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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