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ABSTRACT

THE PHYSIOLOGICAL AND ECOLOGICAL IMPLICATIONS OF RAPID ACCLIMATORY RESPONSES IN INSECTS

by

Josiah D. Gantz

When challenged by brief exposure to sub-lethal stresses, insects and other small ectotherms can make physiological adjustments that enhance their stress tolerance within minutes. Through these rapid acclimatory responses, insects adjust their physiology to track ambient conditions and counter the negative effects of perturbation by abiotic stresses. Although rapid acclimation is best studied as a response to brief chilling, diverse environmental cues, including high temperature, dehydration, and anoxia trigger similar responses. Further, recent evidence suggests that different cues for rapid acclimation trigger distinct mechanistic responses. This dissertation investigated the underpinning physiology and ecological importance of these diverse rapid acclimatory responses in three species of flies.

The first project compared the physiological mechanisms triggered by brief chilling and dehydration in larvae of the freeze-tolerant goldenrod gall fly, *Eurosta solidaginis*. Chilling produced solute accumulation in larval hemolymph and caused the activation of the second messenger p38 MAP kinase, while dehydration caused a redistribution of body water without significant accumulation of solutes and activated both p38 and Erk1/2 MAP kinase. Additionally, though neither treatment differed from control treatments, larvae that were chilled for 2 h maintained a higher metabolic rate at low temperatures than those that were dehydrated.

The second project examined the effects that brief chilling and dehydration have on flight performance and fecundity in the flesh fly *Sarcophaga bullata*. Both triggers for rapid acclimation enhanced distance flown, peak velocity, increased flight time under stressful conditions, and preserved reproductive output. These results suggest that rapid acclimation helps insects to maintain basic behaviors when faced with stress in nature.

The final project investigated the effects of brief exposure to diverse abiotic stresses in larvae of the Antarctic midge, *Belgica antarctica*. These larvae increased their tolerance of freezing after just 2 h of exposure to sub-lethal temperature extremes, osmotic perturbation, acidic and alkaline conditions, UV irradiation, and starvation. Thus, rapid acclimatory responses can be triggered by many more abiotic stresses than were previously recognized.

In summary, diverse abiotic stresses trigger rapid acclimatory responses that enhance stress tolerance in ecologically relevant ways.

THE PHYSIOLOGICAL AND ECOLOGICAL IMPLICATIONS OF RAPID ACCLIMATORY RESPONSES IN INSECTS

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Chapter 1

General introduction

Physiological adaptations to temperature have been extensively studied in insects, though these acclimatory responses mostly focus on slow, seasonal adjustments occurring over weeks or months. In contrast, rapid physiological responses, which are made within minutes to hours, have received comparatively little attention. Rapid cold-hardening (RCH) is an example of such an acclimatory response that enhances survival and function by allowing insects to adjust to quickly changing ambient conditions (Lee et al., 1987; Lee and Denlinger, 2010).

There is substantial evidence that RCH responses are ecologically important. Indeed, cold-induced RCH enhances cold and freezing tolerance, increases resistance to temperature-dependent disruption of ion imbalance and loss of membrane potential, protects courtship and mating behavior, protects the ability to fly, and maintains or enhances fecundity (Lee and Denlinger, 2010). The utility of RCH becomes even clearer when we consider the dramatic changes in body temperature that many insects face. Their small size makes them susceptible to large, rapid fluctuations in temperature, which can be exacerbated by factors such as direct exposure to solar radiation and convective cooling during flight. Amazingly, insect body temperatures can fluctuate 40°C or more during a 24 h thermoperiod, and 10°C in as few as 10 seconds when moving from one microhabitat to another (Stevenson, 1985; Heinrich, 1993). Without appropriate compensatory physiological adaptations, such dramatic changes in temperature have large impacts on biochemical processes, membrane function, and metabolism. Thus, RCH is likely important for insects to overcome the challenges of eurythermia.

Insects use some cues other than chilling to modulate their stress tolerance; as anoxia and high temperature can also rapidly trigger enhanced cold tolerance (Chen et al., 1987; Coulson and Bale, 1991). Recently, desiccation was added to this list of triggers for RCH (Levis et al., 2012). This drought-induced RCH markedly improves cold and freeze tolerance after only 2 h of desiccation and a loss of less than 1% of fresh mass (Gantz and Lee, 2015). This remarkable sensitivity to slight osmotic perturbation may be critical for modifying insects' physiological states as ambient conditions change.

Despite the demonstrated organismal benefits, the underlying mechanisms of RCH are not well characterized. This is especially true of drought-induced RCH, largely because of its recent discovery. Even so, mounting evidence suggests that cold- and drought-induced RCH are distinct responses that operate by different mechanisms. Insects exposed to both desiccation and chilling are more resistant to the effects of severe cold than those exposed to only one (Yi et al. 2017). Further, cold- and drought-induced RCH seem to affect concentrations of cryoprotective solutes differently. Cold-induced RCH often causes modest increases in glycerol or sorbitol and, while hemolymph osmolality increases during drought-induced RCH, concentrations of glycerol, sorbitol, trehalose, or glucose were unchanged (Lee and Denlinger, 2010; Gantz and Lee, 2015).

Since cold- and drought-induced RCH collectively enhance cold tolerance more than either does separately, it is possible that interactions between other rapid physiological responses contribute to stress tolerance. In nature, abiotic stressors rarely occur as single, isolated pressures (Holmstrup et al., 2010). For example, cold fronts often lower humidity as well as temperature (Miles, 1962; Moeller et al., 1993), high ultraviolet light exposure is often coupled with increases in temperature, and when microhabitats flood, temperature fluctuation and hypoxia accompany overhydration from direct exposure to fresh water (Hoback et al., 1998). Because RCH has mostly been investigated as a response to one trigger at a time, we have likely underestimated insects' capacity for modulating their physiological state through the simultaneous induction of multiple, distinct RCH responses.

Our understanding of both the physiological mechanisms and ecological relevance of RCH responses, particularly those not triggered by chilling, is severely lacking. These responses and their interactions are potentially critical to the success of insects in variable environments. Thus, my dissertation examined the physiological and ecological implications of these rapid acclimatory responses in three species of flies.

References

Chen, C. P., Denlinger, D. L., Lee, R. E. (1987). Cold-shock injury and rapid cold-hardening in the flesh fly *Sarcophaga crassipalpis*. *Physiological Zoology, 60*(3), 297–304.

Coulson, S. J., Bale, J. S. (1991). Anoxia induces rapid cold hardening in the housefly *Musca domestica* (Diptera: Muscidae). *Journal of Insect Physiology, 37*(7), 497–501.

Gantz, J. D., & Lee, R. E. (2015). The limits of drought-induced rapid cold-hardening: Extremely brief, mild desiccation triggers enhanced freeze-tolerance in *Eurosta solidaginis* larvae. *Journal of Insect Physiology, 73*, 30-36.

Heinrich, B. (1993) The Hot-Blooded Insects: Strategies and Mechanisms of Thermoregulation (Harvard Univ. Press, Cambridge, MA), p. 1.

Hoback, W. W., Stanley, D. W., Higley, L. G., & Barnhart, M. C. (1998). Survival of immersion and anoxia by larval tiger beetles, *Cicindela togata*. *The American Midland Naturalist, 140*(1), 27-33.

Holmstrup, M., Bayley, M., Pedersen, S. A., Zachariassen, K. E. (2010). Interactions between cold, desiccation and environmental toxins. In: Denlinger, D. L., Lee, R. E. (Eds.), Low Temperature Biology of Insects. Cambridge University Press, Cambridge, pp. 166–187.

Lee, R. E., Denlinger, D. L. (2010). Rapid cold-hardening: ecological significance and underpinning mechanisms. In: Denlinger, D.L., Lee, R.E. (Eds.), Low Temperature Biology of Insects. Cambridge University Press, Cambridge, pp. 35–58.

Lee, R. E., Chen, C. P., Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science, 238*(4832), 1415–1417.

Levis, N. A., Yi, S-X., & Lee, R. E. (2012). Mild desiccation rapidly increases freeze tolerance

of the goldenrod gall fly, *Eurosta solidaginis*: evidence for drought-induced rapid cold-hardening. *Journal of Experimental Biology*, *215*(21), 3768–3773.

Miles, M. K. (1962). Wind, temperature and humidity distribution at some cold fronts over SE. England. *Quarterly Journal of the Royal Meteorological Society*, *88*(377), 286-300.

Moeller, C. C., Huh, O. K., Roberts, H. H., Gumley, L. E., & Menzel, W. P. (1993). Response of Louisiana coastal environments to a cold front passage. *Journal of Coastal Research*, 434-447.

Stevenson, R. D. (1985). Body size and limits to the daily range of body temperature in terrestrial ectotherms. *American Naturalist*, 102-117.

Yi, S.-X., Gantz, J. D., & Lee, R. E. (2017). Desiccation enhances rapid cold-hardening in the flesh fly *Sarcophaga bullata*: evidence for cross tolerance between rapid physiological responses. *Journal of Comparative Physiology B*, *187*(1), 79-86.

Chapter 2

Brief desiccation and chilling trigger mechanistically-distinct rapid cold-hardening responses in larvae of the goldenrod gall fly, *Eurosta solidaginis*

Introduction

Rapid cold-hardening (RCH) is a transient physiological response that insects use to enhance stress tolerance and preserve motor function (Lee et al., 1987; Wang and Kang, 2003, Kelty et al., 1996; Shreve et al., 2004; Larsen and Lee, 1994). RCH mitigates the effects of changing ambient conditions and microhabitats, which can cause drastic changes in body temperature and hydration state (Heinrich, 1993; Lee and Denlinger, 2010). Though most studies have focused on chilling as the trigger for RCH (cold-induced RCH), high temperature, hypoxia, dehydration (drought-induced RCH), and perhaps other environmental stressors can trigger similar physiological responses (Chen et al., 1987; Coulson & Bale, 1991; Goto and Kimura, 1998; Levis et al., 2012).

The underpinning mechanisms of these RCH responses are poorly understood. While brief chilling triggers a series of physiological responses, including cryoprotective sugar and polyol accumulation, heat shock protein expression, desaturation of cellular membranes, enhanced Na^+/K^+ ATPase activity in neurons, reduced apoptotic activity, enhanced recycling of damaged proteins, and cytoskeletal remodeling, these adaptations are not universally-induced during RCH and do not always coincide with the acquisition of stress tolerance (Chen et al., 1987; Li and Denlinger, 2008; Overgaard et al., 2005; Michaud and Denlinger, 2006; Yi et al., 2007; Armstrong et al., 2012; Gerken et al., 2015; Teets and Denlinger, 2016). Therefore, the relative importance of each remains unclear. Further, recent evidence suggests that different types of RCH (i.e. triggered by chilling versus by dehydration) are mechanistically distinct (Yi et al., 2017; Gantz et al., *in review*), though little is known about these differences.

Accumulation of cryoprotective solutes is perhaps the most commonly-used mechanism in RCH (Lee and Denlinger, 2010). In cold-induced RCH, the solute accumulation response is typically limited to only 20-80 mM increases in hemolymph osmolality; however, even these modest changes can enhance cold tolerance non-colligatively by stabilizing proteins and plasma membranes (Carpenter and Crowe, 1988; Yoder et al., 2006; Overgaard et al., 2007). It is unknown whether drought-induced RCH elicits a similar response. In larvae of the goldenrod gall fly *Eurosta solidaginis*, 4 h of desiccation produced a 40 mOsm kg^{-1} increase in hemolymph osmolality that was not attributable to the osmo-concentration of fluids by dehydration, assuming equal loss of water across body compartments (Gantz and Lee, 2015). Interestingly, there was no

corresponding change in the concentrations of glycerol, sorbitol, or trehalose, which often accumulate during chilling, suggesting that drought-induced RCH may result in the accumulation of different cryoprotective compounds than cold-induced RCH does. It is also possible that this increase in solute concentration was not caused by cryoprotectant accumulation, but instead, by directed compartmentalization of body water (Benoit, 2010). Insects can defend the water content of particular tissues and preferentially lose water from other compartments, which could result in disproportionate water loss from hemolymph and explain the observed increase in osmotic pressure (Williams and Lee, 2011).

Cold- and drought-induced RCH may also have different effects on metabolic rates at low temperature. Thermal acclimation, where insects adapt their physiology to correspond with ambient temperatures, often includes increased metabolic rates during chilling (Berrigan et al., 1997; Isobe et al., 2013; Colinet et al., 2017). While this has mostly been investigated following long-term acclimation, recent studies have demonstrated that brief chilling also affects metabolism, as cold-induced RCH preserved metabolic homeostasis and altered activity of ATP synthase β (Overgaard et al., 2007; Teets et al., 2012a; Teets and Denlinger, 2016). Drought-induced RCH may also affect metabolism, as insects lower their metabolic rate under dehydration stress to reduce water loss during respiration (Hadley, 1994). Transcriptomic analyses showed that dehydration triggers a coordinated downregulation of metabolic pathways in a variety of insects, even in as few as 18 h (Benoit and Denlinger, 2007; Matzkin and Markow, 2009; Wang et al., 2011; Teets et al., 2012b).

The signaling cascades that regulate cold- and drought-induced RCH may also differ between responses. These biochemical cascades often begin by phosphorylating mitogen-activated protein kinases (MAP kinases), which are induced by stress and immune signals (Pearson et al., 2001). During chilling, a second messenger cascade is triggered by phosphorylation of p38 MAP kinase, which is an early step in the activation of cold-induced RCH (Fujiwara and Denlinger, 2007, Li et al., 2012). While p38 MAP kinase responds to a variety of environmental stressors, including osmotic shock (Zarubin and Jaihuai, 2005), it is unclear whether drought-induced RCH, which requires only mild dehydration (Gantz and Lee, 2015), activates this second messenger cascade. The Erk1/2 MAP kinase is, perhaps, a better candidate for activation during mild dehydration since it responds to dehydration stress in

mammalian cell lines, though it has not been linked to cold stress (Zhuang et al., 2000; Lu and Xu, 2006). Thus, it is possible that chilling and dehydration trigger different signaling cascades through the activation of p38 and Erk1/2 MAP kinases, respectively.

In this study, we investigated potential mechanistic differences between cold- and drought-induced RCH in larvae of the goldenrod gall fly, *E. solidaginis*. These flies develop and overwinter as larvae in stem galls on goldenrod plants (*Solidago* spp.). In the fall, host plants senesce and die, which acts as a cue to begin preparing for winter (Williams and Lee, 2005). During this transition, larvae acquire extreme freeze tolerance and desiccation resistance (Lee and Hankinson, 2003, Williams et al., 2004). Drought-induced RCH was first reported in larvae in the early stages of this seasonal transition (Levis et al., 2012), and this response is best characterized in this species (Gantz and Lee, 2015). Thus, we used September-collected larvae to examine 1) whether increased hemolymph osmolality during dehydration is caused by the accumulation of solutes or an altered distribution of body water, 2) whether chilling and dehydration cause changes to metabolic rates through thermal acclimation, and 3) whether p38 and Erk1/2 MAP kinases are activated during cold- and drought-induced RCH.

Methods

Field collection

Using *E. solidaginis* to study drought-induced RCH presents a challenge of timing. Through much of the summer, larvae are small and intolerant of many stresses, including dehydration and freezing (Williams et al., 2004; Williams and Lee, 2005). By late autumn, they have acquired freeze tolerance and, more relevant to this study, extreme resistance to dehydration. Winter-acclimatized larvae lose water at rates commensurate with the most xeric-adapted beetles (Ramlov and Lee, 2000), and consequently, do not dehydrate quickly enough to trigger drought-induced RCH. In southwestern Ohio, there is a six-to-eight-week window of opportunity between September and October in which larvae are moderately freeze tolerant (to about -15°C), yet are also susceptible enough to dehydration to trigger drought-induced RCH (Levis et al., 2012). For this study, stem galls were collected from goldenrod plants at Miami

University's Ecology Research Center in Butler County, OH, in late September and early October. Upon collection, galls were stored outside for up to a week before use.

Experimental treatments

After removal from galls, larvae were weighed, transferred to 1.7 ml microcentrifuge tubes and maintained for 1 h, 2 h, and 4 h in one of three treatments: 1) control, at 22°C with a damp paper towel and sealed lid, 2) cold-induced RCH, at 0°C in a circulating cold bath with a damp paper towel and sealed lid, and 3) drought-induced RCH, at 22°C, a dry paper towel, and ~0% relative humidity (RH) over anhydrous calcium sulfate (Drierite®). For our measurements of survival and changes in hemolymph osmolality, we included an additional, milder dehydration treatment at 75% RH over a saturated NaCl solution. For organismal and cellular survival assessments, larvae were exposed to a discriminating freezing treatment at -15°C for 24 h in a circulating cold bath immediately after RCH treatments concluded. To help standardize the temperature at which internal ice began forming, larvae were frozen in contact with a damp paper towel and a small piece of ice, thereby inoculating internal ice formation close to their freezing point. Other experiments in this study began immediately after RCH treatment, and consequently, these larvae were not exposed to subsequent cold shock.

