, respectively, but even these times were longer than the shortest durations used in our study (Table 1). In nature, drying of the gall tissue surrounding larvae occurs over a period of weeks to months as the goldenrod plant senesces in late summer and early autumn (Rojas et al., 1986). Concurrently, larvae begin to acquire freeze tolerance as they prepare to overwinter. Therefore, we were surprised that as little as a 6-h period of desiccation significantly increased freeze tolerance at both organismal and tissue levels. Even our longest period of desiccation lasted only 6 days, which is much shorter than the 2–3 months required for larvae to fully acclimatize for winter (Williams et al., 2004; Williams and Lee, 2005). Although the intensity of the desiccation treatment used in our study was more severe than occurs naturally, these results suggest that larvae are finely tuned to detect and respond to desiccation stress.

A common acclimatory response to cellular dehydration due to freezing or desiccation is the synthesis of cryoprotectants/osmolytes that counteract osmotic challenge and protect membranes and proteins (Ring and Danks, 1994; Williams and Lee, 2008). To investigate this possibility, we measured hemolymph osmolality and found that desiccated larvae had a significantly higher hemolymph osmolality than would be predicted by the amount of water they lost due to desiccation. For larvae that lost 13.7% of their initial body water, the resulting hemolymph concentration would be expected to increase from 544 to 619 mOsm. However, the actual osmolality of desiccated larvae was 720 mOsm (Fig. 5A). This discrepancy of 101 mOsm suggests that desiccated larvae can rapidly modulate their hemolymph osmolality.

In winter, *E. solidaginis* larvae employ a multicomponent cryoprotectant system, primarily composed of glycerol, sorbitol and trehalose, that collectively can produce a hemolymph osmolality of 900 mOsm or higher (Baust and Lee, 1981; Storey et al., 1981; Williams et al., 2004). We chose to measure glycerol levels because it is the predominant cryoprotectant accumulated at this time of the year, and its synthesis is associated with plant senescence and the drying of gall tissues in autumn (Rojas et al., 1986). However, glycerol synthesis could not explain the increased hemolymph osmolality of desiccated larvae because there was no difference in

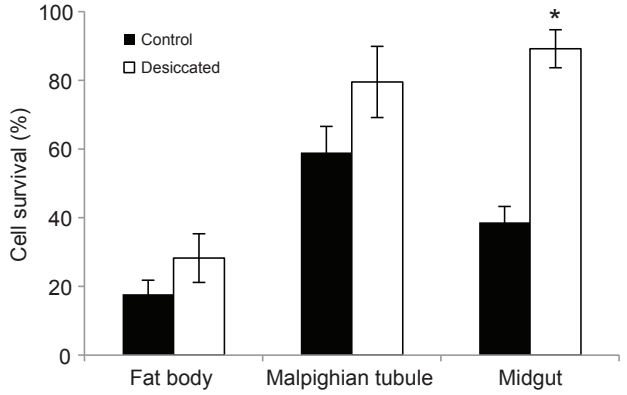

Fig. 4. Cell survival (mean ± s.e.m.; *N*=4) after *ex vivo* freezing at –20°C of *Eurosta solidaginis* larvae collected on 8 and 12 October 2011. Desiccated larvae were dehydrated prior to tissue extraction and freezing. Value marked with an asterisk was significantly higher than the control of the same tissue (*P*<0.05).

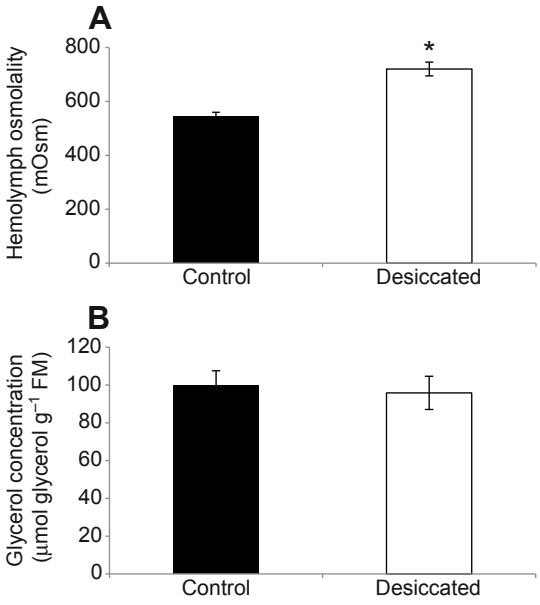

Fig. 5. (A) Desiccated *Eurosta solidaginis* larvae collected on 8 October 2011 had significantly higher hemolymph osmolality compared with controls (**P*<0.05, *N*=10). (B) No difference was found for glycerol concentration of desiccated and control larvae collected on 12 October 2011 (*P*>0.05, *N*=10). FM, fresh mass.

glycerol concentrations between control and desiccated larvae. Sorbitol is a likely candidate because it can be mobilized and detected within 1 hour of larvae being exposed to chilling (Pio and Baust, 1988). In addition, sorbitol increases during brief chilling in the flesh fly, *Sarcophaga crassipalpis* (Michaud and Denlinger, 2007). Trehalose may also play a role because it is elevated in *B. antarctica* during dehydration (Benoit et al., 2009), and in desiccated larvae of the sleeping chironomid *Polypedilum vanderplanki* (Watanabe et al., 2002).

