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# The limits of drought-induced rapid cold-hardening: Extremely brief, mild desiccation triggers enhanced freeze-tolerance in *Eurosta solidaginis* larvae ☆

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ABSTRACT

Rapid cold-hardening (RCH) is a highly conserved response in insects that induces physiological changes within minutes to hours of exposure to low temperature and provides protection from chilling injury. Recently, a similar response, termed drought-induced RCH, was described following as little as 6 h of desiccation, producing a loss of less than 10% of fresh mass. In this study, we investigated the limits and mechanisms of this response in larvae of the goldenrod gall fly *Eurosta solidaginis* (Diptera, Tephritidae). The cold-hardiness of larvae increased markedly after as few as 2 h of desiccation and a loss of less than 1% fresh mass, as organismal survival increased from 8% to 41% following exposure to −18 °C. Tissue-level effects of desiccation were observed within 1 h, as 87% of midgut cells from desiccated larvae remained viable following freezing compared to 57% of controls. We also demonstrated that drought-induced RCH occurs independently of neuroendocrine input, as midgut tissue desiccated *ex vivo* displayed improved freeze-tolerance relative to control tissue (78–11% survival, respectively). Finally, though there was an increase in hemolymph osmolality beyond the expected effects of the osmo-concentration of solutes during dehydration, we determined that this increase was not due to the synthesis of glycerol, glucose, sorbitol, or trehalose. Our results indicate that *E. solidaginis* larvae are extremely sensitive to desiccation, which is a triggering mechanism for one or more physiological pathways that confer enhanced freeze-tolerance.



## 1. Introduction

Insects have a remarkable ability to quickly adjust to changing ambient conditions. For example, rapid cold-hardening (RCH) induces physiological changes within minutes to hours, increasing survival while protecting against damage from cold shock (Chen et al., 1987; Lee et al., 1987; Overgaard et al., 2007). Traditionally, RCH has been studied as a response to brief exposure to sub-lethal chilling; however, RCH-like responses can also be induced by acute exposure to heat and anoxia (Chen et al., 1987; Coulson and Bale, 1991). Recently, drought-induced RCH was described following brief exposure to desiccation stress (Levis et al., 2012; Sinclair and Chown, 2003).

Despite the generality of RCH in insects, little is known about underpinning mechanisms and, due to its novelty, drought-induced RCH is even more poorly understood. However, while investigating drought-induced RCH in *Eurosta solidaginis* larvae, Levis et al. (2012) noted an increase in hemolymph osmolality that was not attributable to either the osmo-concentration of solutes during drying or the synthesis of glycerol. During seasonal cold-hardening, many insects accumulate low molecular mass polyols and sugars that function to stabilize membrane and protein structures, act as a replacement for water, and colligatively depress freezing points and reduce water loss (Crowe et al., 1988; Salt, 1961; Duman, 1977; Duman and Horwath, 1983). Despite the nearly ubiquitous synthesis and accumulation of cryoprotectant compounds during winter cold-hardening, there is no consistent sugar or polyol response associated with RCH (Lee and Denlinger, 2010).

While seasonal cold-hardening is thought to be hormonally regulated, RCH can occur independently of input from the neuroendocrine system, as excised tissues can be treated *ex vivo* to induce RCH (Yi and Lee, 2003, 2004). Further, Teets et al. (2008, 2013) demonstrated that cold-sensing at the cellular level is mediated by temperature-driven calcium flux. Calcium is an activator of the p38 mitogen-activated protein kinase (p38 MAPK) pathway, which modulates cellular activity independently of neuroendo-

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crine input and is activated during RCH induction in the freeze-intolerant flesh fly, *Sarcophaga crassipalpis* (Fujiwara and Denlinger, 2007; Teets et al., 2008). Due to the rapidity of the response, it seems likely that drought-induced RCH may also be sensed and induced at the cellular level in a similar manner.

The goldenrod gall fly, *E. solidaginis*, is widespread in eastern North America, ranging from Florida to Texas and north into southern Canada (Uhler, 1951). Larvae develop and overwinter in stem galls on goldenrod plants (*Solidago* spp.). Throughout the summer and early-to-mid autumn, larvae are susceptible to desiccation if removed from the high humidity environment in their galls (Williams et al., 2004; Williams and Lee, 2005). In winter, cold-hardened larvae are more resistant to desiccation than all but the most xeric-adapted insects (Ramlov and Lee, 2000). During the transition from summer to winter, larvae gradually acquire desiccation tolerance. For a few weeks in September and October, they are hardy enough to readily survive exposure to acute stresses, yet are not so resistant to water loss as to render rapid dehydration impossible (Rojas et al., 1986; Williams et al., 2004; Williams and Lee, 2005).