Organismal and cellular survival

The development of extreme dehydration resistance is regulated by temperature and cues from host plants (Williams and Lee, 2011), meaning there is year-to-year variance in the timing of this response. Thus, we assessed whether larvae were within the critical developmental window for drought-induced RCH by measuring organismal and cellular survival of freezing before starting our mechanistic experiments. For organismal survival, larvae that moved in response to touch 2 h after cold shock were scored as alive (n=30 larvae per treatment). Cellular survival was assessed by a vital dyes assay using a LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA) as adapted by Yi and Lee (2003). After cold shock, fat body and midgut tissues were harvested and incubated in two fluorescent dyes, SYBR-14 and propidium

iodide. SYBR-14 is a membrane-permeating green dye while propidium iodide is a red dye that is excluded by intact cell membranes. After incubation, cells are scored as alive or dead by fluorescent microscopy, where red fluorescence (i.e. a compromised membrane allowing propidum iodide to stain nuclear DNA) indicates freezing damage and death. We scored a minimum of 250 cells for each tissue type, from each larva, to calculate the ratio of live vs. dead cells. To eliminate selection bias, we scored all cells that did not exhibit clear mechanical damage from dissection and staining in randomly-selected excised tissue. These data are presented as means ± SE from five larvae per treatment.

Hemolymph osmolality and distribution of body water

We collected hemolymph from 25 larvae per treatment using a glass microcapillary tube inserted through an incision in the cuticle. Hemolymph was pooled from 2-3 individuals to obtain 20 µl samples, which produced between 9 and 11 measurements of osmolality per treatment using an Advanced® Model 3320 freezing-point depression osmometer (Advanced Instruments Inc., Norwood, MA).

To determine whether larvae preferentially lost water from hemolymph while defending intracellular fluid volume, we measured the amount of water in each compartment (n = 12 larvae per treatment) using methods from Nicolson et al. (1974), which assumes that inaccessible interstitial fluid makes up an insignificant fraction of body water (Bodnaryk and Morrison, 1966; Djajakusumah and Miles, 1966). Briefly, we made an incision in the cuticle and removed hemolymph, first with a glass microcapillary tube, then with absorbent lab tissue. After weighing, the carcass was dried to completion at 65°C and weighed again. Using the wet mass of intact larvae, wet mass of the carcass without hemolymph, and the dry mass of the carcass, it was possible to calculate how body water was distributed between hemolymph and cells. Briefly, we subtracted the mass of the hemolymph from the wet and dry mass measurements, and thus found the intracellular water content.

Metabolic rate

Oxygen consumption, used as a proxy for metabolic rate, was measured in closed-system respirometers according to Lee and Baust (1982). Respirometers were made by sealing 10 µl glass micropipettes into the tip of a 1 ml plastic syringe using hot melt glue. Individual larvae (n=10 per treatment), along with a damp paper towel, were placed into a small space created near the tip of the syringe using the plunger to form a sealed chamber with only the 10 µl micropipette as an outlet. Then, a small plug of 10% KOH solution was added into the end of the micropipette. KOH absorbs CO_2 as it is generated, creating negative pressure in the now-closed system, causing the KOH plug to move along the length of the micropipette. Rates of O_2 consumption, using the change in volume within the closed system, were calculated by measuring the distance the KOH plug travelled during incubation at 1°C. Measurements began after allowing 15 min acclimation to the environment in the chamber and continued for 1 h.

Protein extraction for immunoblotting

Whole larvae (n=4 per treatment) were homogenized in a 1.5 ml Eppendorf tube containing radioimmunoprecipitation assay (RIPA) buffer (pH 7.5) with Halt Protease and Phosphatase Inhibitor mixture (ThermoFisher Scientific) (Yi et al., 2007; Teets et al., 2013). The homogenate was then sonicated with an ultrasonic processor (Cole Parmer, Vernon Hills, IL, USA) and centrifuged twice at 16000 *g* for 15 min at 4°C. The concentration of soluble proteins in the resulting supernatant was determined using NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific).

SDS-PAGE and immunoblotting

SDS-PAGE and Western blotting were conducted according to Yi et al. (2007). Fifty micrograms of protein samples were mixed with Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol and incubated at 95°C for 5 min, and then analyzed by SDS-PAGE on a 4–15% gradient gel (Bio-Rad) using Precision Plus protein standard kit (Bio-Rad) as a reference. Following electrophoresis, proteins were transferred to an Amersham Hybond nitrocellulose blotting membrane (GE Healthcare Life Science). The membrane was stained with 0.2%

Ponceau S staining solution (Sigma Chemical Company, Saint Louis, MO, USA) in 5% acetic acid for 10 min and rinsed with Ultrapure water to verify that proteins were transferred. After they were de-stained for 30 s in a 0.1 N NaOH solution and washed with Ultrapure water, they were used for immunoblotting.

Non-specific protein antigens were blocked at 4°C overnight in 10% non-fat milk Western wash buffer (10 mM Tris, 100 Mm NaCl and 0.1% Tween 20, pH 7.5) on a shaker. The membranes were incubated with the primary antibodies in a 5% non-fat milk solution at room temperature (21°C) for 2 h. Rabbit anti-phospho-p38 (Thr180/Tyr182) (1:1000), rabbit anti-p38 (total, 1:1000), rabbit anti-phospho-Erk1/2 (Thr202/Tyr204) (1:800), and rabbit anti-Erk1/2 (total, 1:1000) were purchased from Cell Signaling Tech. After five, 10-min washes in Western wash buffer, the membranes were incubated for 2 h at room temperature with corresponding secondary antibodies: Goat anti-rabbit IgG-HRP (horseradish peroxidase) conjugates and goat anti-mouse IgG-HRP conjugates (1:2000) (Sigma Chemical Company). Membranes were then washed in Western wash buffer, incubated for 2 min in Amersham Enhanced Chemiluminescence (ECL) Western Blotting Detection Kit (GE Healthcare), and exposed to autoradiography film. Each Western blot was repeated with four to six independent biological replicates. Densitometry of bands were semi-quantified using ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analyses

Organismal survival data were fitted to a generalized linear model with a logit link function and a binomial error distribution. We followed with Tukey's HSD to control for pairwise comparisons (R Foundation for Statistical Computing, Vienna, Austria). Cellular survival, hemolymph osmolality, distribution of body water, metabolic rates, and immunoblotting data were all analyzed using one-way analysis of variance (ANOVA) with the Holm-Sidak method of pairwise comparisons (SigmaPlot 13.0; Systat Software, San Jose, CA).

Results

Cold- and drought-induced RCH enhanced freeze tolerance

Cold- and drought-induced RCH enhanced larval freeze tolerance at both the organismal and cellular levels (Fig. 1, A, B, C). After 1 h treatments, only cold-induced RCH significantly improved organismal survival, with 37 ± 6% of larvae surviving subsequent freezing compared to 10 ± 4% of controls ($p < 0.05$), whereas 15 ± 5% and 23 ± 7% of larvae survived the mild (75% RH) and severe (0% RH) drought-induced RCH treatments, respectively (Fig. 1A). In fat body (Fig. 1B) and midgut (Fig. 1C) tissues, however, all RCH treatments enhanced freeze tolerance within 1 h ($p < 0.01$). Midgut tissue was the most sensitive to RCH, exhibiting a ~3-fold increase in survival across treatments.

When chilling and dehydration treatment lengths were increased to 2 and 4 h, the protective effects of RCH became even more pronounced. Indeed, all three treatments, cold-induced, mild drought-induced, and severe drought-induced RCH, enhanced survival of subsequent freezing in organismal, fat body, and midgut survival. The smallest treatment effect, which was observed in fat body tissue following 2 h in the mild drought-induced RCH treatment, still produced a 2.2-fold increase in survival ($p < 0.05$).

Cold- and drought-induced RCH increase solute concentrations by different mechanisms

Each RCH treatment produced a significant increase in hemolymph osmolality in as little as 1 h (Table 1). The mean hemolymph osmolality for control larvae immediately after being removed from their galls was 485 ± 5 mOsm kg^{-1}, while 1 h of cold-induced, mild drought-induced, and severe drought-induced RCH treatments increased hemolymph osmolalities to 502 ± 6, 509 ± 5, and 506 ± 4 mOsm kg^{-1}, respectively ($p < 0.01$). Even after subtracting the effects of osmo-concentration of body fluids during dehydration, both cold-induced and mild drought-induced RCH produced significant increases in osmolyte concentrations (Table 1; $p < 0.05$). Similar trends were observed after 2 and 4 h as well, as all three RCH treatments produced significant increases in both absolute and adjusted hemolymph osmolality ($p < 0.01$).

A different set of larvae were used to measure the distribution of water between intracellular compartments and hemolymph. In control larvae, intracellular water accounted for

54.4 ± 0.6% of tissue wet mass, which was similar to levels in cold- and drought-induced RCH treatments, at 54.6 ± 0.4 and 53.2 ± 0.9%, respectively (Table 2; $p = 0.33$). When calculated as a fraction of the total body water, larvae in the drought-induced RCH treatment had a higher portion of water in intracellular compartments than controls, 68.4 ± 2.2 versus 61.9 ± 1.6% ($p < 0.05$). In cold-induced RCH larvae, 65.0 ± 1.0% of total body water was in intracellular compartments, which was indistinguishable from either control or drought-induced RCH treatments ($p = 0.31$). Support for the validity of these methods is provided by the fact that our calculated total water volume and intracellular water volumes match closely with previously reported fractions of water among these compartments (Nicolson et al., 1974; Williams and Lee, 2010).

Cold- and drought-induced RCH have opposite effects on metabolic rate

The rate of oxygen consumption in control larvae was 0.25 ± 0.02 µl O_2/mg dry mass/h, which was not significantly different from that of cold-induced RCH, at 0.31 ± 0.04 µl O_2/mg dry mass/h ($p = 0.28$), or drought-induced RCH, at 0.20 ± 0.03 µl O_2/mg dry mass/h (Fig. 2; $p = 0.20$). However, oxygen consumption was significantly lower in drought-induced RCH when compared to cold-induced RCH ($p < 0.05$).

Brief chilling and desiccation had different effects on MAP kinase activation

Both cold- and drought-induced RCH increased the amount of phosphorylated p38 MAP kinase relative to controls without affecting total p38 MAP kinase concentrations, according to semi-quantitative densitometry (Fig. 3 A, B). Dehydration for 1 h produced a 3.3-fold increase in band density corresponding to phosphorylated p38 MAP kinase ($p < 0.01$; Fig. 3A). Prolonging dehydration, in the 2 and 4 h dehydration treatments, resulted in 3.0- and 3.1-fold increases in band density relative to controls ($p < 0.01$). Cold-induced RCH produced similar results, yielding a 2.9-fold increase in band density after 2 h ($p < 0.01$). Total p38 MAP kinase concentrations did not change during any RCH treatment ($p = 0.93$; Fig. 3B), suggesting the observed increases in concentration of phosphorylated p38 MAP kinase was not driven by synthesis of new protein.

Drought-induced RCH increased the phosphorylated portion of Erk1/2 MAP kinase relative to controls, though cold-induced RCH did not have a significant effect (Fig. 3 C, D). Dehydration for 1, 2, and 4 h produced 3.0-, 4.5-, and 3.2-fold increases in band density corresponding to phosphorylated Erk1/2 MAP kinase ($p < 0.05$; Fig. 3C). Chilling for 2 h produced a 2.4-fold increase relative to controls, though this difference was not significant ($p = 0.09$). Further, 2 h of dehydration produced higher concentrations of Erk1/2 MAP kinase than 2 h of chilling did ($p < 0.01$). Total Erk1/2 MAP kinase levels were unaffected by treatments ($p = 0.92$; Fig 3D).

Discussion

Previous studies in *E. solidaginis* have shown that brief dehydration causes increased hemolymph osmolality within a few hours, which suggests that drought-induced RCH triggers the accumulation of solutes (Levis et al., 2012; Gantz and Lee, 2015). However, these same studies did not find an associated increase in the concentrations of four commonly-used cryoprotectants (glycerol, sorbitol, glucose, and trehalose). Here, we found that 1 h of dehydration triggered a significant increase in hemolymph osmolality, which occurred concomitantly with the acquisition of enhanced freeze tolerance (Table 1; Fig. 1). Any increase in osmolality would be expected to enhance freeze tolerance by decreasing the amount of ice formed at a given temperature (Lee, 1991). Dehydration accomplishes this simply by reducing the amount of solvent (water) without adding new solute. Even so, drought-induced RCH increased the hemolymph osmolality beyond what can be explained by water loss, suggesting these results are the product of a directed acclimation response.

Increases in hemolymph osmolality can be attained by numerous mechanisms unrelated to cryoprotectant synthesis, including manipulating water balance among body compartments by altered aquaporin activity and the regulation of osmotic gradients by converting ions, amino acids, and fatty acids between soluble and insoluble forms (Hadley, 1994; Campbell et al., 2008; Benoit, 2010). Thus, we investigated whether the observed increases in hemolymph osmolality could be explained by maintenance of intracellular water volume during brief dehydration. Our results suggest that defense of intracellular water, at the expense of hemolymph water, accounts

for most or all of the increase in solute concentration observed during drought-induced RCH. Control larvae had an initial hemolymph osmolality of 508 ± 11 mOsm kg^{-1}, which increased to 574 ± 15 mOsm kg^{-1} after 2 h of dehydration (Table 2). The dehydration treatment resulted in a 4.2% reduction in body water, which, if we assume water was lost from all compartments evenly, would produce a predicted osmolality of 530 mOsm kg^{-1}. However, after treatment, the fraction of body water in intracellular compartments was 6.5% higher than in controls, though this ratio should not have changed if water loss was distributed evenly. Accounting for 6.5% less water in the hemolymph brings the calculated osmolality to 564 mOsm kg^{-1}, which is within the standard error of our measured value in this treatment. These results suggest preferential retention of intracellular water (Table 2).

In contrast, cold-induced RCH appears to trigger solute accumulation in *E. solidaginis*, as evidenced by an increase in hemolymph osmolality that cannot be explained by body water distribution (Table 2). Though it has not been shown in *E. solidaginis* before, cryoprotectant accumulation is a common (though not universal) feature of cold-induced RCH, which may be driven by temperature-dependent shifts in metabolic pathways (Teets and Denlinger, 2013; Teets and Denlinger, 2016). Additionally, these larvae use a multi-component cryoprotectant response during winter acclimation, which includes rapid accumulation of sorbitol during chilling (Baust and Lee, 1982; Storey and Storey, 1983). Here, larvae in the cold-induced RCH treatment had a hemolymph osmolality of 551 ± 12 mOsm kg^{-1}. Even after accounting for a non-significant increase in the percent of water in intracellular compartments, the predicted hemolymph osmolality in this treatment is only 524 mOsm kg^{-1}. The remaining gap between predicted and observed hemolymph osmolality, along with a history of cryoprotective solute synthesis during cold-induced RCH in other species, and a well-established cryoprotectant response in *E. solidaginis*, suggest that these results are due to solute accumulation.

Hence, it appears that cold- and drought-induced RCH trigger changes in hemolymph osmolality by different mechanisms, with management of water distribution during brief dehydration and solute synthesis during chilling. This may be why solute synthesis was not sufficient to explain the source of increased hemolymph osmolality in previous studies on drought-induced RCH (Levis et al., 2012; Gantz and Lee, 2015; Yi et al., 2017). Even so,

increases in solute concentration likely enhance freeze tolerance, regardless of how they are achieved.

The metabolic rate was significantly lower in drought-induced RCH treatments than in cold-induced RCH, though neither caused changes relative to controls (Fig. 2). Our inability to discern whether chilling, dehydration, or both affected metabolic rate makes it challenging to interpret these data; however, the difference between the two suggests that at least one triggered metabolic adjustment. Long-term adaptation to low temperature in cold-hardy insects often includes thermal acclimation of metabolic enzymes, increases in metabolic rate at a given temperature, and higher concentrations of ATP (Burnell et al., 1991; Berrigan et al., 1997; Takeuchi et al., 2009; Isobe et al., 2013; Williams et al., 2014; 2016; Colinet et al. 2017). Conversely, insects under dehydration stress tend to decrease their metabolic rate to reduce water loss through respiratory transpiration (Nicolson et al., 1974; Edney, 1977; Hadley, 1994; Benoit, 2010). Thus, either or both could have a modest, but ecologically-significant effect on metabolic physiology. Additionally, the differences observed between RCH responses in this study lends further support to the idea that brief chilling and dehydration trigger distinct biochemical mechanisms.

Both chilling and dehydration triggered the activation of p38 MAP kinase relative to controls (Fig. 3). Phosphorylation of p38 MAP kinase was previously linked to cellular cold-sensing and the induction of cold-induced RCH in insects (Fujiwara and Denlinger, 2007; Li et al., 2012). Osmotic stress in yeast and mammalian cells also phosphorylates this protein messenger (Han et al., 1994; Shiozaki and Russell, 1996). Thus, its activation during both cold- and drought-induced RCH is unsurprising. The sensitivity to both stresses suggests that p38 MAP kinase may be an important mediator of cross-tolerance between the physiological responses to dehydration and freezing. One of the primary challenges associated with freezing is increased osmotic stress as ice forms extracellularly, and the physiological adjustments to chilling often improve dehydration tolerance, and vice versa (Yoder et al., 2006; Hayward et al., 2007; Levis et al., 2012).

Conversely, Erk1/2 MAP kinase is more-closely associated with dehydration and osmotic stress in mammalian cells (Wehner et al., 2003), and was activated only during drought-induced RCH in this study (Fig. 3). Additionally, dehydration for 2 h increased Erk1/2 MAP kinase

phosphorylation more than 2 h of chilling did, which indicates that, even though there may be mechanistic overlap between RCH responses modulated by p38 MAP kinase, there are some differences as well. This supports the conclusions of Yi et al. (2017), where we interpreted improved cold tolerance in flies treated with both chilling and dehydration as an interaction between two mechanistically distinct RCH responses.

