Cross-tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*: rapid desiccation increases organismal and cellular freeze-tolerance

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Abstract

A number of similarities exist that support the idea of cross-tolerance between low temperature and desiccation stress responses. Freeze-tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis*, are exposed to extremely dry and cold conditions when they overwinter and serve as a useful model for cross-tolerance of these two stresses. To determine if mild, rapid desiccation can improve freeze-tolerance at the organismal and cellular levels, we assessed survival, hemolymph osmolality, and glycerol concentration of control and desiccated larvae. Desiccated larvae lost 6-10% of their body mass and, to our knowledge, this is the lowest amount of water loss reported to increase freeze-tolerance. Organismal survival significantly increased with mild, rapid desiccation treatment prior to freezing for 24 h at -15°C in September (33.3 ± 6.7 to 73.3 ± 12%) and at -20°C in October (16.7 ± 6.7 to 46.7 ± 3.3%) collected larvae. Similarly, desiccation in as little as 6 h improved *in vivo* survival at -20°C for 24h in fat body, Malpighian tubule, salivary gland, and tracheal cells by ~34%, ~43%, ~38%, and ~17% , respectively. Desiccation treatment induced intracellular changes because improved freeze-tolerance occurred in midgut cells frozen *in vitro* (38.7 ± 4.6 to 89.2 ± 5.5 %). Whereas hemolymph osmolality increased significantly with desiccation treatment (720 ± 26 vs. 544 ± 16 mOsm), glycerol concentration did not differ between control and desiccated groups. The rapidity with which the sub-lethal desiccation stress increased freeze-tolerance resembles the rapid cold hardening (RCH) response that occurs when sub-lethal cold exposure for only minutes or hours enhances freeze-tolerance, and suggests that there may be a link between rapid environmental changes and cold hardening.

Introduction

During the winter, many temperate insects are threatened simultaneously by desiccation and low temperature. Desiccation and freezing of body water due to low temperature challenge cells by creating a hyperosmotic extracellular environment that osmotically draws water out of cells; desiccation increases hemolymph osmolality and establishes an osmotic gradient (Williams and Lee, 2011) and the freeze concentration resulting from extracellular ice formation creates an osmotic gradient that draws water from intracellular compartments (Lee, 1989). In addition, severely desiccating conditions or low temperatures can compromise membranes fluidity and function (Holmstrup et al., 2002; Bennett et al., 1997) and denature proteins (Pestrelski et al., 1993; Hayward et al., 2004; Rinehart et al., 2007). Not surprisingly, the physiological responses to these osmotic challenges also have many similarities. Accumulation of low molecular mass polyols and sugars (cryoprotectants; such as glycerol) functions to colligatively reduce water loss (Ring and Danks, 1994; Williams and Lee, 2008), unsaturation of membrane lipids maintains fluidity and function (Holmstrup et al., 2002; Bennett et al., 2002), and upregulation of chaperone stress proteins enhances stabilization of membranes and other proteins (Hayward et al., 2004; Rinehart et al., 2007). Recently, Michaud et al. (2008) found that similarities in metabolite production in response to desiccation and cold stress in *B. antarctica*; glycerol, erythritol, nonanoic acid, and succinate levels increased and levels serine decreased.

Although similarities between mechanisms conferring enhanced desiccation and low temperature tolerance have been shown in various invertebrate taxa (see references in Bayley et al., 2001), relatively few studies have investigated the existence of cross-

tolerance, physiological adjustments to one stress enhancing the tolerance of another stress, of these two stresses in invertebrates. However, there are some examples that demonstrate a significant relationship between desiccation acclimation and enhanced cold tolerance at the organismal level. Slow dehydration dramatically increased the freeze-tolerance of larvae of the Antarctic midge, *Belgica antarctica* (Hayward et al., 2007). In addition, fruit flies, *Drosophila melanogaster*, selected for increased desiccation tolerance recovered significantly faster from chill coma than controls (Sinclair et al., 2007). Even in a species with relatively moderate levels of cold tolerance, the soil collembolan, *Folsomia candida*, sub-lethal desiccation stress conferred increased tolerance to cold shock (Bayley et al., 2001).

