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ORIGINAL PAPER

Desiccation enhances rapid cold-hardening in the flesh fly *Sarcophaga bullata*: evidence for cross tolerance between rapid physiological responses

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Abstract Many insects use rapid cold-hardening (RCH), a physiological response to sub-lethal exposure to stressors, such as chilling and desiccation, to enhance their cold tolerance within minutes. Recently, drought-induced RCH, triggered by brief, mild desiccation, was described in larvae of the freeze-tolerant gall fly (*Eurosta solidaginis*). However, its prevalence and ecological significance in other insects is not known. Consequently, we used a freeze-intolerant model, the flesh fly, *Sarcophaga bullata*, to investigate the effects and mechanisms of drought-induced RCH. In addition, we investigated how drought- and cold-induced RCH interact by exposing flies to both desiccation and chilling. Desiccation for 3 h increased larval pupariation after cold shock from 28 to 40 %—the first example of drought-induced RCH in both a freeze-intolerant insect and in a non-overwintering life stage. We also found that desiccation and chilling together enhanced the cold hardiness of larvae and adults more than either did separately, suggesting that drought and cold trigger distinct physiological mechanisms that interact to afford greater cold tolerance. These results suggest that drought-induced RCH is a highly conserved response used by insects with diverse life history strategies. Furthermore, the protective interaction between drought- and cold-induced RCH suggests that, in nature, insects use multiple cues and physiological mechanisms to fine-tune their response to changing ambient conditions.

Keywords Rapid cold-hardening · Drought-induced rapid cold-hardening · *Sarcophaga bullata* · Desiccation · Cold tolerance · Cross tolerance

Introduction

Desiccation and cold are closely linked to environmental stressors that elicit similar physiological responses (Holmstrup et al. 2002; Rinehart et al. 2007). Because of their similarity, physiological adjustments made to desiccation stress often confer increased cold tolerance in both freeze-tolerant and freeze-intolerant arthropods (Bayley et al. 2001; Hayward et al. 2007; Sinclair et al. 2013). Most acclimatory responses occur seasonally over weeks or months, and as a result, research on cross tolerance often focuses on relatively slow, seasonal acclimatization (Lee and Denlinger 1991). Yet, many insects and other ectotherms can also "instantaneously" enhance their cold tolerance in response to brief chilling or desiccation in processes known as cold- and drought-induced rapid cold-hardening (RCH) (Lee et al. 1987; Levis et al. 2012). Despite the generality of RCH in insects, little is known about the potential for cross tolerance resulting from brief exposure to desiccation and chilling.

In nature, desiccating conditions and low temperatures often occur concomitantly. Protracted exposure to both stressors occurs during temperate and polar winters, which are characterized by cold or freezing conditions and the limited availability of liquid water (Danks 2000). However, even brief temperature excursions, which occur naturally as a weather front passes, can strongly influence humidity. For example, cold fronts are low-pressure air masses that generally lower relative humidity as well as temperatures (Miles 1962; Moeller et al. 1993). Furthermore, due to their

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small size, insects' body temperatures closely track ambient temperatures (Sinclair et al. 2013). It is likely, then, that temperate and polar insects simultaneously experience rapid chilling and desiccation as a consequence of volatile weather and diurnal thermoperiods.

In insects, cold exposure frequently triggers the accumulation of various types of osmolytes, which serve as cryoprotectants (Lee and Denlinger 1991). Cryoprotectants are often low molecular mass polyols and sugars that mitigate the effects of chilling or freezing (Lee and Denlinger 2010). Many of these compounds also serve as osmoprotectants and are accumulated during desiccation (Holmstrup et al. 2010). This osmolytic response is activated during RCH as well; cold-induced RCH triggers a modest accumulation of cryoprotectants, such as glycerol, sorbitol, and/or glucose, affecting a 10–20 mOsm kg^{-1} increase in hemolymph osmolality (Lee and Denlinger 2010). Similarly, drought-induced RCH causes a ~40 mOsm kg^{-1} increase in the hemolymph osmolality of *Eurosta solidaginis* larvae, though the specific solutes driving this response were not identified (Levis et al. 2012; Gantz and Lee 2015).