In this study, we provided the first direct evidence that, despite some mechanistic overlap, brief chilling and dehydration trigger distinct acclimatory responses. These differences have interesting implications from both physiological and ecological perspectives. Though we only compared the mechanisms of chilling and dehydration, a variety of abiotic stresses induce RCH-like responses, including anoxia and high temperature (Coulson and Bale, 1991; Goto and Kimura, 1998). The mechanisms of these other responses are unknown, and it is possible that the various triggers for RCH all produce distinct physiological responses. Cold- and drought-induced RCH may also interact in ecologically-relevant ways by simultaneously triggering rapid acclimatory responses to enhance stress tolerance more than either does separately (Yi et al., 2017). In nature, these interactions may be important because abiotic stressors rarely occur as single, isolated insults (Holmstrup, 2010). Indeed, both body temperature and hydration state are likely drastically influenced by even modest changes in ambient temperature and humidity, moving among microhabitats, mating, flight, and defensive behavior (Edney, 1977; Gringorten and Friend, 1982; Heinrich, 1993; Hadley, 1994; Nicolson, 1994; Johnson and Gibbs, 2004). Consequently, the interplay among different RCH responses may be critical to insects' success, particularly in temperate and polar regions.

References

Armstrong, G. A., Rodríguez, E. C., & Robertson, R. M. (2012). Cold hardening modulates K+ homeostasis in the brain of *Drosophila melanogaster* during chill coma. *Journal of Insect Physiology*, *58*(11), 1511-1516.

Baust, J. G., & Lee, R. E. (1982). Environmental triggers to cryoprotectant modulation in separate populations of the gall fly, *Eurosta solidaginis* (Fitch). *Journal of Insect Physiology*, *28*(5), 431-436.

Benoit, J. B., Lopez-Martinez, G., Michaud, M. R., Elnitsky, M. A., Lee, R. E., & Denlinger, D. L. (2007). Mechanisms to reduce dehydration stress in larvae of the Antarctic midge, *Belgica antarctica*. *Journal of Insect Physiology*, *53*(7), 656-667.

Benoit, J. B. (2010). Water management by dormant insects: comparisons between dehydration resistance during summer aestivation and winter diapause. In *Aestivation* (pp. 209-229). Springer, Berlin, Heidelberg.

Berrigan, D. (1997). Acclimation of metabolic rate in response to developmental temperature in *Drosophila melanogaster*. *Journal of Thermal Biology*, *22*(3), 213-218.

Bodnaryk, R. P., & Morrison, P. E. (1966). The relationship between nutrition, haemolymph proteins, and ovarian development in *Musca domestica* L. *Journal of Insect Physiology*, *12*(8), 963-976.

Burnell, A. M., Reaper, C., & Doherty, J. (1991). The effect of acclimation temperature on enzyme activity in *Drosophila melanogaster*. *Comparative Biochemistry and Physiology. B, Comparative Biochemistry*, *98*(4), 609-614.

Campbell, E. M., Ball, A., Hoppler, S., & Bowman, A. S. (2008). Invertebrate aquaporins: a review. *Journal of Comparative Physiology B*, *178*(8), 935-955.

Carpenter, J. F., & Crowe, J. H. (1988). The mechanism of cryoprotection of proteins by solutes. *Cryobiology*, *25*(3), 244-255.

Chen, C. P., Denlinger, D. L., & Lee, R. E. (1987). Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Physiological Zoology*, *60*(3), 297-304.

Colinet, H., Renault, D., & Roussel, D. (2017). Cold acclimation allows Drosophila flies to maintain mitochondrial functioning under cold stress. *Insect Biochemistry and Molecular Biology*, *80*, 52-60.

Coulson, S. J., & Bale, J. S. (1991). Anoxia induces rapid cold hardening in the house fly *Musca domestica* (Diptera: Muscidae). *Journal of Insect Physiology*, *37*(7), 497-501.

Djajakusumah, T., & Miles, P. W. (1966). Changes in the relative amounts of soluble protein and amino acid in the haemolymph of the locust, *Chortoicetes terminifera* Walker (Orthoptera: Acrididae), in relation to dehydration and subsequent hydration. *Australian Journal of Biological Sciences*, *19*(6), 1081-1094.

Edney, E. B. (1977). Water loss—respiratory. In *Water Balance in Land Arthropods* (pp. 66-86). Springer, Berlin, Heidelberg.

Fujiwara, Y., & Denlinger, D. L. (2007). p38 MAPK is a likely component of the signal transduction pathway triggering rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Journal of Experimental Biology*, *210*(18), 3295-3300.

Gantz, J. D., & Lee, R. E. (2015). The limits of drought-induced rapid cold-hardening: extremely brief, mild desiccation triggers enhanced freeze-tolerance in *Eurosta solidaginis* larvae. *Journal of Insect Physiology, 73*, 30-36.

Gantz, J. D., Spong, K. E., Seroogy, E., Robertson, R. M., & Lee, R. E. Effects of brief chilling and desiccation on ion homeostasis in the central nervous system of the migratory locust,

Locusta migratoria. *In review, Biology Open.*

Gerken, A. R., Eller, O. C., Hahn, D. A., & Morgan, T. J. (2015). Constraints, independence, and evolution of thermal plasticity: probing genetic architecture of long- and short-term thermal acclimation. *Proceedings of the National Academy of Sciences*, *112*(14), 4399-4404.

Goto, S. G., & Kimura, M. T. (1998). Heat-and cold-shock responses and temperature adaptations in subtropical and temperate species of Drosophila. *Journal of Insect Physiology*, *44*(12), 1233-1239.

Gringorten, J. L., & Friend, W. G. (1982). Water balance in *Rhodnius prolixus* during flight: quantitative aspects of diuresis and its relation to changes in haemolymph and flight-muscle water. *Journal of Insect Physiology*, *28*(7), 573-577.

Hadley, N. F. (1994). *Water relations of terrestrial arthropods*. Academic Press.

Han, J., Lee, J. D., Bibbs, L., & Ulevitch, R. J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, *265*(5173), 808-811.

Hayward, S. A., Rinehart, J. P., Sandro, L. H., Lee, R. E., & Denlinger, D. L. (2007). Slow dehydration promotes desiccation and freeze tolerance in the Antarctic midge *Belgica antarctica*. *Journal of Experimental Biology*, *210*(5), 836-844.

Heinrich, B. (1993). *The hot-blooded insects: strategies and mechanisms of thermoregulation*. Springer Science & Business Media.

Holmstrup, M., Bayley, M., Pedersen, S.A., & Zachariassen, K.E., 2010. Interactions between cold, desiccation and environmental toxins. In: Denlinger, D.L., Lee, R.E. (Eds.), Low Temperature Biology of Insects. Cambridge University Press, Cambridge, pp. 166–187.

Isobe, K., Takahashi, A., & Tamura, K. (2013). Cold tolerance and metabolic rate increased by cold acclimation in *Drosophila albomicans* from natural populations. *Genes & Genetic Systems*, *88*(5), 289-300.

Johnson, R. A., & Gibbs, A. G. (2004). Effect of mating stage on water balance, cuticular hydrocarbons and metabolism in the desert harvester ant, *Pogonomyrmex barbatus*. *Journal of Insect Physiology*, *50*(10), 943-953.

Kelty, J. D., Killian, K. A., & Lee, R. E. (1996). Cold shock and rapid cold-hardening of pharate adult flesh flies (*Sarcophaga crassipalpis*): effects on behaviour and neuromuscular function following eclosion. *Physiological Entomology*, *21*(4), 283-288.

Larsen, K. J., & Lee, R. E. (1994). Cold tolerance including rapid cold-hardening and inoculative freezing of fall migrant monarch butterflies in Ohio. *Journal of Insect Physiology*, *40*(10), 859-864.

Lee, R. E., & Baust, J. G. (1982). Respiratory metabolism of the Antarctic tick, *Ixodes uriae*. *Comparative Biochemistry and Physiology Part A: Physiology*, *72*(1), 167-171.

Lee, R. E., Chen, C. P., & Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science*, *238*(4832), 1415-1417.

Lee, R. E., & Hankison, S. J. (2003). Acquisition of freezing tolerance in early autumn and seasonal changes in gall water content influence inoculative freezing of gall fly larvae, *Eurosta solidaginis* (Diptera, Tephritidae). *Journal of Insect Physiology*, *49*(4), 385-393.

Lee R. E & Denlinger D. L. (eds) (2010). Rapid cold-hardening: ecological significance and underpinning mechanisms. Low temperature biology of insects. Cambridge University Press, New York, pp 35–58.

Levis, N. A., Yi, S.-X., & Lee, R. E. (2012). Mild desiccation rapidly increases freeze tolerance

of the goldenrod gall fly, *Eurosta solidaginis*: evidence for drought-induced rapid cold-hardening. *Journal of Experimental Biology*, *215*(21), 3768-3773.

Li, A., & Denlinger, D. L. (2008). Rapid cold hardening elicits changes in brain protein profiles of the flesh fly, *Sarcophaga crassipalpis*. *Insect Molecular Biology*, *17*(5), 565-572.

Li, F. F., Jun, X., Li, J. M., Liu, S. S., & Wang, X. W. (2012). p38 MAPK is a component of the signal transduction pathway triggering cold stress response in the MED cryptic species of *Bemisia tabaci*. *Journal of Integrative Agriculture*, *11*(2), 303-311.

Lu, Z., & Xu, S. (2006). ERK1/2 MAP kinases in cell survival and apoptosis. *IUBMB Life*, *58*(11), 621-631.

Matzkin, L. M., & Markow, T. A. (2009). Transcriptional regulation of metabolism associated with the increased desiccation resistance of the cactophilic *Drosophila mojavensis*. *Genetics*, *182*(4), 1279-1288.

Michaud, M. R., & Denlinger, D. L. (2006). Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology*, *52*(10), 1073-1082.

Nicolson, S. W., Horsfield, P. M., Gardiner, B. O. C., & Maddrell, S. H. P. (1974). Effects of starvation and dehydration on osmotic and ionic balance in *Carausius morosus*. *Journal of Insect Physiology*, *20*(10), 2061-2069.

Nicolson, S. W. (1994). Water replenishment following reflex bleeding in the blister beetle *Decapotoma lunata* Pallas (Coleoptera: Meloidae). *African Entomology*, *2*(1), 21-23.

Overgaard, J., Sørensen, J. G., Petersen, S. O., Loeschcke, V., & Holmstrup, M. (2005). Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *Journal of Insect Physiology*, *51*(11), 1173-1182.

Overgaard, J., Slotsbo, S., Holmstrup, M., & Bayley, M. (2007). Determining factors for cryoprotectant accumulation in the freeze-tolerant earthworm, *Dendrobaena octaedra*. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*, *307*(10), 578-589.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., & Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine Reviews*, *22*(2), 153-183.

Ramlov, H. A., & Lee, R. E. (2000). Extreme resistance to desiccation in overwintering larvae of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). *Journal of Experimental Biology*, *203*(4), 783-789.

Shiozaki, K., & Russell, P. (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. *Genes & Development*, *10*(18), 2276-2288.

Shreve, S. M., Kelty, J. D., & Lee, R. E. (2004). Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response. *Journal of Experimental Biology*, *207*(11), 1797-1802.

Storey, J. M., & Storey, K. B. (1983). Regulation of cryoprotectant metabolism in the overwintering gall fly larva, *Eurosta solidaginis*: temperature control of glycerol and sorbitol levels. *Journal of Comparative Physiology*, *149*(4), 495-502.

Takeuchi, K. I., Nakano, Y., Kato, U., Kaneda, M., Aizu, M., Awano, W., Yonemura, S., Kiyonaka, S., Mori, Y., Yamamoto, D. & Umeda, M. (2009). Changes in temperature preferences and energy homeostasis in dystroglycan mutants. *Science*, *323*(5922), 1740-1743.

Teets, N. M., Peyton, J. T., Ragland, G. J., Colinet, H., Renault, D., Hahn, D. A., & Denlinger, D. L. (2012a). Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiological Genomics*, *44*(15), 764-777.

Teets, N. M., Peyton, J. T., Colinet, H., Renault, D., Kelley, J. L., Kawarasaki, Y., Lee, R. E. & Denlinger, D. L. (2012b). Gene expression changes governing extreme dehydration tolerance in an Antarctic insect. *Proceedings of the National Academy of Sciences*, *109*(50), 20744-20749.

Teets, N. M., Yi, S.-X., Lee, R. E., & Denlinger, D. L. (2013). Calcium signaling mediates cold sensing in insect tissues. *Proceedings of the National Academy of Sciences*, *110*(22), 9154-9159.

Teets, N. M., & Denlinger, D. L. (2013). Physiological mechanisms of seasonal and rapid cold-hardening in insects. *Physiological Entomology*, *38*(2), 105-116.

Teets, N. M., & Denlinger, D. L. (2016). Quantitative phosphoproteomics reveals signaling mechanisms associated with rapid cold hardening in a chill-tolerant fly. *Journal of Proteome Research*, *15*(8), 2855-2862.

Wang, M. H., Marinotti, O., Vardo-Zalik, A., Boparai, R., & Yan, G. (2011). Genome-wide transcriptional analysis of genes associated with acute desiccation stress in *Anopheles gambiae*. *Plos One*, *6*(10), e26011.

Wang, X., Qi, X., & Kang, L. (2003). Rapid cold hardening process of insects and its ecologically adaptive significance. *Progress in Natural Science*, *13*(9), 641-647.

Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E., & Kinne, R. K. H., 2003. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. Springer, Berlin Heidelberg (pp. 1-80).

Williams, C. M., Watanabe, M., Guarracino, M. R., Ferraro, M. B., Edison, A. S., Morgan, T. J., Boroujerdi, A. F. & Hahn, D. A. (2014). Cold adaptation shapes the robustness of metabolic networks in *Drosophila melanogaster*. *Evolution*, *68*(12), 3505-3523.

Williams, C. M., McCue, M. D., Sunny, N. E., Szejner-Sigal, A., Morgan, T. J., Allison, D. B., & Hahn, D. A. (2016). Cold adaptation increases rates of nutrient flow and metabolic plasticity during cold exposure in *Drosophila melanogaster*. *Proceedings of the Royal Society B*, *283*(1838), 20161317.

Williams, J. B., Ruehl, N. C., & Lee, R. E. (2004). Partial link between the seasonal acquisition of cold-tolerance and desiccation resistance in the goldenrod gall fly *Eurosta solidaginis* (Diptera: Tephritidae). *Journal of Experimental Biology*, *207*(25), 4407-4414.

Williams, J. B., & Lee, R. E. (2005). Plant senescence cues entry into diapause in the gall fly *Eurosta solidaginis*: resulting metabolic depression is critical for water conservation. *Journal of Experimental Biology*, *208*(23), 4437-4444.

Williams, J. B., & Lee, R. E. (2011). Effect of freezing and dehydration on ion and cryoprotectant distribution and hemolymph volume in the goldenrod gall fly, *Eurosta solidaginis*. *Journal of Insect Physiology*, *57*(8), 1163-1169.

Yi, S.-X., & Lee, R. E. (2003). Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes. *Journal of Insect Physiology*, *49*(11), 999-1004.

Yi, S.-X., Moore, C. W., & Lee, R. E. (2007). Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis. *Apoptosis*, *12*(7), 1183-1193.

Yi, S.-X., Gantz, J. D., & Lee, R. E. (2017). Desiccation enhances rapid cold-hardening in the flesh fly *Sarcophaga bullata*: evidence for cross tolerance between rapid physiological

responses. *Journal of Comparative Physiology B, 187*(1), 79-86.

Yoder, J. A., Benoit, J. B., Denlinger, D. L., & Rivers, D. B. (2006). Stress-induced accumulation of glycerol in the flesh fly, *Sarcophaga bullata*: evidence indicating anti-desiccant and cryoprotectant functions of this polyol and a role for the brain in coordinating the response. *Journal of Insect Physiology, 52*(2), 202-214.

Zarubin, T., & Jiahuai, H. A. N. (2005). Activation and signaling of the p38 MAP kinase pathway. *Cell Research, 15*(1), 11.

Zhuang, S., Hirai, S. I., & Ohno, S. (2000). Hyperosmolality induces activation of cPKC and nPKC, a requirement for ERK1/2 activation in NIH/3T3 cells. *American Journal of Physiology-Cell Physiology, 278*(1), C102-C109.

Tables

Table 1. Changes in hemolymph osmolality after chilling and dehydration. 25 larvae per treatment. Hemolymph for osmolality measurement was pooled from 2-3 larvae, as needed. Predicted change in osmolality was calculated assuming 65% of fresh mass was due to water (measured from larvae collected at the same time) and the mass lost during dehydration. Hemolymph osmolality values are presented as mean ± SEM. Statistical significance ($p < 0.05$) is denoted by * and indicates that the change in osmolality is greater than zero.