Larvae of the goldenrod gall fly, *Eurosta solidaginis*, inhabit stem galls of goldenrod plants (*Solidago* spp.) and are dispersed across North America from Florida and Texas to New Brunswick and British Colombia (Uhler, 1951; Miller, 1959; Waring et al., 1990). During the summer, the moist gall tissue provides nutrients for larval growth and development. In autumn, the gall senesces, third-instar larvae cease feeding, and they acquire freeze-tolerance before over-wintering (Uhler, 1951). Gall drying triggers accumulation of the cryoprotectant glycerol that aids in winter survival (Rojas et al., 1986). The gall provides little buffering for *E. solidaginis* against the extreme cold and desiccating conditions of winter (Layne, 1991) and thus makes this species a useful model for cross-tolerance research. *E. solidaginis* larvae prepare for winter by acquiring freezing tolerance and reducing rates of water loss, a correlation that suggests a link between cold tolerance and desiccation resistance (Williams et al., 2004). Consequently, gall fly larvae from higher latitudes have higher levels of cold-hardiness and lower water

loss rates, which suggests that the most cold-hardy populations are also better able to withstand desiccation stress (Williams and Lee, 2008).

Most studies have assessed cross-tolerance by correlating physiological responses to both stresses (Williams et al., 2004; Williams and Lee, 2008) or measuring organismal survival of cold stress after desiccation (Bayley et al., 2001; Hayward et al., 2007). This study extends current cross-tolerance research by evaluating the effect of mild, rapid pre-freezing desiccation on the freeze-tolerance of *E. solidaginis* larvae at the cellular level and by investigating possible mechanistic underpinnings of this response. Based on the relatedness of desiccation and low temperature stress and the similarities of the physiological responses to these stresses, we predict desiccation acclimation will enhance cold tolerance. By using larvae in early autumn when they are not fully cold-hardy, we can isolate desiccation treatment as the cause of any difference in survival between control and treatment groups. Because hemolymph osmolality and glycerol production are important for cold-hardiness in this species, we measured hemolymph osmolality and glycerol content of desiccated and control larvae to determine potential alterations occurring during the development of cross-tolerance.

Methods

Gall collection, body mass, and desiccation resistance

Spherical galls of *E. solidaginis* were collected from goldenrod plants (*Solidago* spp.) at the Miami University Ecology Research Center from July through September 2003 and in October 2011 prior to larval acquisition of the extensive freeze-tolerance found in mid-winter. Galls were stored outdoors and used within two weeks of collection from the field. Larval body mass was determined from July to September 2003 and late-

September to late-October 2011 and water loss rates were determined from late-August to mid-September 2003 and October 2011 according to Ramlov and Lee (2000).

Whole organism viability

Whole larvae (n = 30) were placed in a desiccator at 15°C and dried at 33% or 4% relative humidity (over $CaCl_2$ or Drierite) until they lost ~7% of body mass (Table 1) and then frozen in microcentrifuge tubes for 24h at -15°C or -20°C for September and October larvae, respectively. A lower temperature was needed in October to discriminate substantial mortality between treatments. Control larvae were not desiccated prior to freezing. According to the freezing regimen used by Philip et al. (2008), larvae were cooled at a rate of 0.2°C min^{-1} (4°C to -20°C) and inoculatively frozen at -4°C by spraying the microcentrifuge tubes with Super Friendly Freeze It ® (Fisher Scientific Company, Hanover Park, IL, USA). Viability was assessed by responsiveness to tactile stimuli after thawing for 2h at 22°C.

In vivo *tissue viability*

For assessment of *in vivo* cellular freeze-tolerance, two groups of four larvae from September 2003 collection were used: the treated group was desiccated for 6 h at 33% RH and 15°C before freezing for 24 h at -20°C; and the control group was frozen without desiccation treatment. After a 2-h recovery from freezing at 22°C, fat body (FB), Malpighian tubule (MT), salivary gland (SG) and tracheal (TR) tissue were dissected out and survival was assessed with a LIVE/DEAD sperm viability kit (Molecular Probes, Inc., Eugene, OR) according to Yi and Lee (2003). 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. Survival rates were based on the mean of three counts of 100 cells except in Malpighian tubules where all cells (~65) were counted.

In vitro *tissue viability*

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

Hemolymph osmolality and glycerol content after desiccation

Hemolymph osmolality of October larvae (n =10) was measured for desiccated and control larvae 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.