To better understand drought-induced RCH and to compare chilling and desiccation as RCH-induction triggers, we characterized the thresholds of drought-induced RCH relative to the duration of exposure and magnitude of desiccation in September and October collected *E. solidaginis* larvae. We also examined the effects of acute desiccation in fat body and midgut tissues *ex vivo*. Lastly, we investigated select cryoprotectants to determine the source of the observed increase in hemolymph osmolality.

## 2. Methods

### 2.1. Collection and water content

Spherical galls containing *E. solidaginis* larvae were collected from goldenrod plants in Butler County, Ohio. Galls were collected from early September through mid-October, 2013 and stored outside for up to one week before use. During these few weeks, larvae were an appropriate size for experimentation ($\geqslant$25 mg) and had not yet developed the extreme desiccation resistance that results from winter cold-hardening. At the beginning of each treatment, larvae were removed from their galls and weighed. After treatment, larvae were dried at 65 °C until they reached a constant mass. Water content was determined by the difference between initial mass and dry mass. When appropriate, larvae were flash frozen in liquid nitrogen (−196 °C) and then held at -80 °C until needed.

### 2.2. Freezing-tolerance

To test for differences in freeze-tolerance, larvae were exposed to discriminating cold temperature by placing them in 0.6 ml microcentrifuge tubes in a programmable refrigerated bath. Larvae were placed in contact with a small piece of ice during cooling, thus inoculating internal ice formation near the freezing point of the hemolymph. A critical test temperature was selected such that control larvae experienced ~20% survival after 24 h of freezing. During our study, this temperature decreased from −15 °C to −20 °C as field-collected larvae progressively increased their cold-hardiness in preparation for winter. Survival was determined by response to tactile stimulation following a 2 h recovery at room temperature.

### 2.3. Vital dye assay for cell survival

Fat body and midgut tissues were dissected from larvae in Coast's solution (Coast, 1988). Cell membrane integrity was determined using the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR) as adapted by Yi and Lee (2003). In this assay, tissues were incubated in SYBR-14, a membrane-penetrating green fluorescent dye, and propidium iodide, a non-penetrating red fluorescent dye. Cells with intact membranes excluded the propidium iodide and fluoresced green, while those that sustained membrane damage appeared red–orange. During microscopy, we counted living and dead cells visible within the field of view. Three-to-four frames containing at least 50 cells were counted per tissue and the results were compiled to determine a rate of survival for the tissue in one larva. These results were averaged for 3–4 larvae for each treatment and are expressed as a percentage of the cells that remained impermeable to propidium iodide.

### 2.4. Treatments to induce RCH

Cold-induced RCH larvae were placed in plastic weighing dishes and subjected to a mild cold treatment (0 °C) for 2 h on ice. The thresholds of drought-induced RCH were determined by exposing groups of larvae to desiccation treatments of varying duration and relative humidity. Desiccation treatments lasted between 0.5 h and 12 h. Anhydrous calcium sulfate (Drierite®) and saturated salt solutions were used in sealed desiccating chambers to create different environments: Drierite®(0% RH), $MgCl_2$ (33% RH), NaCl (75% RH), KCl (85% RH), or $K_2SO_4$ (98% RH) (Greenspan, 1977). After treatment, freezing-tolerance was determined as previously described.

### 2.5. Drought-induced RCH ex vivo

To determine if drought-induced RCH can occur independently of the neuroendocrine system, target tissues were rapidly dehydrated *ex vivo*. Hemolymph (80–100 µl, pooled from 10–12 *E. solidaginis* larvae) was collected in microcapillary tubes through an incision in the cuticle. Excised tissues were placed in a small plastic weighing dish and completely submerged in the pooled hemolymph. The dish was transferred to a humidity-controlled desiccation chamber, as described above, and incubated for 30 min, 1 h, 2 h, or 3 h. During incubation, water evaporated from the hemolymph, resulting in the concentration of solutes and exposing the tissue to osmotic stress that mimicked the effect of organismal dehydration. Following desiccation, tissues were placed in a microcentrifuge tube in Coast's solution and frozen at an appropriate discriminating test temperature. The vital dye assay was then performed to assess tissue-level damage as previously described.

### 2.6. Hemolymph osmolality

Hemolymph osmolality was measured by drawing hemolymph into a microcapillary tube through an incision in the larval cuticle. Hemolymph was pooled from 2–3 larvae to obtain a sufficient volume for measurement. Osmolality was measured using a Model 3320 freezing-point depression osmometer (Advanced Instruments Inc., Norwood, MA).

### 2.7. Cryoprotectant content

Whole larvae were weighed and stored at −80 °C until cryoprotectant content was assessed. Larvae were homogenized in 0.6 N perchloric acid (PCA) to extract metabolites and the neutralized extract was used for each of the assays. Glycerol content was determined using the method described by Holmstrup et al. (1999). Briefly, 800 µl of glycerol free reagent (Sigma–Aldrich Chemical Company, Saint Louis, MO #F6428) was reconstituted and added to 200 µl aliquots of PCA extract. Following a 15-min incubation

at 37 °C, absorbance was read at 540 nm on a Jenway model 6705 UV/Vis spectrophotometer.