	Measured osmolality (mOsm kg^{-1})	Percent body water lost	Predicted change in osmolality (mOsm kg^{-1})	Actual change in osmolality (mOsm kg^{-1})	Unexplained change in osmolality (mOsm kg^{-1})
Control	485 ± 5	N/A	N/A	N/A	N/A
RCH 1 h	502 ± 6	0.3	1	17*	16*
DRCH (75% RH) 1 h	509 ± 5	2.7	13	24*	11*
DRCH (0% RH) 1 h	506 ± 4	4.0	19	21*	2
RCH 2 h	509 ± 6	0.6	3	24*	21*

DRCH (75% RH) 2 h	533 ± 5	4.6	22	48*	26*
DRCH (0% RH) 2 h	534 ± 5	6.7	32	49*	18*
RCH 4 h	527 ± 8	1.1	5	42*	37*
DRCH (75% RH) 4 h	533 ± 4	6.7	32	48*	16*
DRCH (0% RH) 4 h	560 ± 7	9.4	46	75*	29*

Table 2. Redistribution of water during chilling and dehydration. Treatments lasted 2 h, with 12 larvae in each group. Hemolymph for osmolality measurement was pooled from 2-3 larvae, as needed. During treatment, control, RCH, and DRCH larvae lost 0.1, 0.4, and 4.2% of their body water, respectively. Intracellular water contents were calculated assuming 65% of fresh mass was due to water (measured from larvae collected at the same time), the mass lost during treatment, intracellular water content of the control treatment, and wet mass of the carcass after removal of hemolymph (data not shown). Hemolymph osmolality and percent water content are presented as mean ± SEM. Statistical significance ($p < 0.05$) is denoted by * and indicates that intracellular water content is different from control values.

	Hemolymph osmolality (mOsm kg^{-1})	Intracellular water, percent tissue mass	Expected intracellular water, percent tissue mass	Intracellular water, percent body water
Control	508 ± 11	54.4 ± 0.6	N/A	61.9 ± 1.6
RCH	551 ± 12	54.6 ± 0.7	54.7	65.0 ± 1.4
DRCH	574 ± 15	53.2 ± 0.9	52.1	68.4 ± 2.2*

Figure Legends

Figure 1. Both cold-induced RCH (RCH) and drought-induced RCH (DRCH) enhanced freezing tolerance at the organismal and cellular levels. Treatments are grouped by duration of exposure to chilling or dehydration on the x-axis. **A.** Organismal survival, assessed after 2 h of recovery from freezing. **B.** Ratio of surviving fat body cells, assessed immediately after freezing. **C.** Ratio of surviving midgut cells, assessed immediately after freezing. Treatments sharing a letter were not statistically distinguishable ($p<0.05$).

Figure 2. Larvae in the cold-induced RCH (RCH) treatment had a higher metabolic rate than larvae in the drought-induced RCH (DRCH) treatment, though neither differed from controls. Treatments sharing a letter were not statistically distinguishable ($p<0.05$).

Figure 3. Cold-induced RCH (RCH) and Drought-induced RCH (DRCH) had different effects on MAP kinase activation without changes to total protein levels. **A.** Both RCH and DRCH increased the phosphorylated fraction of p38 MAP kinase relative to controls. **B.** Neither treatment affected levels of total (both phosphorylated and un-phosphorylated) p38 MAP kinase protein. **C.** DRCH, but not RCH increased the phosphorylated fraction of Erk1/2 MAP kinase relative to controls. **D.** Neither treatment affected levels of total Erk1/2 MAP kinase. Treatments sharing a letter were not statistically distinguishable ($p<0.05$).

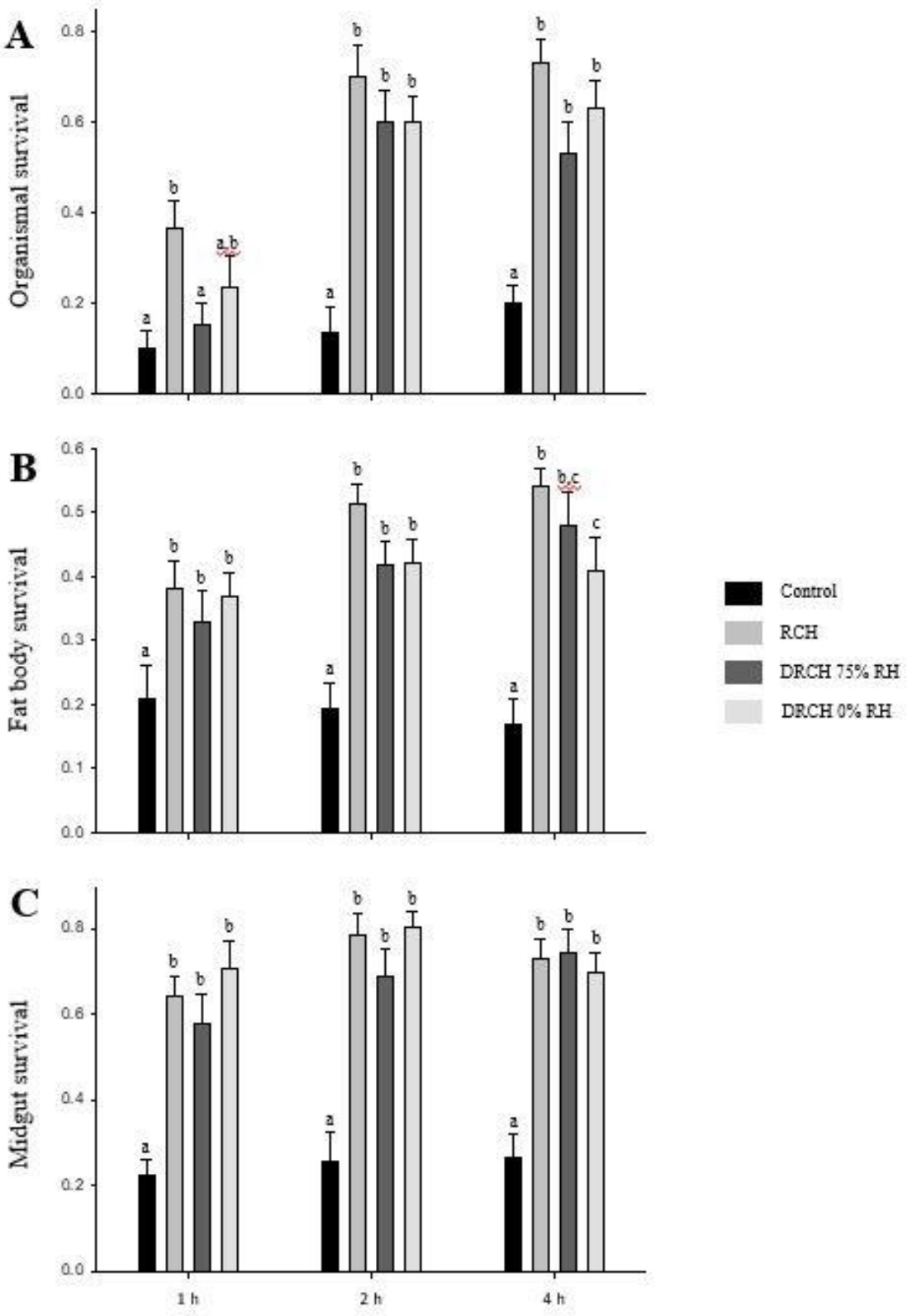
A
Organismal survival
B
Fat body survival
C
Midgut survival
Control
RCH
DRCH 75% RH
DRCH 0% RH
1 h
2 h
4 h

Figure 1

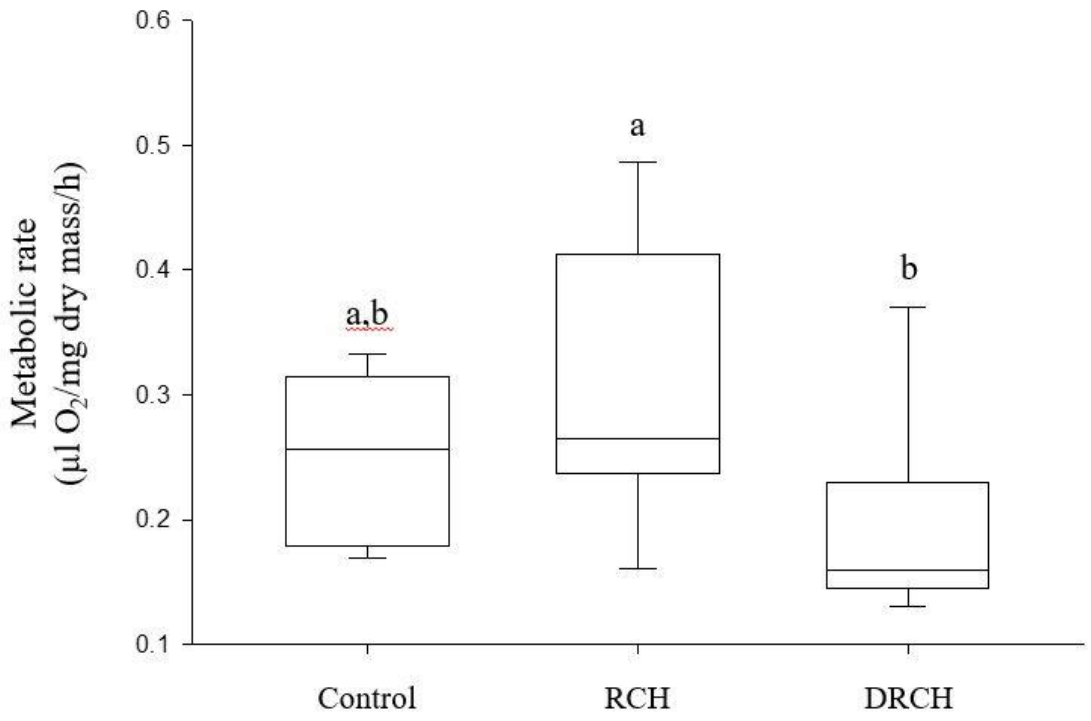
Metabolic rate
(µl O_2/mg dry mass/h)
0.6
0.5
0.4
0.3
0.2
0.1
a
a,b
b
Control
RCH
DRCH

Figure 2

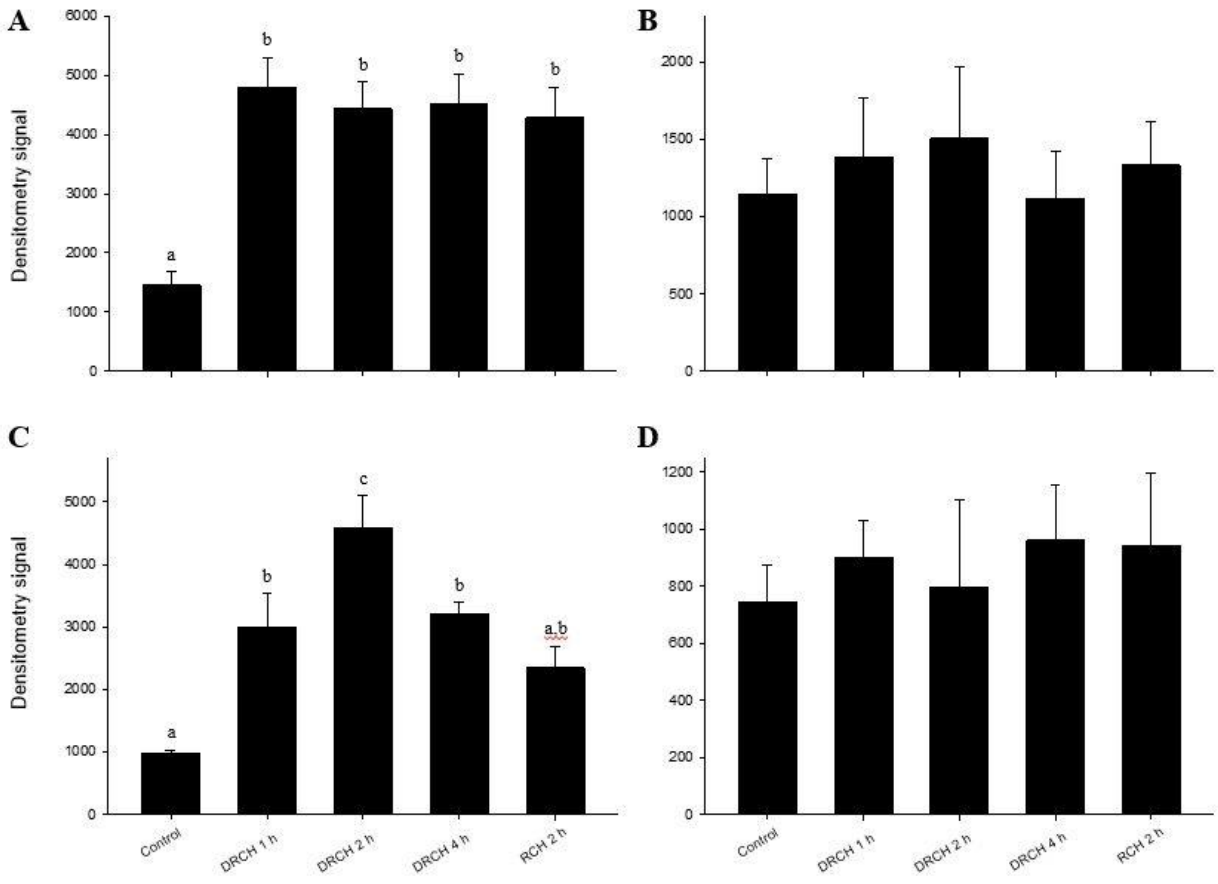
A
B
C
D
Densitometry signal
Densitometry signal
a
b
b
b
b
a
b
c
b
a,b
Control
DRCH 1 h
DRCH 2 h
DRCH 4 h
RCH 2 h

Figure 3

Chapter 3

Rapid acclimatory responses to chilling and dehydration improve flight performance and fecundity in the flesh fly, *Sarcophaga bullata*

Introduction

Many insects make physiological adjustments over weeks or months to prepare for seasonal changes in ambient conditions; however, in nature, they also experience drastic fluctuations in body temperature and hydration state that occur within minutes to hours. Remarkably, because of their small size, an insect's body temperature can change by 40°C or more during a 24 h thermoperiod, and can increase by 10°C in as few as 10 s when moving from shade to sunlight (Stevenson, 1985; Heinrich, 1993). Similarly, water loss during flight can cause a 40% reduction in hemolymph volume in fewer than 4 h (Gringorten and Friend, 1982). As such, physiological adjustments that help to counter these challenges must occur quickly. Insects use environmental cues such as temperature, humidity, and oxygen availability to trigger rapid acclimatory responses that enhance stress tolerance within minutes to hours (Lee et al., 1987; Coulson and Bale, 1991; Goto and Kimura, 1998, Levis et al., 2012). The best known of these responses is cold-induced rapid cold-hardening (RCH), where acclimation is triggered by brief exposure to sub-lethal chilling (Lee and Denlinger, 2010). Cold-induced RCH not only enhances survival at low temperature, it also preserves higher-order processes, such as courtship and mating, when low ambient temperature would otherwise prevent them (Kelty and Lee, 1999; Shreve et al., 2004).

Flight is a similarly complex, higher-order behavior that requires coordination of multiple organs and organ systems (Candy and Wegener, 1997). Excursions above or below critical thermal limits render insects unable to fly (Taylor, 1963), though few studies have investigated whether these disruptions are mitigated by RCH (Lee and Denlinger, 2010). Larsen and Lee (1994) found that severe cold shock prevented flight in migrating monarch butterflies (*Danaus plexipus*); however, cold-induced RCH rescued their ability to fly. Further, brief chilling triggers physiological adjustments that seem likely to enhance flight performance under stressful conditions, including reducing cellular damage (Yi and Lee, 2003), preserving ion homeostasis in nervous and muscle tissue (Powell and Bale, 2006; Armstrong et al., 2012; MacMillan and Sinclair., 2011), and triggering metabolic adaptations to increase ATP availability (Li and Denlinger, 2008; Teets and Denlinger, 2016). Yet it is unclear whether these adjustments improve flight performance in the expected manner.

Automatically-recording flight mills provide a convenient method to measure flight distance and velocity (Chambers et al., 1976). Typically, flight mills use tethered insects to power the movement of the mill while recording each revolution. Though these measurements aren't directly comparable to flight in the wild (Heinrich, 1974; Riley et al., 1997; Taylor et al., 2010; Ribak et al., 2017), they can provide insight about how treatment conditions affect flight in a controlled setting.

The physiological adjustments made during cold-induced RCH are also important for preserving insects' reproductive output (Irwin and Lee, 2003; Powell and Bale, 2006). In addition to immediate, deleterious impacts on survival, cold shock reduces fecundity and longevity (Rinehart et al., 2000; Overgaard and Sorensen, 2008). Rapid acclimation can preserve, or even enhance, fecundity and longevity, though these effects vary markedly among species (Coulson and Bale, 1992; Kelty and Lee, 1999; Rinehart et al., 2000; Broufas and Koveos, 2001; Powell and Bale, 2004; 2005). In the flesh fly, *Sarcophaga crassipalpis*, cold-induced RCH partially restored egg and sperm production following cold shock (Rinehart et al., 2000). Brief exposure to high temperature also reduced the effects of subsequent heat shock on egg production in female flies, suggesting that other RCH responses (i.e. those triggered by different environmental cues) also protect reproduction.

Comparatively little is known about rapid acclimation triggered by cues other than chilling. Brief exposure to dehydration (drought-induced RCH) also enhances stress tolerance, though it seems to do so via different underpinning mechanisms than cold-induced RCH (Yi et al., 2017; Gantz et al., *in review*; Gantz et al., *in preparation*). Most notably, drought-induced RCH may cause a decrease in metabolic rate (Wang et al., 2011; Gantz et al., *in preparation*), thereby reducing the availability of ATP for flight and other energy-expensive behaviors. This begs the question whether these mechanistic differences translate to organismal-level effects, i.e. do distinct underpinning mechanisms cause cold- and drought-induced RCH to affect flight and reproduction differently?