Glycerol content for desiccated and control larvae was determined according to Philip and Lee (2010). Whole larvae collected in October (n = 10) were homogenized using a Teflon pestle in 0.6N perchloric acid (PCA) and incubated on ice for 5 min. The supernatant was retained after centrifugation for 2 min at 16,000 x g. To neutralize the PCA, an equivalent amount of 1M 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 Chemical Company, Saint Louis, MO #F6428) 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 540nm on a spectrophotometer and values reported as micromoles of glycerol per gram of freeze weight (wet mass and mass after dehydration for control and desiccation groups, respectively).

Statistical Analysis

Organismal and tissue survival relationships, hemolymph osmolality and glycerol concentration were evaluated using unpaired t-tests to compare control and desiccated conditions Additionally for tissue survival, a two-way ANOVA was performed with treatment and tissues as the factors the proportion of tissue survival at the response. The response variable was arcsine square root transformed to stabilize the variance.We used a test of interaction to determine if the effect of a treatment differed between tissue types. If this test was significant, then we would look at contrasts between the tissues to see where the differences were. Otherwise, we tested the main effects of tissue and treatment. Analyses were done using SAS and significance was set at $\alpha = 0.05$.

Results

Seasonal changes in body mass and desiccation resistance

We determined body mass from mid-July to mid-September 2003 and from late September to late October 2011. Additionally, water loss rates were calculated for larvae from late-August to mid-September 2003 and from early October to late October 2011 (Table 1). Body mass increased by ~33 mg from mid-July to mid-September and remained relatively constant at ~57 mg from late September to late October (data not shown). Similarly, desiccation resistance demonstrated a seasonal increase. It took as little as three hours in August to achieve similar levels of desiccation that took 10 days in

October. More intense treatment (i.e. longer time and/or lower RH) was required in order to achieve the same level of desiccation as autumn progressed (Table 1). The trend of increased desiccation resistance agrees with findings from previous studies and continues into mid-winter (Williams et al., 2004).

Freeze-tolerance after mild desiccation

As expected, desiccation enhanced freeze-tolerance at the organismal and tissue levels. Larvae were frozen after losing 6-10% body mass through desiccation treatment (Table 1) or being held at 15°C and survival was assessed at the organismal and cellular levels. To our knowledge, this treatment water loss is the lowest amount that has been used to assess cross-tolerance.

Organismal survival of freezing at -15°C in September and -20°C in October for 24h was significantly enhanced by rapid desiccation (Fig. 1; $p < 0.05$). In fact, it took as little as 6 hours of desiccation to confer enhanced freeze-tolerance in September. Survival increased by ~40% (33.3 ± 6.7 to 73.3 ± 12%) and ~30% (16.7 ± 6.7 to 46.7 ± 3.3%) for September- and October-collected larvae, respectively.

Significantly improved survival at the organismal level prompted investigation into *in vivo* cell survival and reflected the organismal results. Fat body (FB), Malpighian tubule (MT), salivary gland (SG), and tracheal (TR) cell freeze-tolerance reflected the organismal trend and was significantly improved when larvae were desiccated prior to *in vivo* freezing (Fig. 2; Fig. 3). All tissues demonstrated significant increase in survival with desiccation treatment ($p < 0.05$), but tracheal cells had a smaller increase than the other tissues (Fig. 3; FB, ~34%; MT, ~43%, SG, ~38%; TR, ~17%;). This is consistent with the findings of Yi and Lee (2003) for this species; tracheal cell susceptibility to

freezing injury was greater than the other three tissues and those three had comparable susceptibilities. There was no interaction between tissue type and treatment ($F=1.25$, $df_1=3$, $df_2=88$, p-value=0.2151) indicating that the effect of treatment did not differ between tissue type. However, treatment and tissue type both have effects on survival ($F=61.62$, $df_1=1$, $df_2=88$, p-value<0.0001 and $F=7.57$, $df_1=3$, $df_2=88$, p-value=0.0001, respectively).