It is widely accepted that insects' physiological responses to cold and desiccation are often similar and that prolonged exposure to one of these stressors confers enhanced tolerance to the other (Sinclair et al. 2013). Though this cross tolerance is primarily known from slow acclimatory responses, acute exposure to sub-lethal desiccation also induces physiological responses that trigger osmolyte accumulation and enhanced cold tolerance (Sinclair and Chown 2003; Benoit et al. 2009; Levis et al. 2012), suggesting that cross tolerance between stressors occurs even during brief exposure to desiccation and chilling. Furthermore, the interplay between temperature and humidity suggests that brief, unpredictable cooling, as occurs when a weather front passes, is prone to concomitantly produce desiccating conditions. Despite this, no study has addressed the potential for cross tolerance between these RCH triggers. Thus, to more fully understand the relationship between the rapid responses to brief desiccation and chilling, we investigated the following questions using larvae and adults of the flesh fly, *Sarcophaga bullata*: does acute desiccation induce RCH in this freeze-intolerant fly? Do the responses to chilling and desiccation interact to increase the rate of organismal recovery from cold shock? Do they also enhance cold tolerance at the tissue-level? And what effect do these have on the osmolytic response?

Materials and methods

Insect culture and treatments

The flesh fly, *S. bullata* Parker (Diptera: Sarcophagidae), was reared following the methods described by Lee and Denlinger (1985). Larvae and adults were used to assess the effects of brief desiccation on cold tolerance, chill coma recovery, and the RCH response. In larvae ($n = 30$), organismal survival was evaluated by whether they successfully pupariated 72 and 96 h after treatment. The same larvae were used for both time points; that is, the 96 h measurement included larvae that pupariated after 72 h as well. Nearly, all the larvae that failed to pupariate after 96 h died without pupariating. In adults ($n = 30$), assessment was made using the rate of recovery from chill coma, determined by return of coordinated movement 20, 30, 40, 60, and 120 min after treatment. Coordinated movement was defined as natural movements as during walking or flying. Four-day-old larvae and 6-day-old adults were treated as follows: control (untreated at 25 °C), cold shocked (CS) (direct transfer to −9 °C and held for 2 h), RCH (pretreated at 0 °C for 2 h followed by cold shock), D + CS (larvae and adults were desiccated for 3 and 4.5 h, respectively, at ~4 % relative humidity over Drierite® at 22 °C, followed by cold shock), and D + RCH (desiccation as described in D + CS followed by RCH treatment, then cold shock). Larval and adult desiccation times were determined by the time it took to achieve a loss of 2–3 % of their fresh mass. We used 2–3 % of fresh mass as a target, because, in *E. solidaginis*, a similar amount of water loss was sufficient to trigger drought-induced RCH (Gantz and Lee 2015).

Assessment of cellular viability

We assessed cellular-level damage caused by cold shock with a vital dye assay using a LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA) as adapted by Yi and Lee (2003). Briefly, isolated larval fat body and adult midgut tissues were harvested from randomly selected, living insects immediately following the termination of the treatment. Tissues were incubated in two fluorescent vital dyes in a two-step process. SYBR-14, a membrane-penetrating green dye, stains nucleic DNA from all cells, while propidium iodide, a red dye, is excluded by intact membranes and only stains the DNA in cells whose membranes are compromised by cold shock. After the dyes were applied, fluorescence microscopy was used to determine the ratio of undamaged to damaged cells. For each tissue, every cell in four or more discrete fields of view during microscopy (minimum of 50 cells) was scored as damaged or undamaged. Mean ± SE were determined by the average ratio of undamaged cells from 3 to 4 larvae per treatment.

Osmolality and polyol measurement

Hemolymph osmolality was measured for control and desiccated larvae ($n = 10$ measurements) by drawing 20 µl of hemolymph into a glass microcapillary pipette.

Hemolymph was pooled from 2 to 3 larvae, and the osmolality was measured using a Model 3320 Osmometer (Advanced Instrument Inc., Norwood, MA, USA). We only measured hemolymph osmolality in larvae, because we were unable to harvest adequate amounts of hemolymph from adult flies.

Cryoprotective sugars and polyols (glycerol, sorbitol, and glucose) were measured in the whole-body extracts of larvae, as described by Gantz and Lee (2015). Each larva was homogenized in a 1.5-ml Eppendorf tube with 0.6 N 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 extract, an equivalent amount of 1.0 M potassium bicarbonate was added to the supernatant and incubated on ice with a vented lid for 15 min. Following brief centrifugation, the supernatant was aliquoted for use in cryoprotectant assays.