In this study, we used the flesh fly, *Sarcophaga bullata*, to investigate how rapid acclimatory responses triggered by chilling and dehydration affect flight performance and fecundity. We compared the effects of cold- and drought-induced RCH on 1) distance flown and peak flight velocity following cold shock, 2) how quickly the ability to fly was recovered

following cold shock, 3) how flight performance was affected by moderate chilling or dehydration (12°C or ~0% relative humidity [RH]), and 4) egg production after cold shock.

Methods

Experimental treatments, general

The flesh fly, *S. bullata* Parker (Diptera: Sarcophagidae), was reared in a laboratory colony at 25°C under 15:9 Light:Dark cycles, according to Lee and Denlinger (1985). Humidity within the rearing chamber was ~75% RH and all colonies were offered water and sugar cubes *ad libitum*.

We collected individuals from the colony in Falcon™ 15mL conical centrifuge tubes, replaced the lids with fine nylon mesh, and exposed flies to treatment conditions in these containers. Treatment conditions were as follows: 1) controls, held separately without food or water under otherwise normal rearing conditions, 2) cold shock, placed in a circulating cold bath at -7°C, 3) cold-induced RCH (RCH), placed in a circulating cold bath with a damp paper towel at 0°C, and 4) drought-induced RCH (DRCH), maintained in a sealed container over saturated $MgCl_2$ (33% RH) at 25°C, which resulted in a loss of ~1% fresh mass. Control, cold shock and RCH treatments lasted 2 h. RCH and DRCH treatments received subsequent cold shock for flight trials under control conditions, but not for flight under stressful conditions.

Flies used on flight mill

For flies that were used in flight mill assays (n = 10-12 per treatment), it was necessary to account for size, age, sex, mated status, dietary protein, and circadian rhythms because each of these can affect flight or RCH (Nakamori and Simizu, 1983; Liquido and Irwin, 1986; Schumacher et al., 1997; Helfrich-förster et al., 1998; Kelty and Lee, 2001; Coleman et al., 2015; Fischbein et al., 2018). We controlled size, sex, and mated status by discarding pupae that were not between 110 to 140 mg and separating males and females upon adult emergence. To control for age among treatment groups, only flies between 2 and 12 days post-emergence were used on

the mills, and each treatment was tested each day. Further, to account for circadian effects on flight, treatments were tested in rotation, the order of which changed each day such that start times for flight assays were evenly distributed throughout a day. Finally, colonies were not provided with a source of dietary protein to avoid confounding issues of reproductive development.

Flight mill design

Our flight mill design was adapted from Naranjo (1990). Briefly, flies were tethered to a balanced, lightweight lever that rotates around a fulcrum. A computer-interfaced infrared beam-break system recorded each rotation, which was used, along with the length of the lever arm, to calculate flight distance and velocity. Each mill was constructed using a Teflon™ bearing suspended above a center post using opposing magnets. The lever arm was created by threading a 22G stainless-steel hypodermic tube through a hole in the Teflon™ bearing, creating a low-friction mill that constrains flight to the horizontal plane. Flies were tethered to the mill by first attaching them to a #2 insect pin using a low-temperature glue gun, then inserting the pin into the opening at the end of the lever arm. Pins fit snugly enough to prevent rotation around their own axis. Once tethered to the mill, we measured duration flying, flight velocity, and total distance flown.

Experimental conditions, flight in control environment

We tested male and female flies separately in this assay. Mills were placed in large, clear plastic containers along with a small container of saturated NaCl to maintain humidity near 75% at 22°C. The plastic containers also reduced the effects of ambient air currents, as might occur when the HVAC system is active. After treatment, flies were immediately attached to the mill and recording was started. We used the elapsed time from the start of recording until the first rotation to measure the recovery time needed before flight became possible (latency to begin flying). Flies were left on the mill for 4 h after flight began.

Experimental conditions, flight in stressful environments

We only used female flies in these assays to remove the effects of differences among sexes (Fischbein et al., 2018). Flight under moderate abiotic stress was achieved as described above for control environments with a few modifications. For flight in a dehydrating environment, the container of saturated NaCl was replaced with anhydrous calcium sulfate (Drierite®) to maintain RH near 0%. For flight at low temperature, the whole apparatus (plastic container and mill) was transferred to a freestanding incubator set to 12°C. Flies were left on the mill for 2 h, during which we measured flight duration, velocity, and distance.

Fecundity

For assessment of fecundity (n = 10 per treatment), only pupae between 110 and 140 mg were used. Females were exposed to treatment conditions within a day of emergence as adults and housed as separate groups thereafter, along with 15 untreated males in each colony. Adults were fed beef liver *ad libitum* from day 1 though day 6 post-emergence. On day 11, females were collected and stored frozen at -20°C until analysis. Fecundity was assessed by proxy using egg production, where eggs were counted during abdominal dissection.

Statistical analyses

All data were analyzed using SigmaPlot 13.0 (Systat Software, San Jose, CA). Parametric data were analyzed using one-way analysis of variance (ANOVA) with the Holm-Sidak method of pairwise comparisons and are reported as mean ± SEM. Our results that failed the Shapiro-Wilk test of normality (male flies under control conditions- peak velocity; female flies under control conditions- distance flown and latency to begin flying; flight at 0% RH- distance flown; and flight at 12°C- distance flown and time spent flying) were analyzed using Kruskal-Wallis one-way ANOVA on ranks with post hoc Tukey test for pairwise comparisons and are reported as medians with their interquartile range (IQR).

Results

Cold- and drought-induced RCH preserved flight performance following cold shock

Cold- and drought-induced RCH significantly affected flight performance following cold shock in both male and female flies (Fig. 1). The cold shock treatment reduced peak flight velocity relative to controls in female flies, 6.6 ± 0.7 vs. 9.0 ± 0.5 m/s ($p < 0.05$; Fig. 1D), without significant effects on peak velocity in males (Fig. 1B), or distance flown in either sex (Fig. 1A,C). Despite few demonstrable costs of cold shock, DRCH enhanced distance flown, 3141 ± 290 m, and flight velocity, 10.2 (IQR = 2.0) m/s, compared to male flies that received only cold shock, at 1189 ± 206 m and 8.2 (IQR = 2.6) m/s, respectively ($p < 0.05$; Fig. 1A,B). In female flies, distance flown was indistinguishable from controls in each of our treatment groups (Fig. 1C). However, cold-induced RCH restored peak velocity to control levels, at 9.0 ± 0.5 and 9.0 ± 0.5 m/s, which were higher than cold shock, at 6.6 ± 0.7 m/s ($p < 0.05$; Fig. 1D).

Cold- and drought-induced RCH hastened recovery of the ability to fly following cold shock

For latency to begin flying, only treatments that received cold shock were included in analysis (i.e. untreated control flies were excluded) since there was no latency to begin flying in untreated controls. Among these treatments, male flies that received the chilling or dehydration treatment recovered the ability to fly faster than those that only received cold shock, at 622 ± 49, and 583 ± 61 vs. 936 ± 101 s, respectively ($p < 0.01$; Fig 2A). In females, cold shocked flies took longer to begin flying than cold-induced RCH flies, at 1950 s (IQR = 980) vs. 630 s (IQR = 1258), respectively ($p < 0.05$). Flies treated for drought-induced RCH began flying after 1070 s (IQR = 1072), which was indistinguishable from cold shock and cold-induced RCH treatments (Fig. 2B).

Cold- and drought-induced RCH enhanced flight ability under stress

Both cold- and drought-induced RCH enhanced flight performance under stressful conditions (Figs. 3 and 4). In desiccating conditions, flies treated for cold- and drought-induced

RCH flew further than controls, at 874 m (IQR = 1584), and 456 m (IQR = 523) vs. 259 (IQR = 209) ($p < 0.01$; Fig. 3A). Cold- and drought-induced RCH both had higher peak velocities than controls, at 7.9 ± 0.3 and 7.9 ± 0.5 m/s, compared to 6.3 ± 0.3 m/s ($p < 0.001$; Fig 3B). In time spent flying, cold-induced RCH increased the duration of flight relative to control and drought-induced RCH flies, 7065 (IQR = 505) vs. 5874 (IQR = 2664) and 6020 (IQR = 2300), respectively ($p < 0.05$; Fig. 3C).

In flight distance trials at 12°C, flies treated for drought-induced RCH flew further than the control treatment, 197 (IQR = 170) vs. 54 m (IQR = 52) ($p < 0.05$), while flies treated for cold-induced RCH were indistinguishable from controls, 139 (IQR = 46) (Fig. 4A). Peak velocity was greater in cold- and drought-induced RCH treatments than in controls, 7.3 ± 0.4 and 7.3 ± 0.3 m/s vs. 5.4 ± 0.3 ($p < 0.05$; Fig. 4B). Additionally, both cold- and drought-induced RCH-treated flies maintained the ability to fly longer than control flies, at 5709 ± 398 and 5459 ± 366 s, compared to 2346 ± 585 s ($p < 0.05$; Fig 4C).

Cold- and drought-induced RCH partially restored fecundity after cold shock

Cold shock significantly decreased egg production relative to control treatments, 54.8 ± 3.2 vs. 77.8 ± 2.6 eggs per female ($p < 0.001$). Cold-induced RCH restored egg production to 69.7 ± 2.0 eggs per female, which was indistinguishable from control flies and significantly higher than in the cold shock treatment ($p < 0.001$). Drought-induced RCH females produced 68.4 ± 2.2 eggs per female, which was fewer than controls ($p < 0.05$), but more than cold shocked flies ($p < 0.01$; Fig. 5).

Discussion

Both cold- and drought-induced RCH preserved or enhanced flight performance relative to appropriate controls in *S. bullata*, the effects of which were particularly noticeable in flight trials under stressful conditions, i.e. at low temperature or low humidity. Similar trends were observed in measurements of fecundity, where brief chilling and dehydration reduced or eliminated the negative effects of cold shock on egg production. Interestingly, there were few

examples of differences between cold- and drought-induced RCH among our results. Indeed, there were only two comparisons in which chilling and dehydration elicited significantly different responses, and only one case where the results trended in different directions (in males flown in control conditions, distance decreased following chilling, but increased following dehydration; Fig. 1A).

These similarities are notable because previous studies indicate that the acclimatory responses to chilling and dehydration are mechanistically distinct (Yi et al., 2017; Gantz et al., *in review*; Gantz et al., *in preparation*). As such, it would not have been surprising for cold- and drought-induced RCH to manifest differently at the organismal level, yet that was not the case since distance flown, peak velocity, resistance to stress, and fecundity were nearly uniformly preserved or enhanced. Previous studies also found that cold- and drought-induced RCH are similar in numerous ways, as both responses were sensitive to very modest cues, enhanced cellular and organismal cold tolerance within an hour, hastened recovery from chill coma (where low temperature exposure makes coordinated movements impossible), and afforded increased resistance to anoxia (Shreve et al., 2004; Gantz et al., 2015; Yi et al., 2017; Gantz et al., *in review*). Thus, despite some underpinning differences, chilling and dehydration seem to trigger general stress responses that are remarkably alike.

Flight is of paramount importance to insects, as it affects several behaviors that are central to survival and fitness, including resource location, migration, predator avoidance, and dispersal. Flight is also a complex process that requires exquisite coordination of oxygen delivery, ATP cycling, and substrate mobilization to muscle and nervous tissues (Kammer and Heinrich, 1978; Candy and Wegener, 1997). Since flying requires the integrated activity of many organs, improvements in flight ability suggest that cold- and drought-induced RCH affect multiple aspects of insect physiology in ecologically-relevant ways. Through our flight mill assays, we investigated two possible, organismal-level effects of these physiological adjustments on flight: 1) whether cold- and drought-induced RCH reduce the amount of tissue damage caused by cold shock, thereby preserving the ability to fly after returning to control conditions, and 2) whether chilling and dehydration produce physiological adjustments that enhance flight performance under moderate stress from dehydration or low temperature.

Cold- and drought-induced RCH had only modest effects on flight distance and velocity following cold shock in male and female flies (Fig. 1), though this is likely because our cold shock treatment was not severe enough to cause tissue damage that significantly impaired flight. Previous studies in monarch butterflies, *Danaus plexippus*, show that low temperature can disrupt flight, presumably by causing tissue damage (Larsen and Lee, 1994); however, flies in our cold shock treatment did not consistently perform worse than untreated controls. This apparent lack of damage may explain the relatively weak effects of RCH observed in this study.

In contrast, chilling and dehydration accelerated the rate of recovery from cold shock (Fig. 2) and increased distance, velocity, and duration of flight in stressful environments (Figs. 3 and 4). These results suggest that cold- and drought-induced RCH enhanced the ability to maintain and restore homeostatic control over the physiological and biochemical processes required for flight. This is consistent with previous studies that found rapid acclimation helps insects maintain homeostasis in critical systems (MacMillan and Sinclair, 2011; Armstrong et al., 2012; Gantz et al., *in review*). Differences in motivation to fly may also affect flight performance (Harrison and Lighton, 1998), presenting a potential confounding variable for these data. However, while motivation may be a factor, our results showing accelerated recovery from cold shock suggest that RCH enhanced underlying physiological processes that restore homeostatic norms. During recovery from chill coma, flies began flying on the mill shortly after they regained the ability to move at all. Thus, the latency to begin flying was likely independent of motivation and, instead, is a measure of the time required before flight was possible. Therefore, a reduction in recovery time indicates that homeostatic control was reestablished more quickly.

Interestingly, motivation to fly and physiological adjustments to enhance stress tolerance may be regulated through a shared mechanism. The neurohormone octopamine enhances tolerance of thermal stress and stimulates physical activity in insects (Farooqui, 2012). Though it has not previously been linked to RCH, octopamine is released within minutes of exposure to high and low temperature (Davenport and Evans, 1984; Adamo et al., 1995). It also triggers the mobilization of lipids to fuel intense activity and is associated with initiating and maintaining flight (Claassen and Kammer, 1986; Orchard et al., 1993; Adamo et al., 1995). Further, octopamine influences mating behavior, memory, and feeding and grooming reflexes (Farooqui, 2012), which are similarly affected by RCH (Lee and Denlinger, 2010). Thus, we suggest that

octopamine is an important regulator of rapid acclimatory responses and helps to modulate the enhanced flight performance observed in this study.

RCH also preserved egg production in female flies after cold shock (Fig. 5). These results are consistent with previous studies that found cold-induced RCH can mitigate the negative effects of cold shock on fecundity (Coulson and Bale, 1992; Kelty and Lee, 1999; Rinehart et al., 2000). In contrast, drought-induced RCH is a recent discovery and its effects on reproduction have not been investigated before now. Even so, it is unsurprising that drought-induced RCH partially restored fecundity in this study because reproductive output is reduced by acute temperature stress (Hutchinson and Bale, 1994), and drought-induced RCH reduces cellular damage caused by cold shock in *S. bullata* and other flies (Gantz and Lee, 2015; Yi et al., 2017).

Clearly, RCH preserves or enhances ecologically-relevant behaviors and processes in laboratory-based assays designed to trigger these responses; however, there is also substantial evidence that rapid acclimation is important in nature. RCH can be triggered by naturally-occurring rates of cooling and dehydration (e.g. 0.1°C per min; loss of less than 1% of fresh mass over 2 h) (Kelty and Lee, 1999; 2001; Gantz and Lee, 2015). This sensitivity to mild stress indicates that insects use rapid acclimation to track ambient conditions and make appropriate physiological adjustments (Lee and Denlinger, 2010). Additionally, due to their small size, ability to move among diverse microhabitats, and the physiological ramifications of intense activity (e.g. significant loss of body water during flight), insects can also experience drastic changes in conditions, where rapid acclimation may be particularly important for the maintenance of fundamental behaviors. Indeed, even moving from the protected environment of a burrow or the underside of an actively-transpiring leaf into the open can cause precipitous changes in temperature and humidity that exceed the severity of some treatments used in this study (Willmer, 1982). Further, behaviors such as mating, flight, and use of chemical defenses produce even more drastic fluctuations in hydration state than we achieved with these treatments (Gringorten and Friend, 1982; Nicholson, 1994; Johnson and Gibbs, 2004). Thus, in nature, cold- and drought-induced RCH likely help to mitigate the disruptions that insects routinely experience throughout a normal day.

References

Adamo, S. A., Linn, C. E., & Hoy, R. R. (1995). The role of neurohormonal octopamine During 'fight or flight' behaviour in the field cricket *Gryllus bimaculatus*. *Journal of Experimental Biology*, *198*(8), 1691-1700.

Armstrong, G. A., Rodríguez, E. C., & Robertson, R. M. (2012). Cold hardening modulates K+ homeostasis in the brain of *Drosophila melanogaster* during chill coma. *Journal of Insect Physiology*, *58*(11), 1511-1516.

Broufas, G. D., & Koveos, D. S. (2001). Rapid cold hardening in the predatory mite *Euseius* (Amblyseius) *finlandicus* (Acari: Phytoseiidae). *Journal of Insect Physiology*, *47*(7), 699-708.

Candy, D. J., Becker, A., & Wegener, G. (1997). Coordination and integration of metabolism in insect flight. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *117*(4), 497-512.

Chambers, D. L., Sharp, J. L., & Ashley, T. R. (1976). Tethered insect flight: A system for automated data processing of behavioral events. *Behavior Research Methods & Instrumentation*, *8*(4), 352-356.

Coleman, P. C., Bale, J. S., & Hayward, S. A. (2015). Meat feeding restricts rapid cold hardening response and increases thermal activity thresholds of adult blow flies, *Calliphora vicina* (Diptera: Calliphoridae). *PloS One*, *10*(7), e0131301.

Coulson, S. J., & Bale, J. S. (1991). Anoxia induces rapid cold hardening in the house fly *Musca domestica* (Diptera: Muscidae). *Journal of Insect Physiology*, *37*(7), 497-501.