Since rapid pre-freeze desiccation significantly improved *in vivo* cell survival rates, we determined if isolated tissues would experience increased freeze-tolerance following desiccation by freezing tissues *in vitro* and removed from the hemolymph. Mild desiccation (6-10% mass loss) prior to *in vitro* freezing significantly increased cell survival only in the midgut, and showed no difference for fat body and Malpighian tubule cells (Fig. 4,). Midgut cell survival in desiccated larvae increased ~50% (38.7 ± 4.6 to 89.2 ± 5.5%). Whereas fat body cells showed relatively low rates of survival in control and desiccated groups, Malpighian tubule cells had survival rates > 50% in both (Fig. 4). Similar to the *in vivo* experiment, there was no interaction between tissue type and treatment ($F=3.49$, $df_1=2$, $df_2=18$, p-value=0.0525), but treatment and tissue type both effect survival ($F=19.90$, $df_1=1$, $df_2=18$, p-value=0.0003 and $F=19.80$, $df_1=2$, $df_2=18$, p-value<0.0001, respectively).

Hemolymph osmolality and glycerol contents

Our initial findings demonstrated significant survival at the organismal and tissue level for rapidly desiccated larvae. Therefore, we investigated possible causes of the increased survival by measuring hemolymph osmolality and organismal glycerol concentration. Fluid compartments of larvae were significantly altered by desiccation

treatment. Hemolymph osmolality was ~175 mOsm higher in desiccated larvae compared to controls (720 ± 26 vs. 544 ± 16 mOsm; $p < 0.05$; Fig. 5A). Surprisingly, whole body glycerol concentrations of control and desiccated larvae showed no difference ($p > 0.05$; Figure 5B) and could not explain the increased rates of survival.

Discussion

The association between water relations and cold tolerance has been thoroughly established by studies investigating cross-tolerance. In *B. antarctica* larvae, a 20-50% mass reduction improved survival at -10°C for 48 h by >90% (Hayward et al., 2007). Likewise, exposure to hyperosmotic (1000mOsm) seawater reduced larvae water content by 30-40% and increased survival to -12°C and -15°C by ~30 and 40%, respectively (Elnitsky et al., 2009). Even in a freeze-intolerant organism, *F. candida*, drought acclimation increased survival by ~60% at -2°C and ~30% at -4°C for 48 h. (Bayley et al., 2001). The mild desiccation treatment of the present study in which only 6-10% loss of body mass was lost (Table 1) is the smallest reduction in water content reported to enhance cold-tolerance. Despite the relatively small amount of water lost, our study demonstrated comparable levels of increased survival at the organismal and tissue levels during two different times (September and October) when larvae were not fully winter cold-hardened. Organismal survival increased by 30-40% and tissue survival by ~20-40% because of a slight (6-10%) desiccation-induced mass reduction. These results suggest that larvae are highly sensitive to slight losses of body water and undergo rapid physiological changes, including enhanced cold tolerance, when loss is detected.

Desiccation-induced increases in cold tolerance of other species occur more slowly than what we found in *E. solidaginis* larvae. For instance, *F. candida* was

dehydrated for 7 d prior to cold exposure in a cross-tolerance study by Bayley et al. (2001). Elnitsky et al. (2009) and Hayward et al. (2007) desiccated *B. antarctica* larvae over a shorter time scale (3 and 2 d, respectively), but even these times were longer than the duration used in our study (Table 1). Drying of the gall tissue surrounding larvae in nature occurs over a period of weeks to months (Rojas et al., 1986) and is correlated with increased cold tolerance. Therefore, it was interesting that as little as a 6-h desiccation exposure significantly increased organismal and tissue survival. Even our maximum desiccation duration lasted only 10 days and was shorter than the natural time-span this species faces. However, it is important to note that the intensity of the desiccation treatment was more severe than what would be felt in nature. The rapidity of response induction resembles the rapid cold-hardening (RCH) response that takes place in minutes to hours and suggests a link between RCH and desiccation hardening.