Glycerol content was determined following the method by Holmstrup et al. (1999). Briefly, a 200 µl aliquot of whole-body extract was added to 800 µl of reconstituted Free Glycerol Reagent (Sigma-Aldrich, St. Louis, MO, USA). Absorbance was read at 540 nm on a Jenway Model 6705 UV/Vis spectrophotometer following a 15-min incubation at 37 °C. Values are reported as micromoles of glycerol per gram of fresh mass.

Sorbitol content was measured using a colorimetric assay adapted from Bergmeyer et al. (1974). A reaction mixture of 333 µl of neutralized whole-body extract, 666 µl of 0.1 M sodium pyrophosphate, and 33 µl of 30 mM NAD was prepared, and the initial absorbance was read at 340 nm. Sorbitol dehydrogenase (16.6 µl, 5 mg protein/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to the reaction mixture and incubated for 1 h at room temperature. The final absorbance was recorded at 340 nm. Sorbitol content was determined by the difference in absorbance from the initial reading to the final reading. Data are expressed as micromoles of sorbitol per gram of fresh mass.

Glucose concentrations were measured by aliquotting whole-body extract into 100 µl portions. Glucose content was then determined using a colorimetric glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA).

Statistical analysis

Cold survival rates at both organismal and tissue-levels, hemolymph osmolality, and polyol contents were evaluated using unpaired *t* tests (SigmaPlot 12.5) to determine differences between treatment groups, with the significance level set to $P < 0.05$ ($n = 30$). Response variables with percentage rates were arcsine-square root transformed to stabilize the variance before comparison. One-way ANOVA (SigmaPlot 12.5) with a post hoc Bonferroni correction was also performed to determine whether desiccation treatment had a significant effect on the RCH response for organismal and cellular survival. All data are expressed as mean ± SE.

Results

Desiccation enhanced organismal cold tolerance in RCH-treated flies

Larvae that were desiccated before being exposed to a discriminating cold temperature (cold shock) pupariated at a higher rate than non-desiccated, cold-shocked larvae, 27.4 ± 5.8–40.2 ± 7.1 % after 72 h; however, this effect was not evident after 96 h (Fig. 1; $P > 0.05$). Pretreatment with desiccation and chilling (D + RCH) further improved the rate of pupariation, which also exceeded that of larvae that were chilled and cold shocked (RCH treatment), 46.1 ± 4.5–63.1 ± 3.9 % and 63.5 ± 5.1–89.9 ± 2.4 %, after 72 and 96 h, respectively (Fig. 1; $P < 0.05$).

D + RCH treatment in adult flies accelerated the rate of recovery from chill coma relative to RCH alone, as 66.7 ± 8.8 % of desiccated flies were capable of movement within 20 min of recovery compared to only 20.0 ± 5.8 % of their non-desiccated partners (Fig. 2; $P < 0.05$). Similarly, after 30 min of recovery, 83.3 ± 8.8 % of desiccated

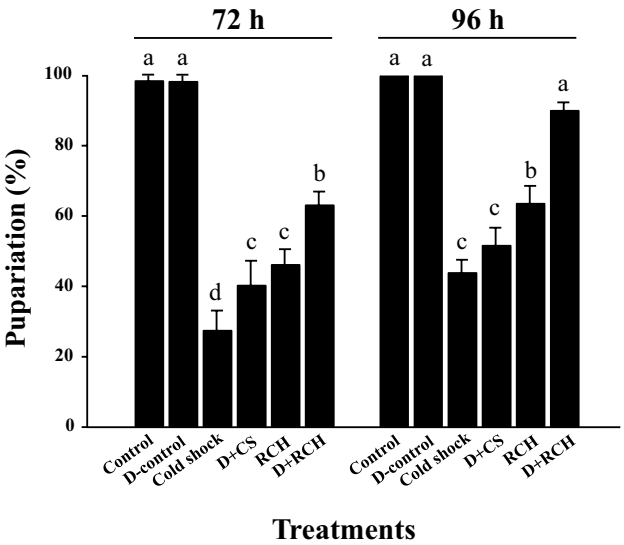

Fig. 1 Desiccation significantly increased pupariation rates (%) in the rapid cold-hardening (RCH)-treated larvae. Control larvae were untreated. D-control treatments were only desiccated without a subsequent cold treatment, while cold-shock (CS) treatments received the discriminating cold exposure alone and RCH treatments received brief chilling. D + CS and D + RCH represent desiccation treatment before CS and RCH, respectively. Pupariation was observed 72 and 96 h after completion of treatment. Groups not sharing letters were significantly different ($P < 0.05$, $n = 30$)