Trehalose and glucose levels were measured by aliquoting PCA extract into 100 µl portions for trehalose and glucose determination. Trehalose samples were treated with trehalase (Sigma Chemical Co. # T8778) diluted to a concentration of 0.2 units/ml in 135 mM citric acid buffer (pH 5.7), and incubated overnight at 37 °C to cleave trehalose molecules into two glucose subunits. The samples were analyzed using a colorimetric glucose assay kit (Sigma Chemical Co. # GAGO20) and absorbance was read at 540 nm. Trehalose content was determined by the difference in absorbance between trehalase-treated and untreated fractions.

Sorbitol content was measured using the enzymatic assay from Bergmeyer et al. (1974). Briefly, a reaction mixture containing 500 µl PCA extract, 1000 µl 0.1 M sodium pyrophosphate, and 50 µl 30 mM NAD was prepared. The absorbance of this mixture was measured at 340 nm, before 25 µl sorbitol dehydrogenase (5 mg protein/ml) was added. Following a 60-min incubation at ~22 °C, absorbance was read again at 340 nm.

### 2.8. Statistical analysis

All data were analyzed using R (R Foundation for Statistical Computing, Vienna, Austria). Organismal and tissue survival rates were analyzed using binomial logistic regression with a post hoc Bonferroni correction. Cryoprotectant levels and changes in hemolymph osmolality passed the Shapiro–Wilk test for normality ($p < 0.05$) and were analyzed with one-way analysis of variance tests with Bonferroni multiple comparisons corrections.

## 3. Results

### 3.1. Drought-induced RCH improved freeze-tolerance

At the organismal level, exposure to acute desiccation significantly improved larval freeze-tolerance in as little as 2 h (Figs. 1 and 2). Survival of freezing improved from ~8% to over 40% in September-collected larvae following 2 h of desiccation at 0, 33 or 75% RH ($p < 0.01$; Fig. 1). In the 2-h treatments at 33% and 75% RH, larvae lost less than 1% of their fresh mass (0.85% and 0.68%, respectively), whereas the 0% RH treatment produced a loss of 3.3% fresh mass. Chilling at 0 °C for 2 h (RCH) and simultaneous chilling and desiccation at 0 °C and 0% RH (D+RCH) did not significantly affect survival (Fig. 1). Among 0% RH treatments, both 2 h and 4 h of desiccation significantly enhanced freeze-tolerance (Fig. 2). Shorter exposure to desiccation did not significantly improve survival. Larvae desiccated for 12 h, resulting in a loss of 7.3% of fresh mass, were no more freeze-tolerant than controls (Fig. 2).

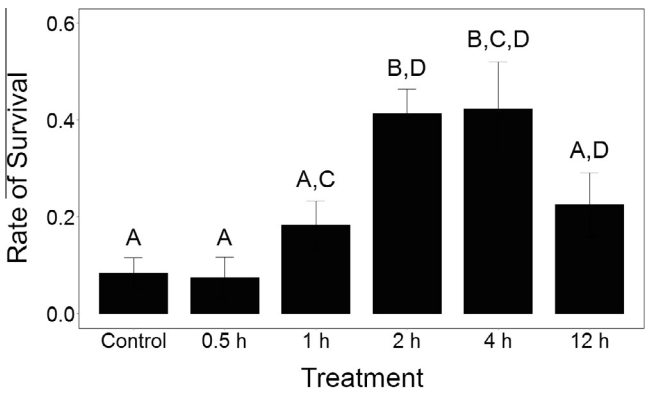


**Fig. 1.** Desiccation for as little as 2 h significantly enhanced larval *E. solidaginis* tolerance of freezing at −18 °C for 24 h. Larvae ($n \geq 26$) were desiccated at 0% RH. Treatments (±SEM) sharing a letter were not statistically distinguishable ($p = 0.05$).

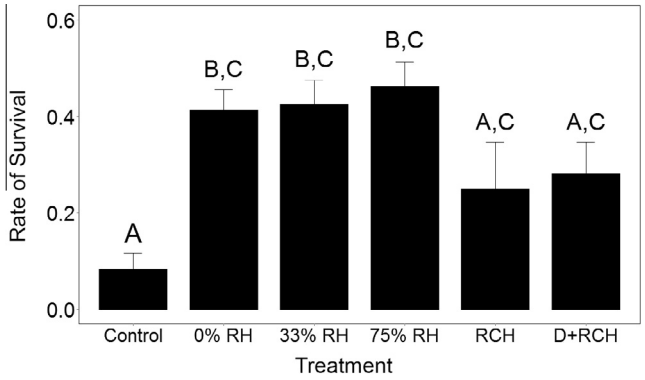


**Fig. 2.** Severity of desiccation had no effect on larval *E. solidaginis* tolerance of freezing at −18 °C for 24 h. Larvae ($n \geq 26$) were exposed to 0 °C, 0% RH and 0 °C simultaneously, and desiccated at 0%, 33%, and 75% RH for 2 h. Treatments (±SEM) sharing a letter were not statistically distinguishable ($p = 0.05$).