The cold-hardening response to mild desiccation resembles the RCH response in insects and suggests that they may be related. Rapid cold-hardening occurs when sub-lethal (mild) low temperature exposure for minutes to hours (rapid) increases survival to a lethal low temperature (Lee, 1989) and has been shown in freeze-tolerant and intolerant species of insect. Lee et al. (2006) found that larvae of *B. antarctica*, a freeze-tolerant insect, are able to rapidly cold-harden and Chen et al. (1987) demonstrated RCH in the flesh fly *Sarcophaga crassipalpis*, a freeze-intolerant insect. Trends similar to RCH where sub-lethal desiccation exposure enhancing desiccation tolerance have been shown in collembolans. For example, sub-lethal desiccation exposure (48 or 96 h; 45% WC loss in 96 h treatment) increased desiccation tolerance for 5 days in *Cryptopygus antarcticus* (Elnitsky et al., 2008). Similarly, *F. candida* had higher desiccation tolerance if

acclimated to drought conditions for 6 days prior (Sjursen et al., 2001). Our study ties together these data because exposure to sub-lethal levels of desiccation for only hours increased the likelihood larvae would survive a lethal low temperature. Both RCH and desiccation are associated with cellular dehydration that increases hemolymph osmolality (Chen et al., 1987; Elnitsky et al., 2009) and synthesis of cryoprotectants that counteract the osmotic stress (Chen et al., 1987; Ring and Danks, 1994; Williams and Lee, 2008).

We investigated the potential mechanism of the rapid response to desiccation by measuring hemolymph osmolality and found that desiccated larvae had significantly higher hemolymph osmolalities than what 7% water loss would predict. If larvae only lost 7% of their body water, then the resulting hemolymph concentration should have been 582 mOsm. However, the hemolymph osmolality of desiccated larvae was 720 mOsm, which leaves a 138 mOsm discrepancy. This species is known to accumulate cryoprotectants as part of its winter cold-hardening strategy so we expected these compounds to account for osmolality difference. Although *E. solidaginis* larvae employ a multicomponent (glycerol, sorbitol, trehalose) cryoprotectant system (Morrissey and Baust, 1976, Storey et al., 1981; Baust and Lee, 1981,1982), we only measured glycerol because it is the predominant cryoprotectant accumulated this time of year and its synthesis is associated with gall drying (Rojas et al., 1986).

Interestingly, glycerol synthesis could not explain the increased osmolality of desiccated larvae. Because there was no difference in concentration within control and desiccated larvae, it is likely that synthesis of another osmolyte occurred. Since this species uses a multicomponent cryoprotectant system, it is possible that sorbitol was synthesized during the response. Pio and Baust (1988) found that sorbitol concentration

could reach measurable levels in as little as 1 h of chilling. In addition, if our response is similar to that of RCH, then sorbitol is still a likely possibility because sorbitol levels increased during RCH in *S. crassipalpis* (Michaud and Denlinger, 2007). Amino acids are also synthesized during the RCH response and could possibly be responsible for the unaccounted osmolality of desiccated larvae. Alanine is sythesized from pyruvate during RCH in the flesh fly (Michaud and Denlinger, 2007) and may function colligatively (like glycerol) to reduce osmotic stress. Similarly, glutamine may function non-colligatively to enhance cold tolerance (Phanvijhitsiri et al., 2005; Fuchs and Bode, 2006) and because it is upregulated during RCH, it may contribute to the osmolality difference. Finally, synthesis of osmolytes may not be entirely responsible for the extra 138 mOsm in the hemolymph of desiccated larvae; instead, movement of molecules between compartments may be altering hemolymph concentrations. Aquaporins are upregulated during freezing, facilitate the movement of water and glycerol in and out of cells (Ishibashi et al., 1994; Philip et al., 2008), and may have allowed for the change of hemolymph composition.

A variety of rapid environmental fluctuations can induce the RCH response and the same mechanism may be occurring in our study. For example, heat and cold can induce RCH in *S. crassipalpis* (Chen et al., 1987; Lee et al., 1987). In addition, Coulson and Bale (1991) found that RCH could be induced by anoxia in the house fly. Our results suggest the possibility that desiccation may also induce the RCH response in insects. The exact physiological mechanism of this rapid response to stress remains somewhat unclear, but a study by Teets et al. (2008) found that calcium plays an important role. Calcium is involved as a second messenger in many stress responses (Mahajan and Tuteja, 2005) and it allows the messenger protein calmodulin to interact with other signal