Coulson, S. C., & Bale, J. S. (1992). Effect of rapid cold hardening on reproduction and survival of offspring in the housefly *Musca domestica*. *Journal of Insect Physiology*, *38*(6), 421-424.

Davenport, A. P., & Evans, P. D. (1984). Stress-induced changes in the octopamine levels of insect haemolymph. *Insect Biochemistry*, *14*(2), 135-143.

Farooqui, T. (2012). Review of octopamine in insect nervous systems. *Open Access Insect Physiology*, *4*, 1-17.

Fischbein, D., Villacide, J. M., De La Vega, G., & Corley, J. C. (2018). Sex, life history and morphology drive individual variation in flight performance of an insect parasitoid. *Ecological Entomology*, *43*(1), 60-68.

Gantz, J. D., Spong, K. E., Seroogy, E., Robertson, R. M., & Lee, R. E. Effects of brief chilling and desiccation on ion homeostasis in the central nervous system of the migratory locust, *Locusta migratoria*. *In review, Biology Open.*

Gantz, J. D., Yi, S-X, & Lee, R. E. Brief desiccation and chilling trigger mechanistically-distinct rapid cold-hardening responses in larvae of the goldenrod gall fly, *Eurosta solidaginis*. *In preparation*.

Goto, S. G., & Kimura, M. T. (1998). Heat-and cold-shock responses and temperature adaptations in subtropical and temperate species of Drosophila. *Journal of Insect Physiology*, *44*(12), 1233-1239.

Gringorten, J. L., & Friend, W. G. (1982). Water balance in *Rhodnius prolixus* during flight: quantitative aspects of diuresis and its relation to changes in haemolymph and flight-muscle water. *Journal of Insect Physiology*, *28*(7), 573-577.

Harrison, J. F., & Lighton, J. R. (1998). Oxygen-sensitive flight metabolism in the dragonfly *Erythemis simplicicollis*. *Journal of Experimental Biology*, *201*(11), 1739-1744.

Heinrich, B. (1974). Thermoregulation in endothermic insects. *Science*, *185*(4153), 747-756.

Heinrich, B. (1993). *The hot-blooded insects: strategies and mechanisms of thermoregulation*. Springer Science & Business Media.

Helfrich-förster, C., Stengl, M., & Homberg, U. (1998). Organization of the circadian system in insects. *Chronobiology international*, *15*(6), 567-594.

Hutchinson, L. A., & Bale, J. S. (1994). Effects of sublethal cold stress on the aphid *Rhopalosiphum padi*. *Journal of Applied Ecology*, 102-108.

Irwin, J. T., & Lee Jr, R. E. (2003). Cold winter microenvironments conserve energy and improve overwintering survival and potential fecundity of the goldenrod gall fly, *Eurosta solidaginis*. *Oikos*, *100*(1), 71-78.

Johnson, R. A., & Gibbs, A. G. (2004). Effect of mating stage on water balance, cuticular hydrocarbons and metabolism in the desert harvester ant, *Pogonomyrmex barbatus*. *Journal of Insect Physiology*, *50*(10), 943-953.

Kammer, A. E., & Heinrich, B. (1978). Insect flight metabolism. In *Advances in Insect Physiology* (Vol. 13, pp. 133-228). Academic Press.

Kelty, J. D., & Lee Jr, R. E. (1999). Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. *Journal of Insect Physiology*, *45*(8), 719-726.

Kelty, J. D., & Lee, R. E. (2001). Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophiladae) during ecologically based thermoperiodic cycles. *Journal of Experimental Biology*, *204*(9), 1659-1666.

Larsen, K. J., & Lee, R. E. (1994). Cold tolerance including rapid cold-hardening and inoculative freezing of fall migrant monarch butterflies in Ohio. *Journal of Insect Physiology*, *40*(10), 859-864.

Lee, R. E., & Denlinger, D. L. (1985). Cold tolerance in diapausing and non-diapausing stages of the flesh fly, *Sarcophaga crassipalpis*. *Physiological Entomology*, *10*(3), 309-315.

Lee, R. E., Chen, C. P., & Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science*, *238*(4832), 1415-1417.

Lee R. E & Denlinger D. L. (eds) (2010). Rapid cold-hardening: ecological significance and underpinning mechanisms. Low temperature biology of insects. Cambridge University Press, New York, pp 35–58.

Levis, N. A., Yi, S.-X., & Lee, R. E. (2012). Mild desiccation rapidly increases freeze tolerance of the goldenrod gall fly, *Eurosta solidaginis*: evidence for drought-induced rapid cold-hardening. *Journal of Experimental Biology*, *215*(21), 3768-3773.

Li, A., & Denlinger, D. L. (2008). Rapid cold hardening elicits changes in brain protein profiles of the flesh fly, *Sarcophaga crassipalpis*. *Insect Molecular Biology*, *17*(5), 565-572.

Liquido, N. J., & Irwin, M. E. (1986). Longevity, fecundity, change in degree of gravidity and lipid content with adult age, and lipid utilisation during tethered flight of alates of the corn leaf aphid, *Rhopalosiphum maidis*. *Annals of Applied Biology*, *108*(3), 449-459.

MacMillan, H. A., & Sinclair, B. J. (2011). Mechanisms underlying insect chill-coma. *Journal of Insect Physiology*, *57*(1), 12-20.

Nakamori, H., & Simizu, K. (1983). Comparison of flight ability between wild and mass-reared melon fly, *Dacus cucurditae* Coquillett (Diptera: Tephritidae), using a flight mill. *Applied Entomology and Zoology*, *18*(3), 371-381.

Naranjo, S. E. (1990). Comparative flight behavior of *Diabrotica virgifera virgifera* and

Diabrotica barberi in the laboratory. *Entomologia Experimentalis et Applicata*, *55*(1), 79-90.

Nicolson, S. W. (1994). Water replenishment following reflex bleeding in the blister beetle *Decapotoma lunata* Pallas (Coleoptera: Meloidae). *African Entomology*, *2*(1), 21-23.

Powell, S. J., & Bale, J. S. (2004). Cold shock injury and ecological costs of rapid cold hardening in the grain aphid *Sitobion avenae* (Hemiptera: Aphididae). *Journal of Insect Physiology, 50*(4), 277-284.

Powell, S. J., & Bale, J. S. (2005). Low temperature acclimated populations of the grain aphid *Sitobion avenae* retain ability to rapidly cold harden with enhanced fitness. *Journal of Experimental Biology, 208*(13), 2615-2620.

Powell, S. J., & Bale, J. S. (2006). Effect of long-term and rapid cold hardening on the cold torpor temperature of an aphid. *Physiological Entomology*, *31*(4), 348-352.

Ribak, G., Barkan, S., & Soroker, V. (2017). The aerodynamics of flight in an insect flight-mill. *PloS One, 12*(11), e0186441.

Riley, J. R., Downham, M. C. A., & Cooter, R. J. (1997). Comparison of the performance of Cicadulina leafhoppers on flight mills with that to be expected in free flight. *Entomologia Experimentalis et Applicata, 83*(3), 317-322.

Rinehart, J. P., Yocum, G. D., & Denlinger, D. L. (2000). Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sarcophaga crassipalpis*. *Physiological Entomology*, *25*(4), 330-336.

Schumacher, P., Weyeneth, A., Weber, D. C., & Dorn, S. (1997). Long flights in *Cydia*

pomonella L. (Lepidoptera: Tortricidae) measured by a flight mill: influence of sex, mated status and age. *Physiological Entomology*, *22*(2), 149-160.

Shreve, S. M., Kelty, J. D., & Lee, R. E. (2004). Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response. *Journal of Experimental Biology*, *207*(11), 1797-1802.

Stevenson, R. D. (1985). Body size and limits to the daily range of body temperature in terrestrial ectotherms. *The American Naturalist*, *125*(1), 102-117.

Taylor, L. R. (1963). Analysis of the effect of temperature on insects in flight. *The Journal of Animal Ecology*, 99-117.

Taylor, R. A., Bauer, L. S., Poland, T. M., & Windell, K. N. (2010). Flight performance of *Agrilus planipennis* (Coleoptera: Buprestidae) on a flight mill and in free flight. *Journal of Insect Behavior*, *23*(2), 128-148.

Teets, N. M., & Denlinger, D. L. (2016). Quantitative phosphoproteomics reveals signaling mechanisms associated with rapid cold hardening in a chill-tolerant fly. *Journal of Proteome Research*, *15*(8), 2855-2862.

Wang, M. H., Marinotti, O., Vardo-Zalik, A., Boparai, R., & Yan, G. (2011). Genome-wide transcriptional analysis of genes associated with acute desiccation stress in *Anopheles gambiae*. *Plos One*, *6*(10), e26011.

Willmer, P. G. (1982). Microclimate and the environmental physiology of insects. In *Advances in Insect Physiology* (Vol. 16, pp. 1-57). Academic Press.

Yi, S.-X., & Lee, R. E. (2003). Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes. *Journal of Insect Physiology*, *49*(11), 999-1004.

Yi, S.-X., Gantz, J. D., & Lee, R. E. (2017). Desiccation enhances rapid cold-hardening in the flesh fly *Sarcophaga bullata*: evidence for cross tolerance between rapid physiological responses. *Journal of Comparative Physiology B, 187*(1), 79-86.

Figure Legends

Figure 1. Cold-induced RCH (RCH) and drought-induced RCH (DRCH) reduce the negative costs of cold shock in male and female flies. Results are from 4 h on flight mill. **A** Distance flown, males. **B** Peak velocity, males. **C** Distance flown, females. **D** Peak velocity, females. Treatments sharing a letter were not statistically distinguishable ($p<0.05$).

Figure 2. RCH and DRCH reduced the recovery time needed to begin flying after cold shock. Time elapsed between when flies were tethered to the flight mill and flying began in **A** males and **B** females. Treatments sharing a letter were not statistically distinguishable ($p<0.05$).

Figure 3. Both RCH and DRCH enhanced flight performance at 0% RH. Results are from 2 h on flight mill. **A** Distance flown. **B** Peak velocity. **C** Duration of flight. Treatments sharing a letter were not statistically distinguishable ($p<0.05$).

Figure 4. Both RCH and DRCH enhanced flight performance at 12°C. Results are from 2 h on flight mill. **A** Distance flown. **B** Peak velocity. **C** Duration of flight. Treatments sharing a letter were not statistically distinguishable ($p<0.05$).

Figure 5. RCH and DRCH reduced the negative effects of cold shock on fecundity. Total number of eggs contained within female flies 11 days after adult emergence. Treatments sharing a letter were not statistically distinguishable ($p<0.05$).

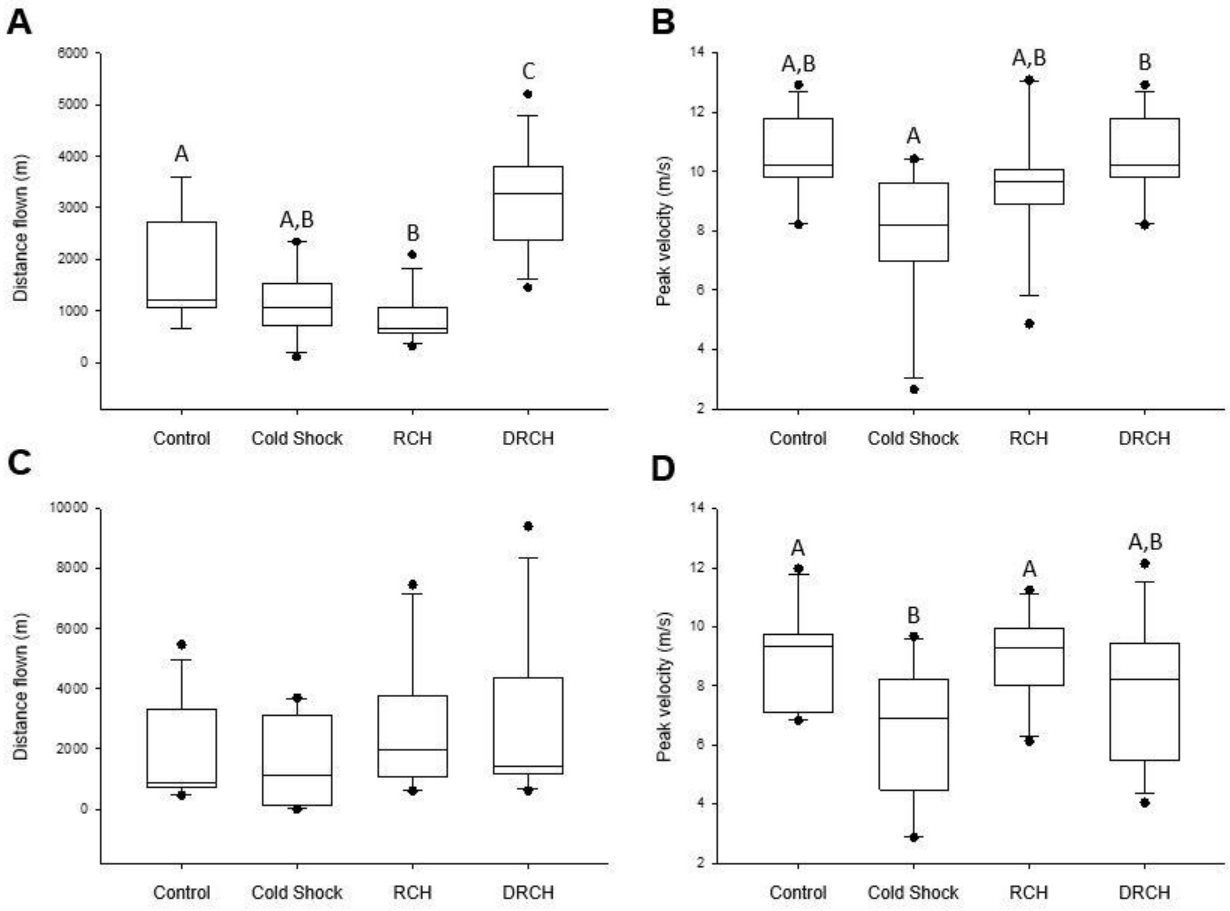
A
Distance flown (m)
6000
5000
4000
3000
2000
1000
0
A
A,B
B
C
Control
Cold Shock
RCH
DRCH
B
Peak velocity (m/s)
14
12
10
8
6
4
2
A,B
A
A,B
B
Control
Cold Shock
RCH
DRCH
C
Distance flown (m)
10000
8000
6000
4000
2000
0
Control
Cold Shock
RCH
DRCH
D
Peak velocity (m/s)
14
12
10
8
6
4
2
A
B
A
A,B
Control
Cold Shock
RCH
DRCH

Figure 1

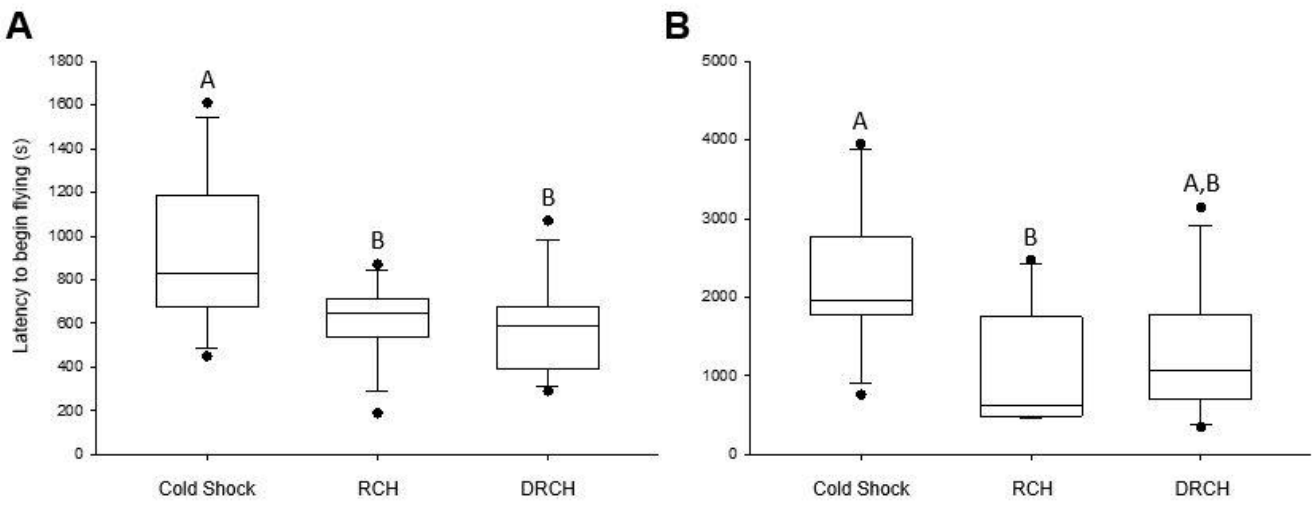
A
Latency to begin flying (s)
1800
1600
1400
1200
1000
800
600
400
200
0
A
B
B
Cold Shock
RCH
DRCH
B
5000
4000
3000
2000
1000
0
A
B
A,B
Cold Shock
RCH
DRCH