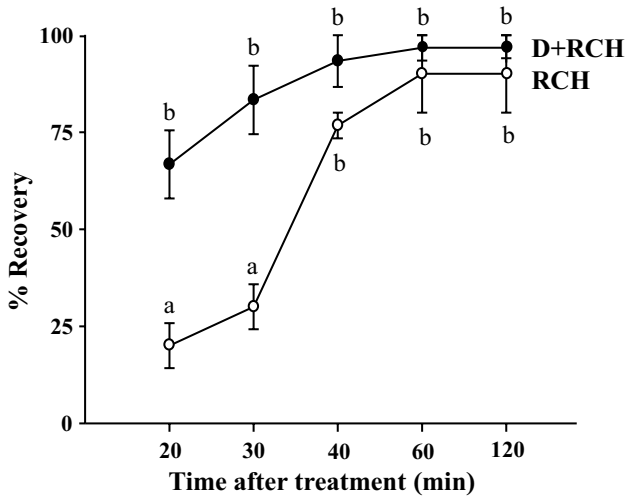

Fig. 2 Comparison of the recovery rates (%) of adults between RCH treatment alone (RCH, *open circles*) and desiccation plus RCH treatment (D + RCH, *closed circles*). The addition of desiccation to RCH treatments accelerated recovery from cold shock. Flies capable of coordinated movement were scored as recovered. Data points not sharing *letters* were significantly different ($P < 0.05$, $n = 30$)

flies could move compared to 30.0 ± 5.8 % of non-desiccated flies (Fig. 2; $P < 0.05$). Slight desiccation enhanced the cold-induced RCH response, suggesting that brief, mild desiccation triggers a physiological response that is distinct from that of chilling. Interestingly, the protective effects of desiccation alone were only evident in larvae 72 h after treatment (Fig. 1; $P < 0.05$). In contrast, desiccation alone did not have a significant effect on the rate of recovery compared to untreated controls at any time point between 20 and 120 min (data not shown).

Desiccation enhanced cellular cold tolerance

Brief desiccation enhanced cellular cold tolerance of both larval fat body and adult midgut cells in cold-shocked flies (Figs. 3, 4). Compared to their non-desiccated counterparts, desiccation increased cell survival by 24.9 % (CS 23.93 ± 6.1 % vs. D + CS 48.8 ± 6.0 %) in the larval fat body (Fig. 3b; $P < 0.03$) and by 45.0 % (CS 13.4 ± 5.8 % vs. D + CS 58.5 ± 8.0 %) in the adult midgut (Fig. 4b, $P < 0.003$).

In addition, cellular viability assays using larval (Fig. 3) and adult (Fig. 4) fat body and midgut tissues corroborated the organismal assessments, as D + RCH treatments enhanced cold tolerance more than RCH alone. In larval fat body, only 23.9 ± 6.1 % of cells remained viable following cold shock. Cold-induced RCH treatment increased the ratio of viable cells to 82.1 ± 2.3 % (Fig. 3b; $P < 0.01$), while 3 h of desiccation preceding cold-induced RCH