Cells from desiccated larvae frozen *in vivo* at –20°C had higher rates of survival than controls (Figs 2, 3). Desiccated larvae had a hemolymph osmolality of 720 mOsm, which is predicted to yield an ice content of 63.3%, a value similar to the 65.0% for controls with an osmolality of 544 mOsm (Clausen and Costanzo, 1990) (bound water has been estimated to be 30%; J. B. Williams, unpublished). Consequently, the difference in survival rates cannot be explained solely by the observed differences in hemolymph osmolality. The fact that midgut cells frozen *ex vivo*, without hemolymph, showed increased freezing survival (Fig. 4) suggests that desiccation caused the intrinsic cellular cold tolerance to increase. This result is consistent with seasonal increases in intracellular cold tolerance in *E. solidaginis* larvae that occur independently of cryoprotectants (Bennett and Lee, 1997). One change that could enhance cellular freezing tolerance is upregulation of aquaporins (AQPs) and aquaglyceroporins that are associated with increased cellular freeze tolerance and increase seasonally as autumn progresses in this species (Philip et al., 2008; Philip and Lee, 2010). In particular, AQP3 is upregulated in response to desiccation, which would facilitate movement of water and glycerol during freezing and thawing (Philip et al., 2008; Ishibashi et al., 1994). Another possibility is rapid upregulation of chaperone proteins or heat shock proteins (Hsps). Zhang et al. (Zhang et al., 2011) reported elevation of various Hsps during autumn and winter in *E. solidaginis* larvae. Hsps are upregulated in some species during rapid cold-hardening (RCH) (Li and Denlinger, 2008; Koštál and Tollarová-Borovanská, 2009), but have not been reported in *E. solidaginis* as part of the RCH response. Further study is needed to determine the role of mild desiccation in cellular cold sensing and signal transduction associated with RCH (Teets et al., 2008; Lee and Denlinger, 2010).

Although initially described only in freeze-intolerant insects (Lee et al., 1987; Lee and Denlinger, 2010), the RCH response is now known in freeze-tolerant species (Lee et al., 2006). Laboratory studies of RCH most frequently use brief chilling (i.e. minutes to hours) to significantly enhance survival at previously lethal temperatures, although heat (Chen et al., 1987; Sinclair and Chown, 2003) and anoxia (Coulson and Bale, 1991) can also induce the response. We determined organismal survival 2 h after thawing; however, RCH can have various long-term effects (Lee and Denlinger, 2010). For example, rapidly cold-hardened females of *Musca domestica* had increased cold tolerance, but a shorter life span, reduced rates of oviposition and lower rates of adult emergence among their progeny compared with flies that were not cold-shocked (Coulson and Bale, 1992). Rinehart et al. (Rinehart et al., 2000) found that RCH partially prevents the reduced fecundity induced by cold-shock in *S. crassipalpis*. The swiftness of the drought-induced cold-hardening response we observed closely resembles the RCH response. Similar to our findings, Sinclair and Chown (Sinclair and Chown, 2003) reported that mild desiccation induced RCH in a freeze-tolerant caterpillar; 6 h of desiccation at 10°C decreased body mass by only 12.1%, yet significantly increased survival of freezing at –7.9°C. Consequently, mild desiccation can also trigger RCH at least in some freeze-tolerant insects.

The rapidity with which mild desiccation induced RCH at organismal and cellular levels suggests that physiological adaptations to cold and drought are closely linked and that rapid acclimatory responses are critical to fine-tune organismal response to even slight changes in environmental conditions (Shreve et al., 2004).

ACKNOWLEDGEMENTS

We thank Hays Cummins, Paul Schaeffer, J. D. Gantz, Jason Williams and Nick Teets for critically reading this manuscript, Ashley Miller and members of the Cryobiology Laboratory for assistance with gall collection, and Michael Hughes for help with statistical analysis.

FUNDING

This research was supported by undergraduate research grants from Miami University, including the DUOS program, a Howard Hughes summer internship and the Dean's Scholar Program (all to N.A.L.), and the National Science Foundation (grant IOS-0840772 to R.E.L.).

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