Figure 2

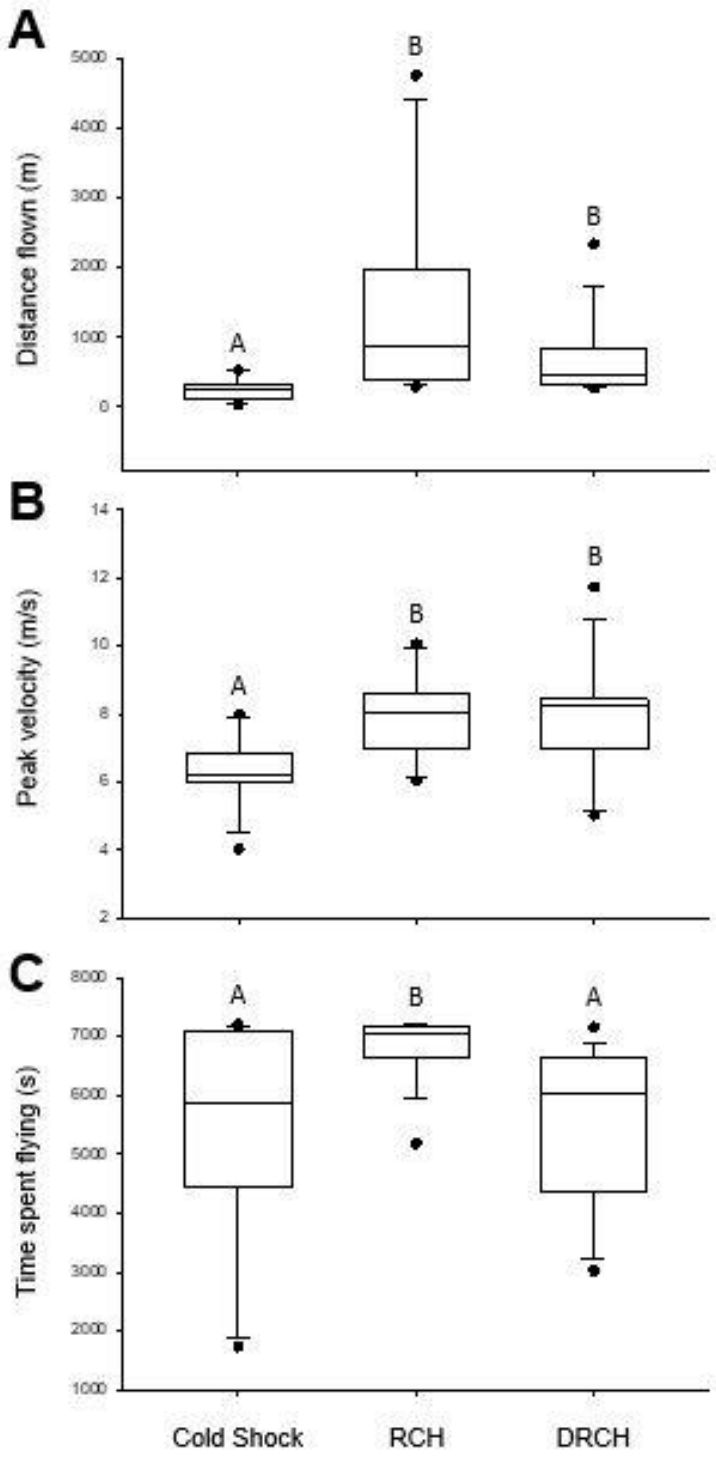
A
Distance flown (m)
5000
4000
3000
2000
1000
0
A
B
B
B
Peak velocity (m/s)
14
12
10
8
6
4
2
A
B
B
C
Time spent flying (s)
8000
7000
6000
5000
4000
3000
2000
1000
A
B
A
Cold Shock
RCH
DRCH

Figure 3

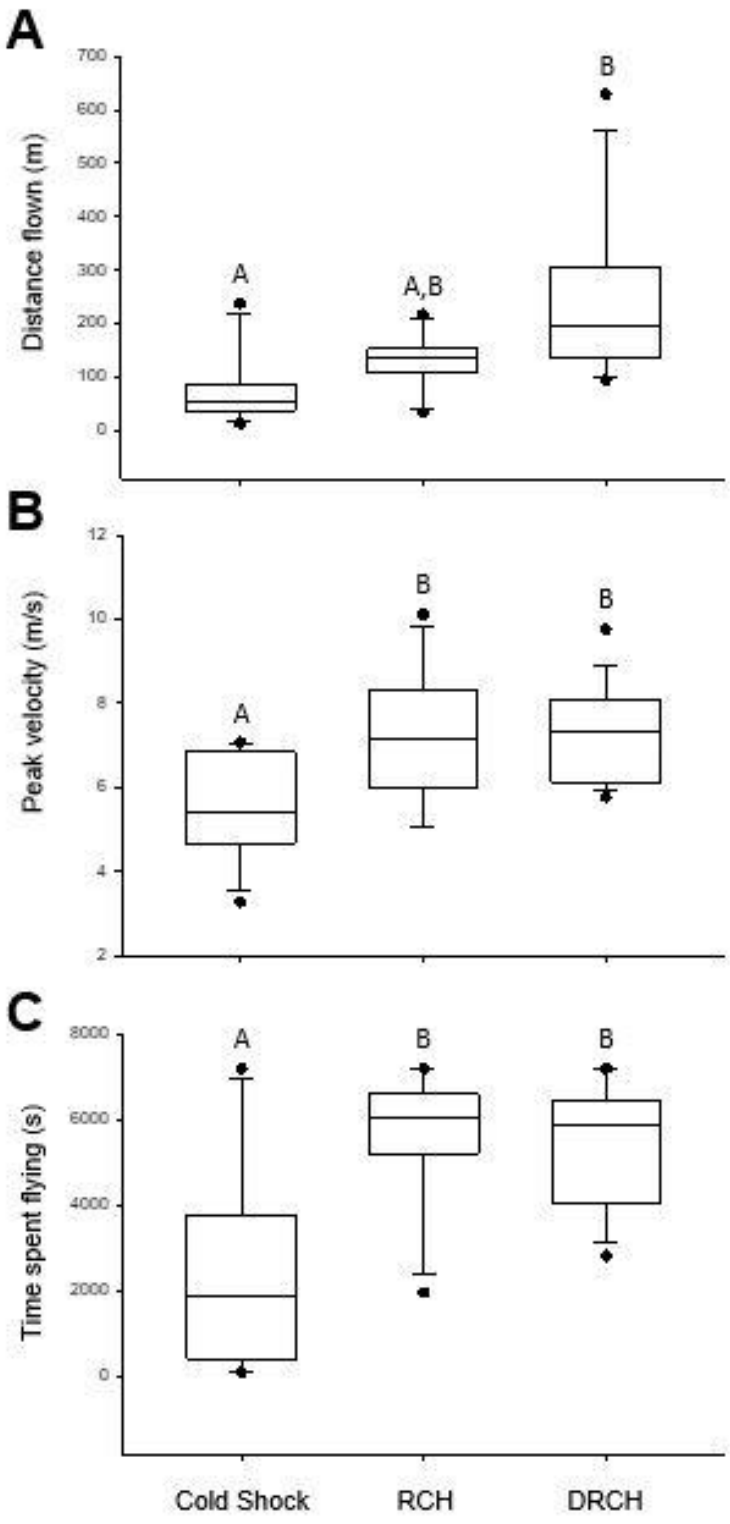
A
Distance flown (m)
700
600
500
400
300
200
100
0
A
A,B
B
B
Peak velocity (m/s)
12
10
8
6
4
2
A
B
B
C
Time spent flying (s)
8000
6000
4000
2000
0
A
B
B
Cold Shock
RCH
DRCH

Figure 4

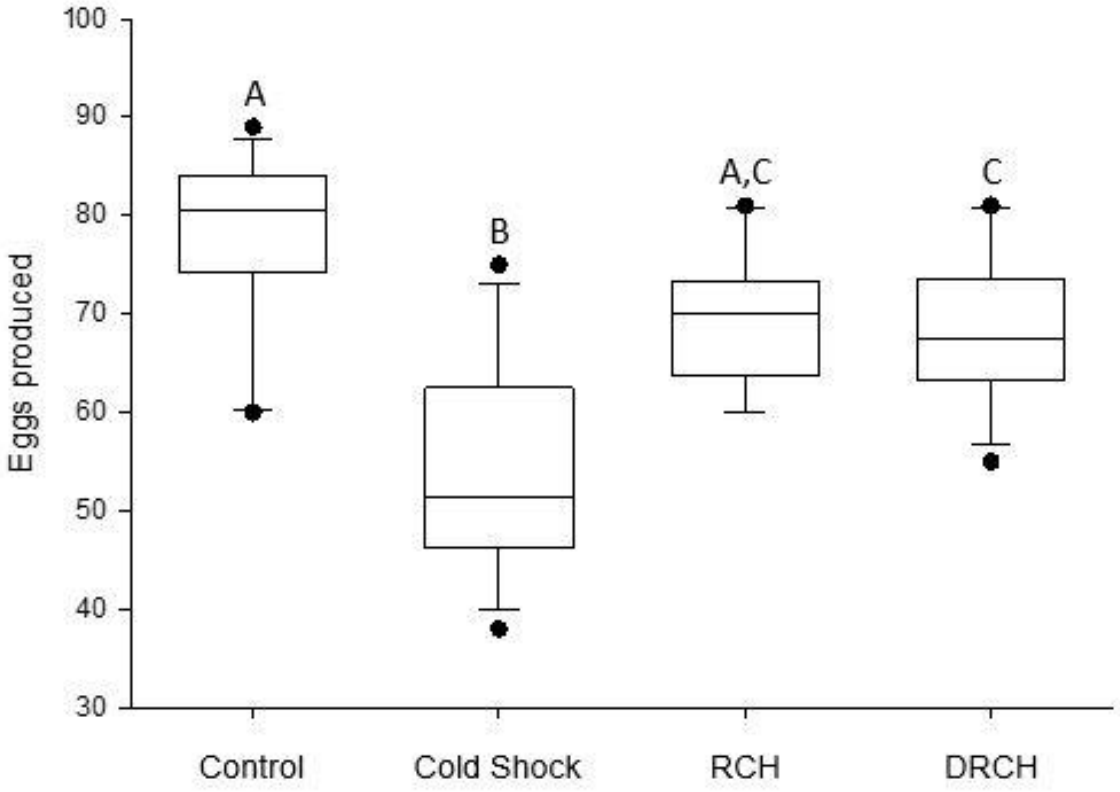
100
90
80
70
60
50
40
30
Eggs produced
A
B
A,C
C
Control
Cold Shock
RCH
DRCH

Figure 5

Chapter 4

Brief exposure to a diverse range of environmental stress enhances freeze tolerance in the polyextremophilic Antarctic midge, *Belgica antarctica*: evidence for a novel generalized rapid acclimatory response

Introduction

Insects can enhance their stress tolerance within minutes in a process called rapid cold-hardening (RCH) (Lee and Denlinger, 2010). Though the name 'rapid cold-hardening' highlights the role of cold, we have come to understand that these responses are broadly-applicable to a multiplicity of environmental cues and can exert effects independently of temperature perturbation (Coulson and Bale, 1991; Yoder et al., 2006; Levis et al., 2012; Gantz et al., *in review*, Gantz et al., *in preparation* a). Indeed, these rapid acclimatory responses can also be triggered by modest changes in humidity, and by hypoxia (Coulson and Bale, 1991; Levis et al., 2012). They protect insects from a variety of insults by enhancing tolerance of high temperature, low temperature, freezing, and anoxia, while also increasing resistance to dehydration (Lee et al. 1987; Chen et al., 1987; Coulson and Bale, 1991, Yoder et al., 2006; Lee et al., 2006; Gantz et al., *in review*). The protection afforded by RCH manifests in numerous ways, including reducing cell death, increasing organismal survival, and preserving normal reflexes and behaviors (Lee et al., 1987; Kelty et al., 1996; Kelty and Lee, 1999; Yi and Lee, 2003; Shreve et al., 2004).

In nature, insects can experience abrupt changes in temperature, hydration state, and oxygen availability (Stevenson, 1985; Gringorten and Friend, 1982; Heinrich, 1993; Hoback et al., 1998), suggesting that rapid acclimation is important to overcome the challenges associated with these fluctuations. Even modest decreases in temperature can render insects incapable of flight, courtship, and mating, and RCH helps to mitigate these negative effects (Taylor, 1963; Heinrich, 1972; Shreve et al., 2004; Gantz et al., *in preparation* a). In addition to temperature, dehydration, and anoxia, there are many abiotic stresses that have not yet been investigated as potential triggers for RCH. Naturally-occurring habitats can present a variety of stresses, including osmotic extremes (Herbst et al., 1988; Nicholson, 1998; Benoit and Denlinger, 2010), acidic and alkaline environments (Winterbourn, 1998; Aislabie et al., 2004; Spitzer and Danks, 2006; Slessarev et al., 2016), starvation and nutrient deprivation (Scott et al., 2004; Simpson et al., 2006), and UV irradiation (Mazza et al., 1999). Since arthropods have successfully colonized nearly all terrestrial habitats on Earth, it is likely that many species are frequently exposed to these less-studied abiotic stresses. Further, many insects can move among habitats readily, suggesting that they experience dramatic fluctuations in these conditions simply by moving into or out of the environments that present these stresses.

The Antarctic midge, *Belgica antarctica*, lives on the western Antarctic Peninsula in environments where they may be exposed to a variety of abiotic stresses at any time. Because of their small size and relative immobility, these midges have few options to deal with environmental stresses other than to tolerate them (Lee and Denlinger, 2014). Though temperatures are relatively mild, freezing remains a possibility any day of the year (Lee and Denlinger, 2014). Hydric stress is an even greater challenge for terrestrial arthropods in Antarctica (Kennedy, 1993; Worland and Block, 2003). During the austral summer, fresh water from rain and snowmelt forms pools that inundate many habitats, leading to hypoosmotic conditions and overhydration (Baust and Lee, 1982). Midges also face dehydration and hyperosmotic stress throughout the year (Baust and Lee, 1987; Elnitsky et al., 2009). Water is frozen and thus biologically-unavailable during the non-summer months, as temperatures are constantly below zero. Additionally, meltwater pools can become hyperosmotic as they receive effluent from nearby penguin rookeries and seal wallows; the effects of which are exacerbated by evaporation, which increases solute concentrations as water is lost (Lee and Denlinger, 2014). This same detrital effluent can also cause drastic changes in pH, whereby midges have been found in pools with a pH as low as 4 (Baust and Lee, 1983).

B. antarctica is a polyextremophile that tolerates a wide-range of environmental stresses in nature, including freezing to -13°C at any time of the year, dehydration to 35% of their original mass, and prolonged exposure to anoxia, hypoosmotic conditions by immersion in fresh water, hyperosmotic conditions by immersion in 0.5 M NaCl, and exposure to pH ranges between 3 and 12 (Lee and Denlinger, 2014). Consequently, we assessed whether diverse abiotic stresses triggered rapid acclimation by measuring organismal and cellular survival of injurious freezing after brief exposure to the following cues: starvation, chilling and freezing at modest sub-zero temperatures, moderate and extreme high temperature, moderate and extreme dehydration, hypoosmotic stress in ultrapure water, hyperosmotic stress in seawater, low pH, high pH, and ultraviolet irradiation.

Methods

Insect collection

B. antarctica larvae were collected from Torgersen Island, near Palmer Station, Antarctica (64° 46’ S, 64° 04’W) in December 2017. They were maintained in their native substrate at 4°C and constant light for a minimum of 1 week before experimentation. Fourth instar larvae were separated from the substrate by hand immediately before exposure to pretreatment conditions.

Experimental treatments: general

Injurious freezing treatments were taken directly from their substrate and frozen at -15°C for 2 h in a programmable cold bath. Larvae were placed in 1.7 ml microcentrifuge tubes with 200 µl of water and a small piece of ice to inoculate ice formation near their freezing point (Lee et al., 2006). Additionally, this injurious freezing protocol was applied to all pretreatment groups immediately after the respective pretreatments concluded. Each group contained 30 larvae for assessment of organismal survival and 12 for assessment of cellular damage.

Experimental treatments: food deprivation, temperature, and dehydration

Larvae in the starvation treatment were maintained on damp filter paper in a sealed, plastic petri dish at 4°C. Since larvae were deprived of food in all treatment conditions, we also assessed survival in a second control condition where larvae were deprived of food for 2 h without exposure to additional treatment. RCH supercooled was exposed to -5°C in a dry 1.7 ml microcentrifuge tube so that larvae were maintained below their freezing point in the absence of ice formation (Teets et al., 2011), and RCH frozen was exposed to -5°C with water and a small piece of ice to inoculate freezing. Dehydration treatments were placed in 1.7 ml tubes with fine, nylon mesh in place of the lid, and maintained in sealed desiccating chambers over saturated salt solutions of KCl (85% RH) for moderate dehydration and MgCl for severe dehydration (33% RH) (Rockland, 1960). Each treatment lasted 2 h.

Experimental treatments: iso-osmotic, hypoosmotic, hyperosmotic, and pH

Iso-osmotic controls were immersed in Coast's solution (Coast, 1988) that was diluted to match the osmotic pressure of *B. antarctica* hemolymph at 400 mOsm kg^{-1} (Elnitsky et al., 2009). Hypoosmotic treatments were immersed in ultrapure water (~0 mOsm kg^{-1}), and hyperosmotic treatments were immersed in seawater (~1000 mOsm kg^{-1}). Low and high pH solutions were created by titrating Coast's solution to a pH of 3 and 12 using HCl and NaOH, and diluting the resulting solution to 400 mOsm kg^{-1}. Each treatment lasted 2 h.