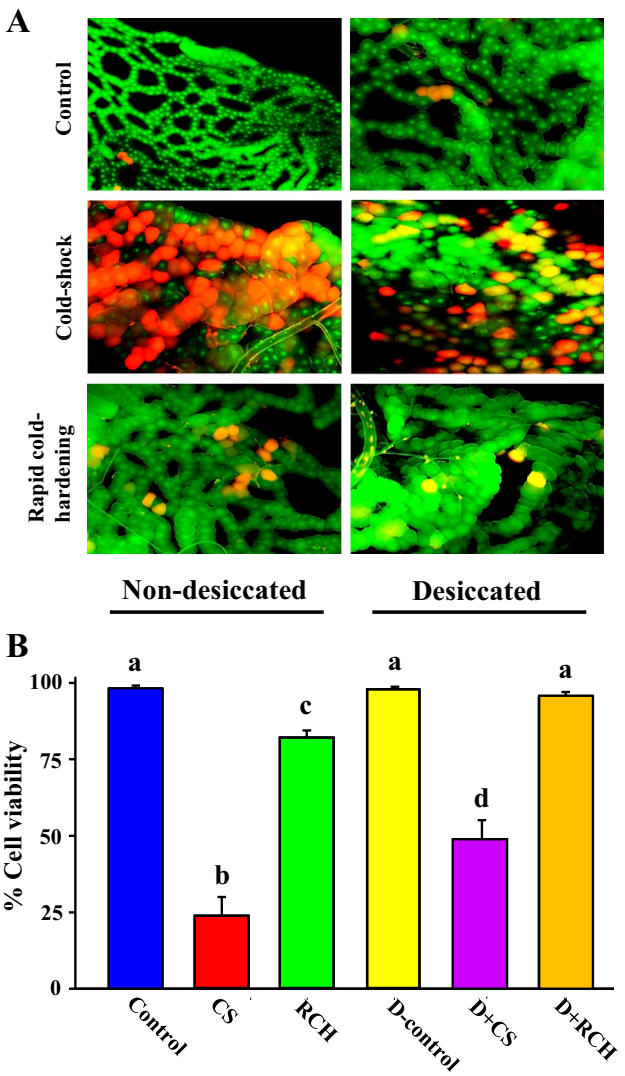

Fig. 3 Effect of desiccation on cell viability of larval fat body. **a** Vital dyes images of larval fat body tissue. *Green* cells were scored as alive, while red cells were scored as dead. Desiccation treatment significantly improved cellular survival in both the D + CS and D + RCH groups. Non-desiccated control photo was taken at ×40 magnification; all other photos were taken at ×100 magnification. **b** Percent of viable cells counted from vital dye-stained larval fat body. Control larvae were untreated. Desiccation treatments were only desiccated without a subsequent cold treatment, while cold-shock (CS) treatments received the discriminating cold exposure alone. RCH treatments received brief chilling. D + CS and D + RCH represent desiccation treatment before CS and RCH, respectively. Groups not sharing *letters* were significantly different ($P < 0.05$) (color figure online)

treatment further increased the percentage of viable cells to 95.5 ± 1.5 % (Fig. 3b; $P < 0.01$). In adult midgut tissues, a 4.5-h desiccation treatment increased the percentage of viable cells in RCH-treated adults to 96.7 ± 0.6 % compared to 77.5 ± 1.8 % after RCH treatment alone (Fig. 4b; $P < 0.001$).

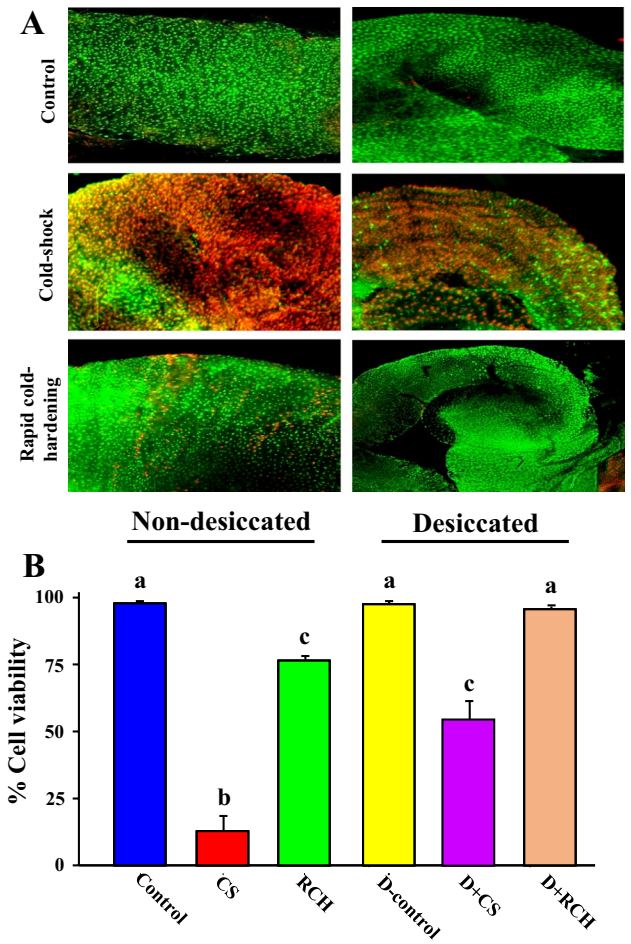

Fig. 4 Effect of desiccation on cell viability of adult midguts. **a** Vital dyes' images of adult midgut tissue. *Green* cells were scored as alive, while red cells were scored as dead. Desiccation treatment significantly improved cellular survival in both the D + CS and D + RCH groups. Non-desiccated control and desiccated rapid cold-hardening photos were taken at ×40 magnification; all other photos were taken at ×100 magnification. **b** Percent of viable cells counted from adult midgut. Control larvae were untreated. Desiccation treatments were only desiccated without a subsequent cold treatment, while cold-shock (CS) treatments received the discriminating cold exposure alone. RCH treatments received brief chilling. D + CS and D + RCH represent desiccation treatment before CS and RCH, respectively. Groups not sharing *letters* were significantly different ($P < 0.05$) (color figure online)