These organismal-level observations were corroborated by measures of cell viability in fat body and midgut tissues (Figs. 3 and 4). Survival rates of midgut cells from larvae desiccated for 2 h at 0, 33 and 75% RH improved from 57% to 94, 95, and 92%, respectively ($p < 0.01$; Fig. 3). Further, 2 h RCH and D+RCH treatments improved cellular survival to 85 and 90%, respectively ($p < 0.01$). Fat body cells exhibited similar, but less dramatic improvements in survival.

Midgut cells from larvae exposed to 0% RH displayed significantly improved freezing tolerance following as little as 1 h ($p < 0.01$) and the difference remained significant following treatments as long as 12 h ($p < 0.05$; Fig. 4). While 12 h of desiccation at 0% RH improved survival relative to controls, cold-hardiness was significantly lower in this treatment group than in larvae desiccated for 2 h at 0% RH ($p < 0.01$).

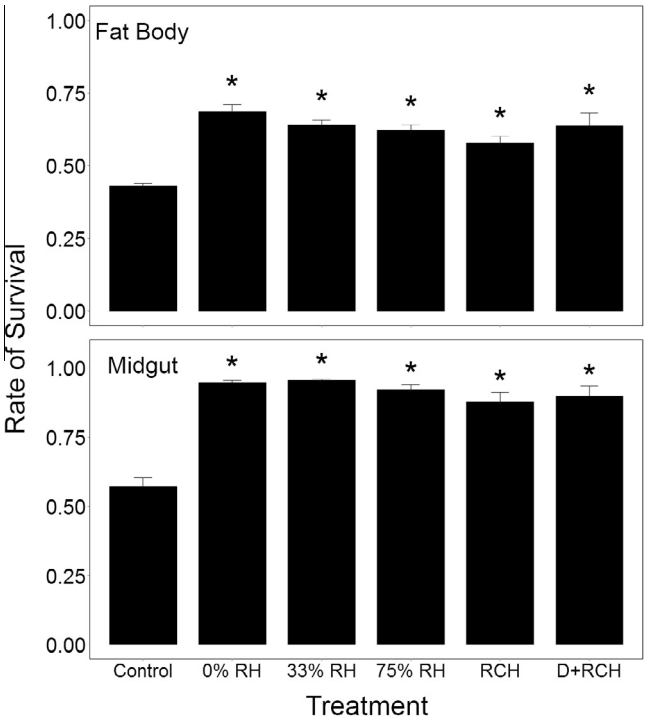


**Fig. 3.** Severity of desiccation had no effect on freezing tolerance of larval *E. solidaginis* fat body or midgut tissue. Larvae ($n \geq 3$) were exposed to 0 °C, 0% RH or 0 °C simultaneously, and desiccated at 0%, 33%, or 75% RH for 2 h following which the appropriate tissues were excised and frozen at -18 °C for 24 h. Analysis was carried out within tissue types. * denotes $p \leq 0.05$.

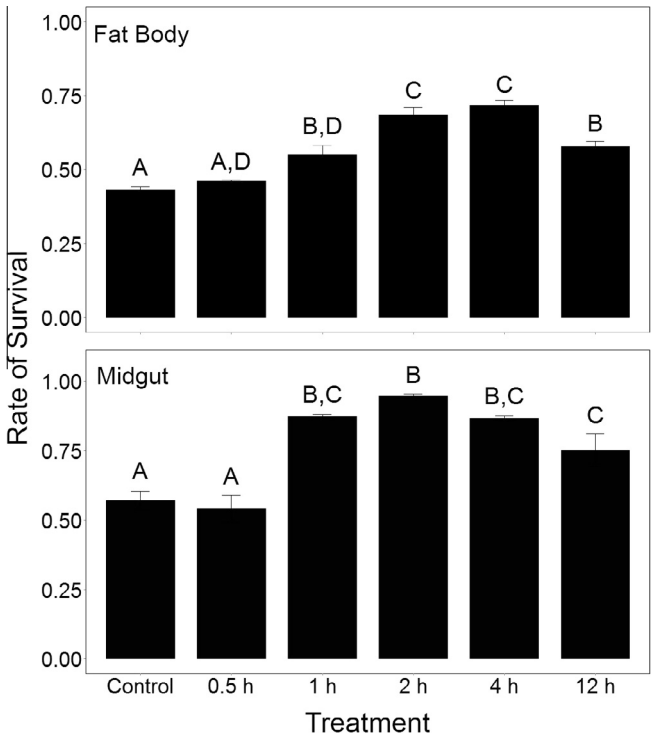


**Fig. 4.** Desiccation for as little as 1 h significantly enhanced larval *E. solidaginis* fat body and midgut tissue tolerance of freezing. Larvae ($n \geqslant 3$) were desiccated at 0% RH for various durations, following which tissues were excised and frozen at −18 °C for 24 h. Treatments (±SEM) sharing a letter were not statistically distinguishable ($p = 0.05$).