proteins by inducing a conformational change (Ikura, 1996). Since cell survival was significantly reduced in isolated RCH cells when calcium was excluded from the bathing solution or chelated with BAPTA, or when calmodulin was inhibited (Teets et al., 2008), a calcium-signaling pathway leading to activation of transcription factors is likely an important part of the RCH response. In addition, the glycolytic pathway seems to be important because pyruvate (the end product of this pathway) is upregulated during RCH in the flesh fly (Michaud and Denlinger, 2007). It is known that rapid cold-hardening occurs intracellularly without neuroendocrine input because isolated cells are capable of demonstrating the response (Teets et al., 2008; Yi and Lee, 2004) Finally, p38 MAPK is phosphorylated in as little as 10 mins in the RCH induction temperature range (Fujiwara and Denlinger, 2007) and likely plays a role in the signal transduction pathway. Because there is evidence linking our rapid response to desiccation and RCH, we hypothesize that they operate under similar mechanisms.

Conclusion

We demonstrated that mild, rapid desiccation prior to freezing significantly increases survival at the organismal and cellular levels. We found a higher hemolymph osmolality in desiccated larvae, but no difference in glycerol concentration between desiccated and control groups. Lack of glycerol production because of desiccation suggests that other osmolytes are synthesized and/or intrinsic mechanisms must be occurring to cause the higher survival rates. The rapidity with which freeze-tolerance was conferred creates a link between our desiccation stress response and the RCH response and the possibility that they operate under similar mechanisms. Our results support the hypothesis that insect responses to desiccation and cold stress are potentially derived

from the same ancestral stress response, but further study is needed to elucidate the exact mechanism.

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Table 1. Mass loss due to desiccation treatment of *E. solidaginis* larvae at 15°C. Date refers to the starting date of the desiccation treatment and RH is relative humidity. Decreased RH and/or increased duration was needed to account for increased desiccation resistance acquired by larvae this time of year. Water loss rates calculated according to Ramlov and Lee (2000).

Date	RH (%)	Duration (h)	Percent mass loss (mean ± SEM)	Water loss rate (ug mm^{-2} h^{-1})
2003				
8/26	33	3	8.3 ± 0.8	29.9
9/5	33	6	6.5 ± 0.8	12.7
9/11	33	6	4.3 ± 0.3	9.1
2011				
10/7	33	29	6.3 ± 0.7	3.2
10/8	33	168	8.4 ± 1.1	0.7
10/12	4	144	7.6 ± 1.3	0.7
10/23	4	240	9.4 ± 2.0	0.5

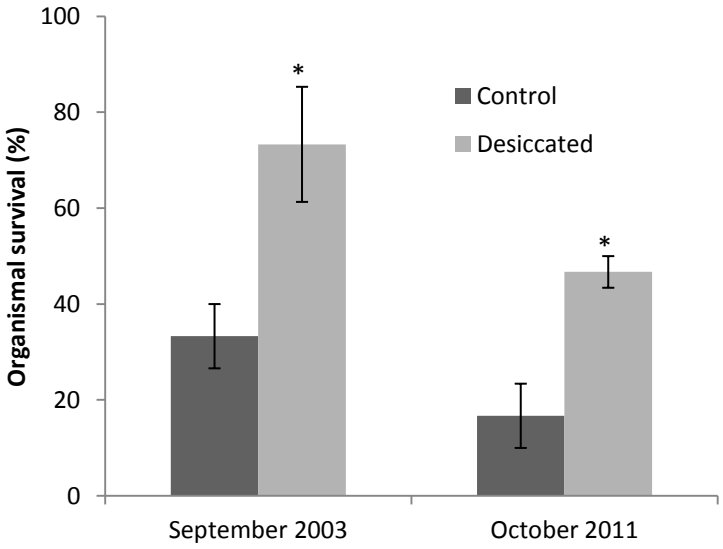

Fig. 1 Desiccation treatment significantly increased organismal survival of larvae after freezing at -15°C (September) or -20°C (October) for 24h ($p < 0.05$; n = 30). Means (± SEM) marked with (*) were significantly higher than the control of the same year.

Fig. 2 Cell survival (mean ± SEM; n = 4) after freezing *in vivo* at -20°C for 24h was significantly higher for desiccated larvae than controls in September 2003. Values marked with (*) were significantly higher than controls of the same tissue following a t-test ($p < 0.05$).