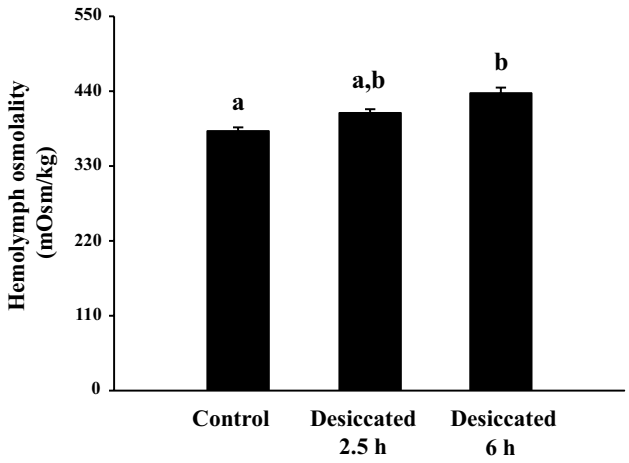

Fig. 5 Changes of osmolality in larval hemolymph before and after desiccation. Larvae were starved for 14 h for all groups prior to desiccation treatment. Groups not sharing *letters* were significantly different ($P < 0.05$)

Desiccation elevated hemolymph osmolality in larvae

To better understand the physiological mechanisms by which desiccation enhances the RCH response, we measured changes in larval hemolymph osmolality following desiccation treatments (Fig. 5). Brief desiccation caused significant increases in hemolymph osmolality in a time-dependent manner: 381.3 ± 5.5 mOsm kg^{-1} in untreated controls, 408.0 ± 5.5 mOsm kg^{-1} after 2.5-h desiccation, and 437.0 ± 8.2 mOsm kg^{-1} after 6-h desiccation ($P < 0.05$). Though we did not directly measure water loss during these experiments, we can calculate the expected effect of water loss on hemolymph osmolality using dehydration rates established during drought-induced RCH experiments and expected organismal water contents. Water content of *S. bullata* larvae is ~71 % (Yi, unpublished data), and water loss rates were close to 1 % fresh mass per hour over Drierite®. Dehydration treatments of 2.5 and 6 h would be expected to cause 13 and 32 mOsm kg^{-1} increases hemolymph osmolality, respectively. Thus, dehydration accounts for about half of the observed increases in hemolymph solute concentration.

Glycerol, sorbitol, and glucose levels did not explain changes in hemolymph osmolality

Glycerol and sorbitol levels remained unchanged across all treatment groups, though there were slight, but significantly increases in glucose concentrations following cold-induced RCH, desiccation, desiccation and cold shock (D + CS), and desiccation with cold-induced RCH (D + RCH) (Table 1).

Discussion

Drought-induced RCH is a novel response that has only been reported in three freeze-tolerant insects (Sinclair and Chown 2003; Benoit et al. 2009; Levis et al. 2012). In this study, we investigated whether drought-induced RCH also occurs in non-overwintering stages (larval and adult) of the freeze-intolerant flesh fly, *S. bullata*. In larvae, pre-treatment with 3 h of desiccation significantly enhanced

Table 1 Cryoprotectant levels in the body extracts of larvae (mean ± SEM, μmol/g FM)

	Glycerol	Sorbitol	Glucose
Non-desiccation			
Control	3.23 ± 0.20	3.89 ± 0.30	13.02 ± 0.44[a]
Cold shock (CS)	3.73 ± 0.02	4.43 ± 0.63	14.92 ± 0.53[a,b]
RCH	2.85 ± 0.24	4.15 ± 0.22	16.04 ± 0.34[b]
Desiccation pretreatment			
Desiccated only	3.84 ± 0.46	3.40 ± 0.45	16.98 ± 0.35[b]
Cold shock (D + CS)	3.41 ± 0.05	4.19 ± 0.40	15.81 ± 0.57[b]
RCH (D + RCH)	3.52 ± 0.09	2.37 ± 0.29	15.82 ± 0.23[b]

Data with different letters in the same column indicate a significant difference ($P < 0.05$) between treatment groups