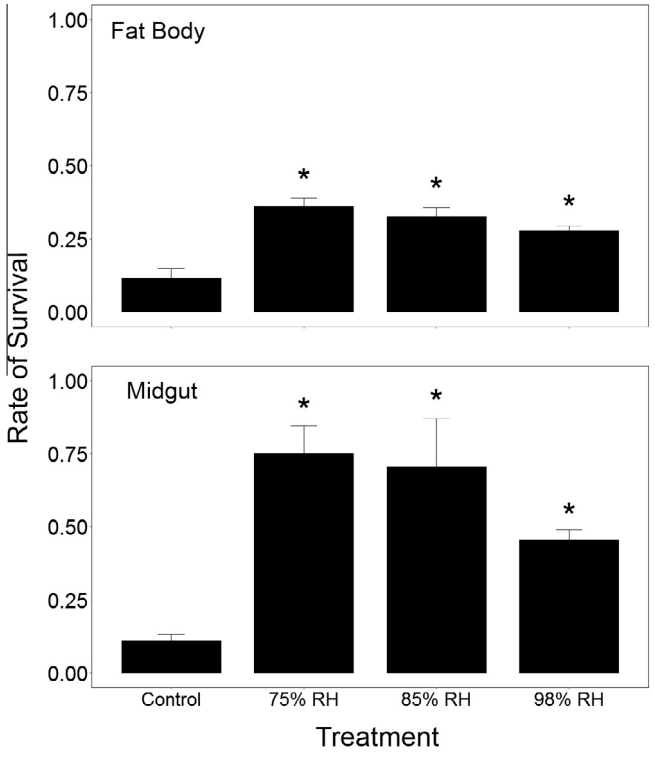


**Fig. 5.** Severity of desiccation had no effect on freezing tolerance of larval *E. solidaginis* fat body or midgut tissue cold-tolerance. Tissues from 3–5 larvae were excised and dehydrated *ex vivo* at 75, 85, and 98% RH for 2 h following which they were frozen at −18 °C for 24 h. Analysis was carried out within tissue types. * denotes $p \leqslant 0.05$.

### 3.2. Drought-induced RCH enhanced tissue cold-hardiness ex vivo

Due to mechanical damage to tissues caused by handling, we were not able to measure the amount of water lost per tissue sample during *ex vivo* desiccation. In lieu of a direct measure of tissue water loss, we measured changes in the osmotic pressure of the hemolymph in which the tissues were bathed. Since this mirrors natural conditions by relying on the increasing concentration of solutes in the hemolymph to dehydrate the tissues, we used this method as a proxy for organismal dehydration. The increases in hemolymph osmolality were approximately linear during our 3 h desiccation treatments, averaging ~100, 61, and 25 mOsm $kg^{-1}$ per hour at 75, 85, and 98% RH, respectively. Assuming an initial hemolymph osmolality of ~600 mOsm $kg^{-1}$, these rates translate to 16.7, 10.2, and 4.2% water loss per hour, respectively.

Fat body and midgut tissues desiccated *ex vivo* rapidly cold-hardened in the absence of a connection to the neuroendocrine system. Freezing-tolerance of midgut tissue desiccated at 75% RH for 2 h improved from 11 to 78% relative to controls ($p < 0.05$; Fig. 5). Desiccation for 2 h at 85 and 98% RH improved survival of midgut cells from 11% to 74 and 45%, respectively ($p < 0.05$). Again, fat body tissues exhibited similar, but less pronounced improvements in cold-hardiness. Further, fat body tissues showed significantly improved cold-hardiness following pre-treatments of 1 h, 2 h, and 3 h at 75% RH ($p < 0.05$), while midgut tissues had significantly enhanced survival after 2 h and 3 h ($p < 0.01$; Fig. 6). In independent experiments, incubation in hemolymph for 2 h at 100% RH produced no change in the freeze-tolerance of fat body or midgut tissue (data not shown).