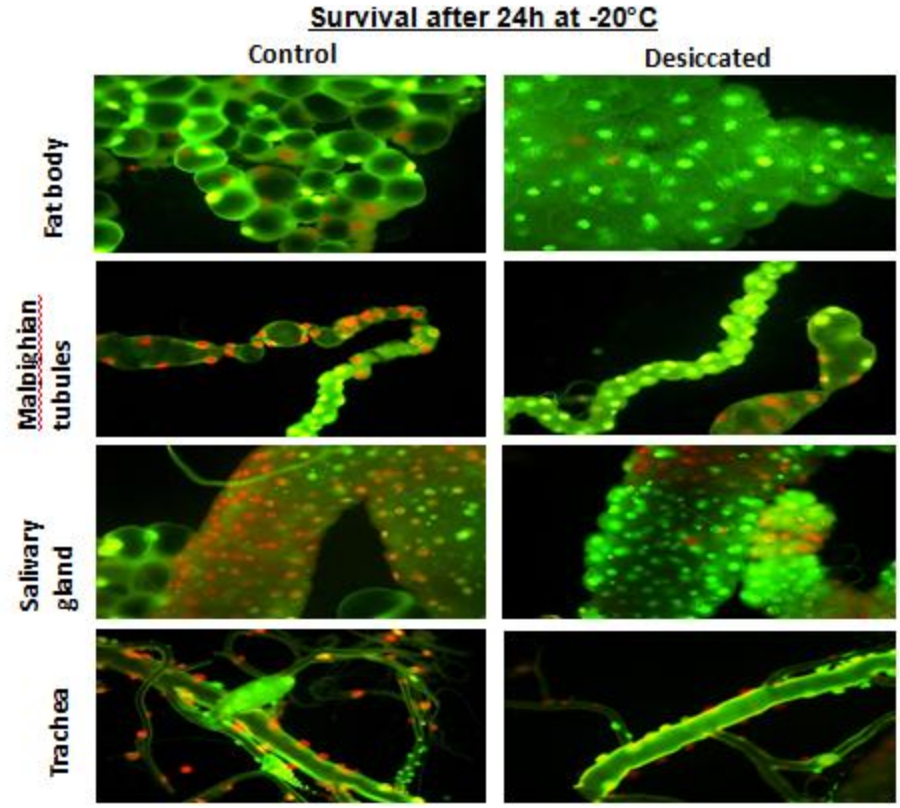

Fig. 3 Desiccation treatment improved *in vivo* cellular freeze tolerance in fat body, Malpighian tubules, salivary gland, and trachea of larvae collected in September 2003. Larvae were either non-desiccated (left panel) or desiccated at 33% RH and 15°C, 6 h (right panel) followed by freezing at -20°C for 24h. Cell viability of each tissue was assessed using the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR) as modified by Yi and Lee (2003).

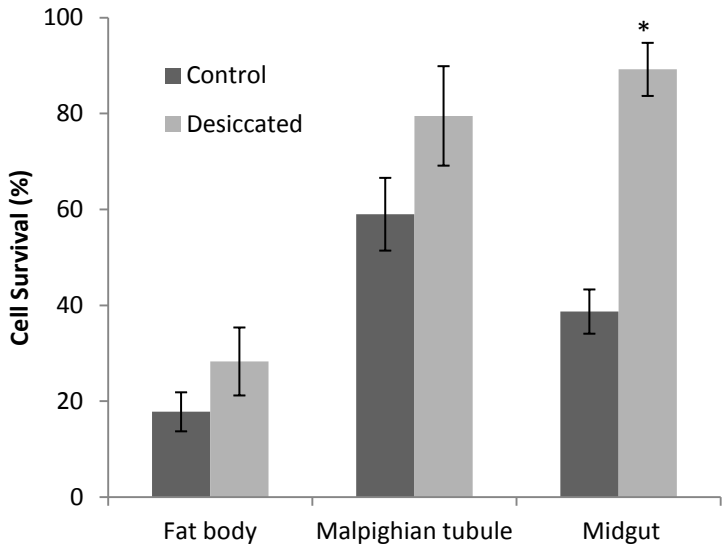

Fig. 4 Cell survival (mean ± SEM; n=4) after *in vitro* freezing at -20°C of larvae collected in October 2011. Desiccated larvae were dehydrated prior to tissue extraction and freezing. Values marked with (*) were significantly higher than controls of the same tissue following an unpaired t-test ($p < 0.05$).