CS cold shock, *D + CS* desiccation prior to cold-shock treatment, *D + RCH* desiccation prior to RCH treatment, *RCH* rapid cold-hardening

the pupariation rate 72 h after cold shock, though this effect was not evident after 96 h. In adults, 4.5 h desiccation had no significant effect on the rate of recovery from chill coma. However, using cellular-level measurements of cold tolerance, we found evidence of drought-induced RCH in both the larval and adult life stages. The discrepancy between organismal and cellular-level assessments of cold hardiness may be due to the subtle nature of RCH responses; that is, organismal-level assessments may be too coarse to detect the effects of RCH. Despite these limitations, our results are the first example of drought-induced RCH in a freeze-intolerant insect.

Though we found only modest effects of drought-induced RCH in this study, cold-induced RCH was strongly enhanced by desiccation pretreatment. This result does not indicate that there is synergy between drought- and cold-induced RCH; indeed, this study was not designed to determine if these are synergistic, additive, or even antagonistic effects. Yet, desiccation and chilling together enhance cold tolerance more than either does separately, suggesting that drought- and cold-induced RCH use different mechanisms that interact to enhance cold tolerance. Differences in cryoprotectant accumulation during brief chilling and desiccation further suggest that these triggers elicit distinct responses. Many insects, including *S. bullata*, accumulate glycerol and/or sorbitol during brief chilling (Yoder et al. 2006; Lee and Denlinger 2010). In contrast, brief desiccation does not cause an accumulation of glycerol, sorbitol, trehalose, or glucose in *E. solidaginis* larvae (Levis et al. 2012; Gantz and Lee 2015), and in this study, there were no changes in glycerol or sorbitol levels in *S. bullata* larvae. Thus, future research should focus on determining which cellular pathways are activated by acute desiccation. Since the first steps of cold-induced RCH include calcium flux caused by changes in plasma membrane permeability followed by downstream phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), both calcium flux and the relative abundance of phosphorylated p38MAPK seem like good starting points for mechanistic studies of drought-induced RCH (Fujiwara and Denlinger 2007; Teets et al. 2008, 2013).

Though little is known about the underpinning mechanisms of drought-induced RCH, the accumulation of cryoprotectants appears to be important. In *E. solidaginis* larvae, brief desiccation causes a ~30 mOsm kg^{-1} increase in hemolymph osmolality beyond what is explained by water loss during dehydration, suggesting an accumulation of cryoprotective solutes (Levis et al. 2012; Gantz and Lee 2015). Similarly, we found that desiccating *S. bullata* larvae for 2.5 and 6 h resulted in ~27 and ~55 mOsm kg^{-1} increases in hemolymph osmolality, respectively. Water loss during desiccation only explains ~10 and ~23 mOsm kg^{-1} of the increases in hemolymph osmolality, suggesting a modest accumulation of osmolytes during acute dehydration to account for the remaining increase in osmotic pressure. The slight increase in hemolymph osmolality during desiccation is consistent with the magnitude of the cryoprotectant response during cold-induced RCH, which usually elicits a 10–20 mOsm kg^{-1} increase in hemolymph osmolality (Overgaard et al. 2007).

To investigate the cause of the increase in osmotic pressure during desiccation, we measured the concentrations of three common cryoprotective sugars and polyols: glycerol, sorbitol, and glucose. While our analyses uncovered some significant increases in glucose concentration, none of these were substantial enough to significantly alter hemolymph osmolality or likely to account for increased cold tolerance. However, caution should be used when interpreting these results, because we calculated cryoprotectant concentrations using fresh mass rather than dry mass, and the slight decrease in fresh mass (~2 %) resulting from water loss in desiccation treatments may influence our results. In addition, it is possible that only measuring cryoprotectant levels from whole-body extracts mask the effects of redistribution of these sugars from one tissue or body compartment to another and that biologically meaningful changes in the distribution of cryoprotectants were not detected. Nonetheless, these results suggest that the cryoprotectant response to brief desiccation is driven by the accumulation of solutes other than glycerol, sorbitol, or glucose. Recently, free amino acids have gained recognition as a prominent part of the cold-hardening response in some insects (Koštál et al. 2011, 2012; Yi and Lee 2016). Furthermore, Gantz and Lee (2015) speculated that increased hemolymph osmolality during drought-induced RCH was a result of increased autophagic proteolysis yielding increased concentrations of free amino acids. Not only would this liberate amino acids, it would actively decrease available body water, as proteolysis is a hydrolytic