### 3.3. Hemolymph osmolality and cryoprotectant levels

Brief desiccation elevates *E. solidaginis* hemolymph osmolality beyond the effects of osmo-concentration of solutes; however,

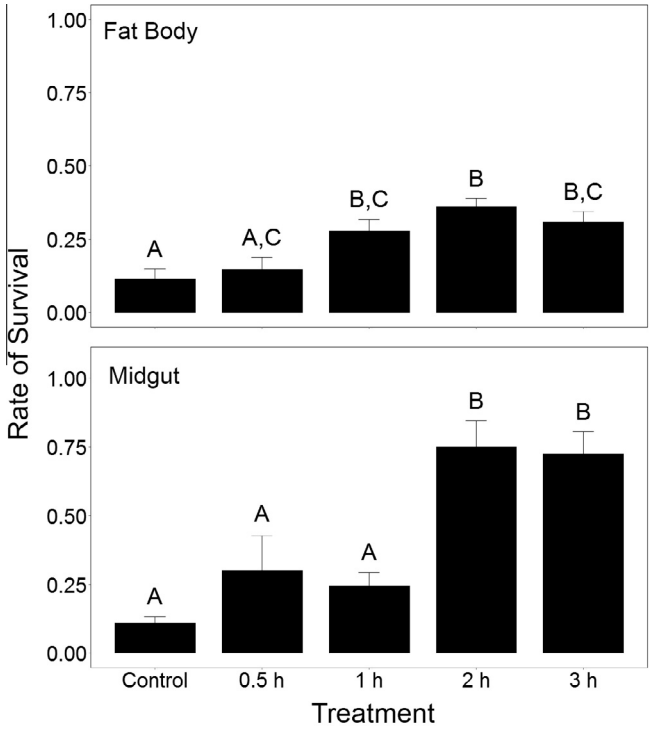


**Fig. 6.** Desiccation for as little as 1 h significantly enhanced larval *E. solidaginis* fat body tissue tolerance of freezing. Tissues from 3–5 larvae were excised and dehydrated *ex vivo* at 75% RH, following which the tissues were frozen at −18 °C for 24 h. Treatments (±SEM) sharing a letter were not statistically distinguishable ($p = 0.05$).

**Table 1**
Changes in hemolymph osmolality elicited by drought-induced RCH in larvae collected and treated in mid-October. Hemolymph was pooled from 2–3 larvae as needed; $n = 6$ per treatment. All treatments lasted 2 h unless noted. Control larvae were untreated. Predicted osmolality is calculated based on the control group measured hemolymph osmolality and the percent body water lost. Measured hemolymph osmolality values are mean ± SEM. Statistical significance ($p \leqslant 0.05$) is denoted by * and indicates the mean measured hemolymph osmolality of the treatment is distinguishable from the control.

| | Control | Hydrated, cold (98% RH, 0 °C) | Mildly dehydrated, warm (75% RH, 22 °C) | Dehydrated, warm (0% RH, 22 °C) | Dehydrated, cold (0% RH, 0 °C) | Dehydrated, prolonged, warm (0% RH, 4 h, 22 °C) |
|---|---|---|---|---|---|---|
| Body water lost (percent) | 0 | 0.3 | 0.7 | 1.8 | 2.0 | 4.0 |
| Predicted osmolality (mOsm $kg^{-1}$) | 573 | 574 | 576 | 583 | 584 | 593 |
| Measured hemolymph osmolality (mOsm $kg^{-1}$) | 573 ± 14 | 588 ± 7 | 616 ± 13 | 620 ± 14 | 632 ± 14* | 656 ± 8* |
| Unexplained Δ osmolality (mOsm $kg^{-1}$) | 0 | 14 | 40 | 37 | 48 | 63 |

**Table 2**
Cryoprotectant concentrations (μmol $ml^{-1}$) in larvae collected and treated in mid-October. All treatments ($n = 6$) lasted 2 h, unless noted. Control larvae were untreated. No treatment elicited significant differences from controls ($p \leqslant 0.05$).

| | Control | Frozen (−15 °C) | Dehydrated (0% RH) | Moderately dehydrated (33% RH) | Mildly dehydrated (75% RH) | Dehydrated, prolonged (0% RH, 4 h) |
|---|---|---|---|---|---|---|
| Glucose | 1.2 ± 0.6 | 1.8 ± 0.7 | 1.5 ± 0.8 | 2.1 ± 1.1 | 0.8 ± 0.5 | 1.2 ± 0.7 |
| Trehalose | 2.4 ± 0.2 | 2.6 ± 0.3 | 2.3 ± 0.5 | 2.5 ± 0.3 | 2.3 ± 0.2 | 2.2 ± 0.4 |
| Sorbitol | 1.7 ± 0.5 | 2.1 ± 0.6 | 1.1 ± 0.3 | 2.4 ± 0.8 | 1.2 ± 0.3 | 0.7 ± 0.2 |
| Glycerol | 473 ± 51 | 443 ± 45 | 545 ± 44 | 383 ± 78 | 407 ± 67 | 441 ± 50 |

the solute(s) driving this increase is not known (Levis et al., 2012). In our study, we found an ~80 mOsm $kg^{-1}$ increase in hemolymph osmolality within 4 h of desiccation, whereas the effects of dehydration only explained a change of ~20 mOsm $kg^{-1}$ ($p < 0.01$; Table 1). Further, hemolymph osmolality was significantly elevated following 2 h of simultaneous exposure to low temperature and desiccation (0 °C and 0% RH) ($p < 0.05$; Table 1). In order to determine the cause of the increase in hemolymph osmolality, we measured the levels of four commonly accumulated cryoprotectants: glucose, trehalose, sorbitol, and glycerol. Acute desiccation did not elicit significant changes in the level of any of these compounds (Table 2).