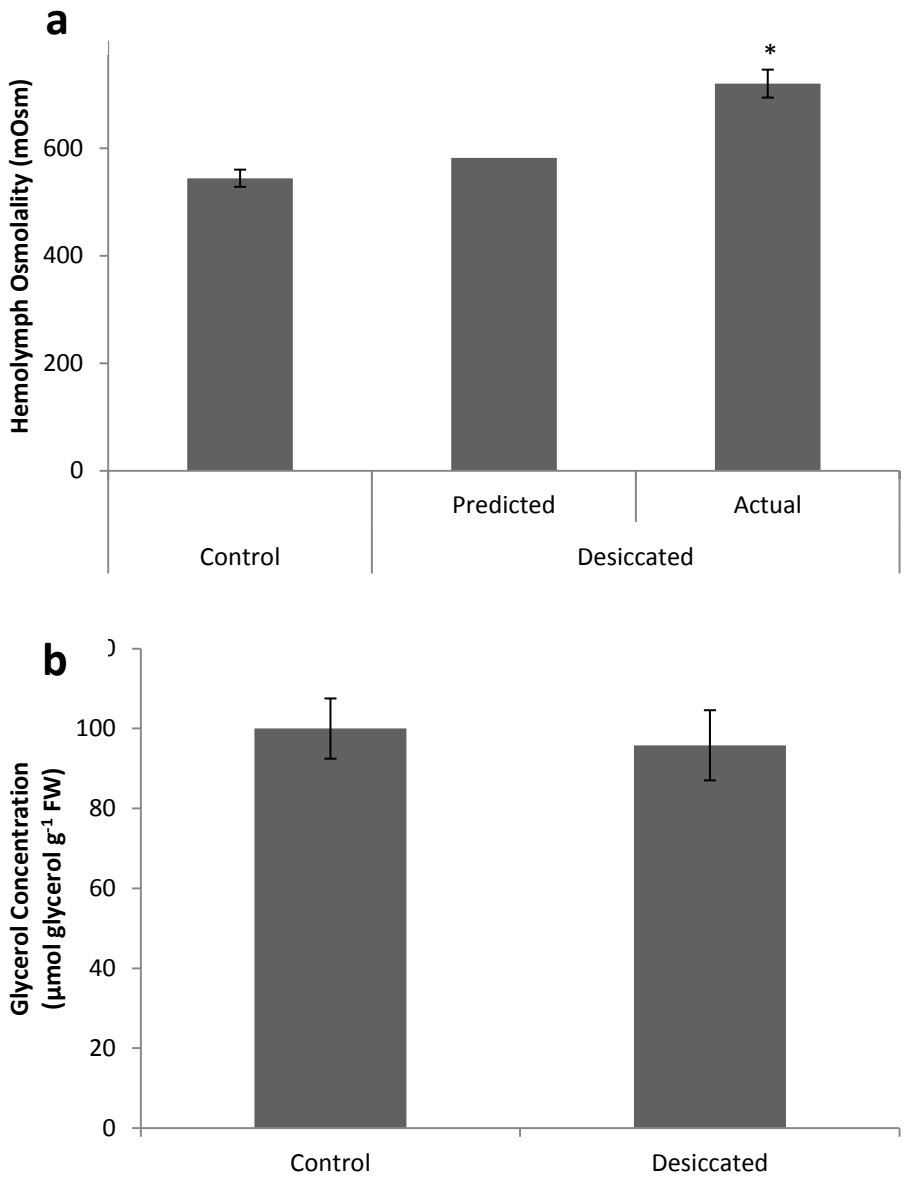

Fig. 5 a) Desiccated larvae collected in mid-October had significantly higher hemolymph osmolality compared to controls and predicted osmolality values for 7% water loss ($p < 0.05$; n = 10). b) No difference was found for glycerol concentration of desiccated and control larvae ($p = 0.7221$; n = 10; FW indicates fresh weight). The ~140mOsm discrepancy between the predicted and actual values for the osmolality of desiccated larvae cannot be explained by glycerol synthesis.

Cross-tolerance in larvae of the goldenrod gall fly, *Eurosta solidaginis*: rapid desiccation increases organismal and cellular freeze-tolerance

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