process (Blommaart et al. 1997). Reducing body water content through proteolysis could have adaptive significance, since a reduction of solvent would increase the solute concentration, which would have the same effect as synthesizing new solutes. Furthermore, autophagy could conceivably be a part of the RCH response, because it can quickly and dramatically increase its activity (Blommaart et al. 1997). In *Drosophila melanogaster* fat body, 3 h incubation in protein deficient media triggered starvation-induced autophagy, causing a significant increase in the number of autophagosomes (Scott et al. 2004), and in perfused rat livers, autophagic proteolysis can degrade up to 5 % of cytosolic protein per hour (Mortimore et al. 1989). Together, these results suggest that the autophagic response is rapid enough and sufficiently robust to significantly contribute to drought-induced RCH.

At the ecological level, the RCH response allows insects to fine-tune their physiological state to match ambient conditions. Indeed, RCH is manifested in a variety of ways that improve survival and reproductive success, including increasing survival of sub-zero temperatures, enhancing freeze-tolerance, decreasing the temperature required to induce chill coma, suppressing water loss during desiccation, as well as preserving flight, mating, courtship behavior, learning, fecundity, and longevity (see review by Lee and Denlinger 2010). Importantly, RCH is induced by ecologically relevant rates of chilling (Shreve et al. 2004), though drought-induced RCH has not yet been investigated under field conditions.

Furthermore, it is possible that RCH is a more robust response than is currently known because of the combined effects of multiple RCH responses triggered by natural conditions. It is likely that insects commonly face simultaneous exposure to multiple stressors, since natural conditions rarely present only one challenge at a time (Holmstrup et al. 2010). For example, cold fronts often cause abrupt decreases in relative humidity as well as temperature (Moeller et al. 1993), suggesting that brief desiccation and chilling are likely to be experienced concomitantly. The interaction between drought- and cold-induced RCH, which resulted in a marked decrease in recovery time and mortality rate in this study, could allow insects to remain active during the spring and autumn seasons, especially in temperate regions, when the weather is particularly unstable. With this in mind, future research should focus on whether drought-induced RCH is triggered in nature and on the effects of simultaneous exposure to multiple RCH triggers under field conditions.

In addition, it would be interesting to investigate the interactions between rapid physiological responses triggered by cues other than chilling and dehydration. RCH can be triggered by high temperature and anoxia, while other stressors, such as overhydration, exposure to ultraviolet light, and oxidative stress also induce rapid physiological responses (Coulson and Bale 1991; Rinehart et al. 2000; Lopez-Martinez et al. 2008, 2009). Characterizing the extent of cross tolerance between distinct rapid physiological responses, especially focusing on ecologically relevant combinations of cues, may lead to a greater appreciation of the significance of rapid acclimatory responses in insects. Many stressors are closely linked and might be expected to frequently occur concomitantly, such as high temperature and UV irradiation, anoxia and freezing, hypoxia and overhydration, and UV irradiation and oxidative stress (Storey and Storey 1988; Hoback and Stanley 2001; Thieden et al. 2006; Meng et al. 2009). Thus, multiple rapid responses may be activated simultaneously under natural conditions. Perhaps, the ecological importance of rapid physiological adaptation has been underestimated by not accounting for the interaction between these responses?

In conclusion, we present the first evidence of (1) drought-induced RCH in a non-overwintering stage of a freeze-intolerant insect and (2) interactions between cold- and drought-induced RCH to further enhance stress tolerance. Insects use cold-induced RCH to fine-tune their physiological state in any life stage, during any season (Lee and Denlinger 2010). In contrast, drought-induced RCH has only been investigated in the overwintering larvae of a freeze-tolerant moth, *Pringleophaga marioni*, and a freeze-tolerant fly, *E. solidaginis* (Sinclair and Chown 2003; Levis et al. 2012). Our finding that drought-induced RCH used by *S. bullata* adults suggests that this response may be as pervasive, as cold-induced RCH is among insect taxa. In addition, the interaction between drought- and cold-induced RCH indicates that desiccation and chilling trigger distinct physiological mechanisms, though these mechanisms are poorly understood. These results raise numerous questions about the interaction between distinct RCH responses in nature. How similar are the mechanisms triggered by desiccation and cold? Which rapid acclimatory responses interact to further enhance stress tolerance? And how important is the interplay between these rapid responses to the success of insects?

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Compliance with ethical standards

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

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