## 4. Discussion

Freezing and dehydration present many of the same physiological challenges; thus, adjustments made to contend with desiccation stress often increase cold-tolerance as well (Block, 1996; Danks, 2000; Ring and Danks, 1994; Williams et al., 2004). For example, long-term drought-acclimation decreases the lower lethal temperature of the soil collembolan *Folsomia candida* by ~6 °C (Bayley and Holmstrup, 1999) and slow desiccation over the course of 2 d enhances freeze-tolerance in *B. antarctica* (Hayward et al., 2007). The interplay between desiccation and cold-tolerance is usually attributed to the induction of seasonal cold-hardening and to the effects of osmo-concentration of solutes within the body (Holmstrup et al., 2010). However, as few as 6 h of dehydration significantly enhances freezing tolerance in larvae of *E. solidaginis* and *Pringleophaga marioni*, suggesting that brief desiccation can trigger an RCH-like response (Levis et al., 2012; Sinclair and Chown, 2003). In this study, we defined the minimum amount of dehydration required to trigger RCH in *E. solidaginis* larvae. Desiccation for 1 h markedly improved freeze-tolerance in fat body and midgut tissues and increased organismal freezing tolerance within 2 h. Further, the protection afforded by dehydration was maximized within 2 h; that is, the rate of survival was not further improved by desiccating larvae for longer periods. These results closely resemble the timing of chilling-induced RCH with respect to the rate of induction and the time required to fully activate this response (Lee and Denlinger, 2010).

Reduced body water content decreases the amount of damage sustained during freezing by concentrating solutes within the body, which colligatively depresses the freezing point, tempers ice formation, and helps to stabilize membrane proteins (Holmstrup et al., 2010). Even slight increases in solute concentrations (i.e. 10–30 mM) can be beneficial during bouts of low temperature (Carpenter and Crowe, 1988). Sugars and polyols accumulated in low concentrations do not have significant effects on the colligative properties of hemolymph; however, they can improve cold-tolerance by stabilizing membrane and proteins structures and acting as a replacement for water (Carpenter and Crowe, 1988; Crowe et al., 1988). Thus, it was unclear whether drought-induced RCH is a result of the effects of modest osmo-concentration of body water or if a physiological response is triggered by slight desiccation. To answer this question, we investigated how the amount of water lost during dehydration affects freeze-tolerance. Survival rates were nearly identical among treatment groups desiccated for 2 h at 0, 33, or 75% RH. Remarkably, larvae desiccated at 75% RH lost only 0.68% of their fresh mass on average and survived at the same rate as larvae desiccated at 0% RH, which lost 3.34% of their fresh mass (Figs. 2 and 3). If the protective effects of dehydration are a result of the concentration of solutes via the removal of body water alone, survival rates should be influenced by the severity of dehydration. Since our data demonstrate that the efficacy of drought-induced RCH was independent of the amount of water lost, we conclude that this is a physiological response that is triggered by slight desiccation.

Drought-induced RCH is similar to chilling-induced RCH in that it can occur independently of the neuroendocrine system. Brief chilling enhances the cold-tolerance of isolated tissues, indicating that RCH operates, at least in part, independently of neuroendocrine input (Watanabe et al., 2002; Yi and Lee, 2003, 2004). In our study, *ex vivo* desiccation at 75% RH enhanced freeze-tolerance in isolated fat body tissues within 1 h and in midgut tissues within 2 h (Fig. 6).

To respond to such external stressors without mediation from the neuroendocrine system, tissues must be able to intrinsically sense the stress and respond appropriately. Calcium flux, likely caused by temperature-dependent changes in cellular ion transport or membrane permeability, is the first step in cold-sensing

at the cellular level (Teets et al., 2008, 2013). Dehydration-sensing at the cellular level may occur in a similar manner. The hydration status of a membrane affects its permeability and can alter the functioning of associated proteins (Hazel and Williams, 1990; M'Baye et al., 2008). Since a concentration gradient of calcium ions is maintained across cellular membranes via active transport by integral proteins, small changes in hydration could affect protein or membrane activity and result in an influx of calcium ions into the cell, triggering the RCH response.

Beyond the role of calcium, we have limited understanding of the mechanisms involved in RCH. Although cryoprotective sugars and polyols are accumulated to counteract long-term desiccation and low temperature stress (Koštál et al., 2001; Lee and Denlinger, 1991; Salt, 1961), there is no consistent polyol or sugar response among species exhibiting RCH (Lee and Denlinger, 2010). While it is unclear what role cryoprotectants play in rapid physiological responses, Levis et al. (2012) found a significant increase in hemolymph osmolality during drought-induced RCH in *E. solidaginis* larvae. We observed similar increases following desiccation as brief as 4 h. October-collected larvae lost an average of 3.6% of their body water during 4 h desiccation at 0% RH (Table 1). This treatment yielded an 83 mOsm $kg^{-1}$ increase in hemolymph osmolality, whereas we would expect only a 21 mOsm $kg^{-1}$ increase based on the effects of dehydration alone. Such an increase in hemolymph osmolality could be explained by the production of cryoprotectants; however, concentrations of glucose, trehalose, glycerol, and sorbitol, the four primary cryoprotective compounds used by *E. solidaginis*, were unaffected by acute desiccation (Table 2).

The increase in hemolymph osmolality associated with drought-induced RCH might be explained by elevated levels of free amino acids that are known to affect cold and freezing tolerance (Karow, 1991). Alanine and glutamine concentrations increase nearly twofold during RCH in flesh flies (Michaud and Denlinger, 2007) and a proline-augmented diet allows the normally freeze-intolerant fruit fly, *Drosophila melanogaster*, to tolerate freezing of ~50% of its body water (Koštál et al., 2012). Levels of free amino acids can be regulated by autophagy, a cellular process that degrades damaged or unnecessary macromolecules and organelles, freeing their components for use elsewhere (Maiuri et al., 2007; Meijer and Dubbelhuis, 2004; Onodera and Ohsumi, 2005; Rabinowitz and White, 2010). Autophagy is constitutively active in most cells (Hosokawa et al., 2006; Rabinowitz and White, 2010). Thus, modest increases in its activity would not require the production of new cellular machinery, which is important since RCH occurs even when protein synthesis is inhibited (Misener et al., 2001). Autophagic proteolysis is triggered by even small changes in cell volume, can degrade up to 5% of cytosolic protein per hour, and is upregulated during long-term desiccation in *B. antarctica* (Prick et al., 2006; Teets et al., 2012). It could further contribute to increased freeze-tolerance by inhibiting apoptosis and quickly removing damaged macromolecules (Maiuri et al., 2007).

Drought-induced RCH displays remarkable similarities to RCH triggered by chilling, which begs the question: Does acute desiccation trigger the same physiological response that is activated by chilling, or is drought-induced RCH one of multiple, subtly different responses activated by stressors such as heat, chilling, desiccation, or anoxia? As discussed above, cold-activated calcium flux is the putative first step in the RCH signaling cascade (Teets et al., 2008, 2013). Calcium mediates the activation of p38 MAPK, a class of mitogen-activated protein kinases that responds to a variety stresses, and chilling stimulates the phosphorylation of p38 MAPK within 10 min in *S. crassipalpis* (Fujiwara and Denlinger, 2007; Teets et al., 2013). Under hypertonic conditions, both p38 MAPK and the related, stress-responsive ERK1/2 MAPK are activated within 10 min in mammalian systems (Itoh et al., 1994; Shrode et al., 1998; Wehner et al., 2003). This suggests that both chilling and desiccation may trigger the same second-messenger cascades. It must be noted, however, that cellular signaling processes are complex; often, many pathways are activated simultaneously with extensive interplay among them (Wehner et al., 2003). Thus, we cannot rule out the possibility that MAPK activation is a common characteristic of two distinct mechanisms.

Our results show that very brief exposure to desiccating conditions elicits a physiological response that significantly enhances *E. solidaginis* freeze-tolerance. Fall-collected larvae were exquisitely sensitive to dehydration, as a loss of less than 1% of their fresh mass was sufficient to trigger RCH. This extreme sensitivity to dehydration suggests that insects track changes in humidity, perhaps using such cues as a means to select favorable microhabitats for temporarily stressful conditions or overwintering. Additionally, insects might alter their cold-hardiness in response to humidity changes as they move from one microhabitat to another. While *E. solidaginis* larvae do not change microhabitats, the conditions they are exposed to within a gall change dramatically during autumn. For most of their development, larvae are protected from desiccating conditions by the hydrated, living gall tissue surrounding them; however, during fall, the host plant senesces and begins to dry (Rojas et al., 1986; Williams and Lee, 2005). By winter, when the desiccated plant tissue offers little protection from the elements, larvae have entered diapause and acquired extreme resistance to evaporative water loss (Rojas et al., 1986; Williams et al., 2004; Williams and Lee, 2005). Between the early stages of senescence of the host plant and acquisition of maximal desiccation resistance; however, larvae are under dehydration stress. While we measured drought-induced RCH as a function of damage accrual and survival under extreme conditions, RCH also protects fecundity and speeds up recovery from chill coma during ecologically relevant cooling rates. Thus, drought-induced RCH may be an important component of the successful transition from summer growth and development to winter-hardened, diapausing larvae.

## Conflicts of interest

The authors have no conflicts of interest to report.

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