Experimental treatments: UV

Larvae in UV treatments were maintained in glass jars with damp filter paper and placed outside in direct sunlight. Jars were submerged up to their openings in an ice bath to control for temperature. The jar used for the UV control treatment was wrapped in duct tape and covered with an opaque lid. The experimental treatment did not receive an opaque wrapping and, instead of a lid, the opening was covered with fine nylon mesh. Treatment lasted 4 h.

Assessing survival

Organismal survival was assessed 2 h after removal from the injurious freezing treatment. Larvae were scored as alive if they could be stimulated to move by touch. Cellular survival was assessed by adapting a LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR, USA) to discriminate between undamaged and freezing-damaged cells (Yi and Lee, 2003). Briefly, excised fat body and midgut tissues were incubated in a membrane-penetrating green nuclear stain (SYBR-14) and an impermeant red nuclear stain (propidium iodide), and numbers of red and green cells were counted via fluorescent microscopy. Cells with membranes that were compromised by freezing damage fluoresced red and were scored as dead, while cells with intact membranes fluoresced green and were scored as alive. We then scored a minimum of 100 cells for each tissue type, from each larva, and calculated the ratio of live vs. dead cells.

Statistical analyses

Organismal survival data were fitted to general linear models (three models, one for each respective control), each with a logit link function and a binomial error distribution. We followed each with Tukey's HSD to control for pairwise comparisons (R Foundation for Statistical Computing, Vienna, Austria). However, we only report significance ($\alpha = 0.05$) for Tukey-corrected pairwise comparisons to our three controls, respectively. Cellular survival data were analyzed using a one-way analysis of variance (ANOVA) on the mean ratio of damaged-to-undamaged cells per larva. The Holm-Sidak method was used for post-hoc pairwise comparisons, and data are reported as mean ± SEM. Cellular survival distributions that failed the Shapiro-Wilk test of normality were analyzed using Kruskal-Wallis one-way ANOVA on ranks with post hoc Tukey test for pairwise comparisons and are reported as medians with their interquartile range (IQR).

Results

Organismal survival

When only exposed to the injurious freezing treatment, the rate of survival was 0.1, which was not significantly different compared to starvation for 2 h, at 0.2 (Fig. 1A). Pretreatment with RCH supercooled, RCH frozen, moderate dehydration, and severe dehydration enhanced organismal survival relative to starved controls, at 0.83, 0.80, 0.70, and 0.73 respectively ($p < 0.01$; Fig. 1A). While survival in iso-osmotic control larvae was 0.17, immersion in hypoosmotic and hyperosmotic solutions increased the rate to 0.63 and 0.83 ($p < 0.01$; Fig 1B). Low and high pH solutions did not significantly increase the rate of organismal survival, at 0.33 and 0.5, respectively (Fig. 1B). Finally, larvae that experienced UV irradiation survived at a rate of 0.33 compared to 0.03 in the UV controls ($p < 0.05$; Fig. 1C).

Cellular survival: starvation, temperature, and dehydration

Starvation for 2 h enhanced the rate of survival in both midgut and fat body tissue, at 0.28 (IQR = 0.1) and 0.22 ± 0.04 compared to control treatments, at 0.19 (IQR = 0.1) and 0.08 ± 0.03, respectively ($p < 0.05$; Fig. 2). Since the other treatments within this group were starved while

exposed to pretreatment conditions, we compared the rates of survival for each treatment to the starved group rather than to those that were only frozen at -15°C without pretreatment. Midgut and fat body tissues exhibited similar trends among all of these treatments (RCH supercooled, RCH frozen, moderate high temperature, extreme high temperature, moderate dehydration, and severe dehydration; Figs. 3 and 4). Moderate high temperature was the only treatment that did not enhance freeze tolerance in either tissue type, and severe dehydration was the only treatment that increased survival in one tissue (midgut) and not in the other (fat body). Each of the remaining treatments enhanced freeze tolerance relative to starved controls, with 2-3-fold increases in the rates of survival of RCH supercooled, RCH frozen, and mild dehydration in both tissue types ($p < 0.001$).

Cellular survival: hypoosmotic, hyperosmotic, and pH

Midgut and fat body cells in iso-osmotic control treatments survived freezing at rates of 0.32 ± 0.03 and 0.12 ± 0.03, respectively. In midgut tissue, immersion in ultrapure water (hypoosmotic) and seawater (hyperosmotic) caused 2.25- and 1.8-fold increases in survival ($p < 0.001$; Fig. 5). The effects of these treatments were even larger in fat body tissue, where ultrapure water and seawater produced 4.67- and 4.25-fold increases in the rate of survival, respectively ($p < 0.001$; Fig. 5)). Immersion in an acidic solution caused a 1.6-fold increase in survival in midgut tissue ($p < 0.05$), though this had no effect on fat body survival (Fig. 6). Immersion in an alkaline solution had no effect on the survival rate of midgut tissue while producing a 4.2-fold increase in fat body survival ($p < 0.001$; Fig. 6).

Cellular survival: UV

Cellular survival rates in midgut and fat body tissues in the UV control treatment were 0.63 ± 0.06 and 0.09 ± 0.04, respectively. UV exposure had no effect on survival in midgut tissue, but caused a 4.1-fold increase in survival rate of fat body cells (0.37 ± 0.02; $p < 0.001$; Fig. 7).

Discussion

Each of the stresses we tested enhanced freeze tolerance in *B. antarctica* larvae, as evidenced by increased organismal survival or decreased cellular damage following exposure to injurious freezing. While some of these stresses are known triggers of RCH (i.e. temperature and dehydration), others, such as overhydration, pH, UV irradiation, and starvation have not previously been linked to rapid acclimatory responses, indicating that insects can make rapid acclimatory adjustments during exposure to more environmental cues than we previously recognized.

Numerous physiological and biochemical mechanisms are quickly-activated by abiotic stresses and may contribute to rapid acclimatory responses, though few of them have previously been linked specifically to RCH. For example, starvation for as few as 3 h increases autophagic proteolysis in *Drosophila melanogaster* (Scott et al., 2004), UV-B irradiation enhances total antioxidant capacity within 30 min in two species of noctuid moths (Meng et al., 2009; Karthi et al., 2014), and dehydration increased trehalose and proline synthesis in *B. antarctica* (Teets et al., 2012). Further, these mechanistic responses often confer enhanced tolerance to other abiotic stresses. In addition to starvation, autophagy may be an important part of the responses to brief chilling and dehydration (Teets et al., 2013; Teets and Denlinger, 2013; Gerken et al., 2015). Proline accumulation enhances freeze tolerance in insects (Koštál et al., 2011), and also increases resistance to damage from UV-B irradiation, osmotic stress, and acidosis in plants (Heuer, 1994; Kurkdjian and Guern, 1989; Alexieva et al., 2001). UV-B irradiation, dehydration, and freezing all trigger enhanced antioxidant capacity (Joanisse and Storey, 1998; França et al., 2007; Meng et al., 2009; Karthi et al., 2014). Thus, there is likely mechanistic overlap among different types of RCH.

This overlap in mechanisms induced by different environmental cues helps to explain the cross-tolerance observed among these responses. Previous work in *B. antarctica* and many other insects demonstrates that exposure to one stress can enhance tolerance of another, which is particularly well-characterized between osmotic perturbation and low temperature (Bayley et al., 2001; Yancey, 2005; Elnitsky et al., 2009; Benoit et al., 2009; Kawarasaki et al., 2013). Yet, in this study, we detected significant effects of different rapid acclimatory responses by measuring freeze tolerance to many environmental cues, where even those that were unrelated to

temperature or osmotic perturbation enhanced freeze tolerance. These results suggest that brief exposure to diverse environmental cues elicit generalized acclimatory responses that enhance tolerance of a multiplicity of stresses.

Interestingly, though there are similarities among rapid acclimatory responses and their underlying physiological mechanisms, recent evidence suggests that the RCH responses triggered by brief chilling (cold-induced RCH) and dehydration (drought-induced RCH) are mechanistically distinct (Yi et al., 2017; Gantz et al., *in review*; Gantz et al., *in preparation* b). Cold- and drought-induced RCH appear to have different effects on second messenger cascades, osmolyte accumulation, Na^+ K^+ ATPase activity, and metabolic rate (Gantz et al., *in review*; Gantz et al., *in preparation* b). It is also possible that many other rapid acclimatory responses trigger distinct mechanisms. If so, these distinct physiological responses could presumably be induced simultaneously and may have additive effects on stress tolerance (Yi et al., 2017).

Due to their small size and correspondingly large surface area, insects are vulnerable to changing ambient conditions. Modest fluctuations in temperature can have dramatic effects on insects' body temperature, which can, in turn, disrupt essential physiological processes (Stevenson, 1985; Heinrich, 1993). Even during brief periods of cloud cover, thoracic temperatures of foraging bumblebees can decrease enough to prevent flight (Heinrich, 1972). Further, abiotic stressors rarely occur as single, isolated pressures in nature (Holmstrup, 2010). For example, moving from shade to direct sunlight is associated with elevated UV irradiation, increased temperature, and lower relative humidity (Nicolson and Louw, 1982; Hadley, 1994), and, when microhabitats flood, temperature fluctuation and hypoxia accompany overhydration from direct exposure to fresh water (Hoback et al., 1998). Thus, concomitant induction of multiple rapid acclimatory responses may be ecologically important. A recent study in the flesh fly, *Sarcophaga bullata*, demonstrated that triggering both cold- and drought-induced RCH decreased the lower lethal temperature and hastened recovery of the righting reflex following cold shock (Yi et al., 2017). Because RCH has traditionally been investigated as a response to only one trigger at a time, we have likely underestimated insects' capacity for modulating their physiological state through simultaneous induction of multiple, generalized, acclimatory responses.

In addition to naturally-occurring stresses that include freezing, dehydration, osmotic challenges, and fluctuations in pH (Lee and Denlinger, 2014), *B. antarctica* larvae are now having to overcome serious challenges from anthropogenic activity and climate change (Vaughan et al., 2003). The western Antarctic Peninsula is warming at a rate that is nearly 10 times greater than the global average, which has severe, cascading effects across trophic levels (Smith, 1994; Convey et al., 2002; Fraser and Hofmann, 2003; Smith et al., 2003; Clarke et al., 2007; Boyce et al., 2010). Warming trends are accompanied by increases in UV-B radiation, precipitation, and an elevated risk of invasion by non-native plants and animals (Convey et al., 2002; Chown et al. 2012). The Antarctic midge is small and unable to fly, therefore the potential for southward range expansion to colder regions of the Antarctic Peninsula is limited (Lee and Denlinger, 2014). Thus, tolerance of diverse abiotic stresses may be particularly important for their survival on the Antarctic Peninsula.

We used the polyextremophilic larvae of *B. antarctica* for this study because they exhibit a strong RCH response and experience a variety of stresses in nature (Lee and Denlinger, 2014). We surmised that they were likely able to make rapid acclimatory adjustments to these cues, and our results support this conclusion. Now, the answers to some critical follow-up questions are needed to assess the ecological relevance of these remarkable responses on a broader scale. Namely, are these rapid acclimatory responses 1) highly conserved throughout insect taxa, and 2) generalized in the protection they provide against other abiotic stresses? Cold-induced RCH is a highly conserved response that is found in diverse arthropod taxa (Lee and Denlinger, 2010). Its nearly-ubiquitous presence in terrestrial invertebrate ectotherms suggests that it is ecologically important to the success of these groups, which is supported by accumulating evidence in the literature (Kelty and Lee, 1999; 2001; Shreve et al., 2004; Kelty, 2007; Rajamohan and Sinclair, 2009; Gantz et al., *in preparation* b). Other types of rapid acclimatory responses, however, are less well known, and examining their prevalence will provide clues about their ecological relevance. Exploring the question of generality will improve understanding of the extent of cross tolerance among diverse stresses and may reveal unknown or little-appreciated effects of these responses. For example, since brief heating enhances resistance to insecticides in two species of mosquitoes (Patil et al., 1996), might other types of rapid acclimation have similar effects?

The results from this study support a growing body of evidence that the term "rapid cold-hardening" is too restrictive for the far-reaching effects of these responses. Indeed, rapid acclimation seems to trigger a multiplicity of responses, many of which can be altogether independent of temperature (Coulson and Bale, 1991; Patil et al. 1996; Yoder, et al., 2006; Gantz et al., *in review*; Gantz et al., *in preparation* b). Thus, we propose that the term "rapid cold-hardening" is replaced by "generalized rapid acclimatory response" to better capture their diverse and under-appreciated effects.

References

Aislabie, J. M., Balks, M. R., Foght, J. M., & Waterhouse, E. J. (2004). Hydrocarbon spills on Antarctic soils: effects and management. *Environmental Science & Technology*, *38*(5), 1265-1274.

Alexieva, V., Sergiev, I., Mapelli, S., & Karanov, E. (2001). The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell & Environment*, *24*(12), 1337-1344.

Baust, J. G., & Lee, R. E. (1982). Adaptive potential of terrestrial invertebrates: maritime Antarctica. *Antarctic JUS*, *16*, 175-176.

Baust, J. G., & Lee, R. E. (1983). Population differences in antifreeze/cryoprotectant accumulation patterns in an Antarctic insect. *Oikos*, 120-124.

Baust, J. G., & Lee, R. E. (1987). Multiple stress tolerance in an Antarctic terrestrial arthropod: *Belgica antarctica*. *Cryobiology*, *24*(2), 140-147.

Bayley, M., Petersen, S. O., Knigge, T., Köhler, H. R., & Holmstrup, M. (2001). Drought acclimation confers cold tolerance in the soil collembolan *Folsomia candida*. *Journal of Insect Physiology*, *47*(10), 1197-1204.

Benoit, J. B., Lopez-Martinez, G., Elnitsky, M. A., Lee, R. E., & Denlinger, D. L. (2009). Dehydration-induced cross tolerance of *Belgica antarctica* larvae to cold and heat is facilitated by trehalose accumulation. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *152*(4), 518-523.

Benoit, J. B., & Denlinger, D. L. (2010). Meeting the challenges of on-host and off-host water balance in blood-feeding arthropods. *Journal of Insect Physiology*, *56*(10), 1366-1376.

Boyce, D. G., Lewis, M. R., & Worm, B. (2010). Global phytoplankton decline over the past

century. *Nature*, *466*(7306), 591.

Chen, C. P., Denlinger, D. L., & Lee, R. E. (1987). Cold-shock injury and rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Physiological Zoology*, *60*(3), 297-304.

Chown, S. L., Huiskes, A. H., Gremmen, N. J., Lee, J. E., Terauds, A., Crosbie, K., Frenot, Y., Hughes, K.A., Imura, S., Kiefer, K., & Lebouvier, M. (2012). Continent-wide risk assessment for the establishment of nonindigenous species in Antarctica. *Proceedings of the National Academy of Sciences*, *109*(13), 4938-4943.

Clarke, A., Murphy, E. J., Meredith, M. P., King, J. C., Peck, L. S., Barnes, D. K., & Smith, R. C. (2007). Climate change and the marine ecosystem of the western Antarctic Peninsula. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *362*(1477), 149-166.

Coast, G. M. (1988). Fluid secretion by single isolated Malpighian tubules of the house cricket, *Acheta domesticus*, and their response to diuretic hormone. *Physiological Entomology*, *13*(4), 381-391.

Convey, P., Pugh, P. J. A., Jackson, C., Murray, A. W., Ruhland, C. T., Xiong, F. S., & Day, T. A. (2002). Response of Antarctic terrestrial microarthropods to long-term climate manipulations. *Ecology*, *83*(11), 3130-3140.

Coulson, S. J., & Bale, J. S. (1991). Anoxia induces rapid cold hardening in the house fly *Musca domestica* (Diptera: Muscidae). *Journal of Insect Physiology*, *37*(7), 497-501.

Elnitsky, M. A., Benoit, J. B., Lopez-Martinez, G., Denlinger, D. L., & Lee, R. E. (2009). Osmoregulation and salinity tolerance in the Antarctic midge, *Belgica antarctica*: seawater exposure confers enhanced tolerance to freezing and dehydration. *Journal of Experimental Biology*, *212*(17), 2864-2871.

França, M. B., Panek, A. D., & Eleutherio, E. C. A. (2007). Oxidative stress and its effects during dehydration. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *146*(4), 621-631.

Fraser, W. R., & Hofmann, E. E. (2003). A predator's perspective on causal links between climate change, physical forcing and ecosystem response. *Marine Ecology Progress Series*, *265*, 1-15.

Gantz, J. D., Spong, K. E., Seroogy, E., Robertson, R. M., & Lee, R. E. Effects of brief chilling and desiccation on ion homeostasis in the central nervous system of the migratory locust, *Locusta migratoria*. *In review, Biology Open.*

Gantz, J. D., Yi, S-X, & Lee, R. E. (a). Rapid acclimatory responses to chilling and dehydration improve flight performance and fecundity in the flesh fly, *Sarcophaga bullata*. *In preparation*.

Gantz, J. D., Yi, S-X, & Lee, R. E. (b). Brief desiccation and chilling trigger mechanistically-distinct rapid cold-hardening responses in larvae of the goldenrod gall fly, *Eurosta solidaginis*. *In preparation*.

Gerken, A. R., Eller, O. C., Hahn, D. A., & Morgan, T. J. (2015). Constraints, independence, and evolution of thermal plasticity: probing genetic architecture of long-and short-term thermal acclimation. *Proceedings of the National Academy of Sciences*, *112*(14), 4399-4404.

Gringorten, J. L., & Friend, W. G. (1982). Water balance in *Rhodnius prolixus* during flight: quantitative aspects of diuresis and its relation to changes in haemolymph and flight-muscle water. *Journal of Insect Physiology*, *28*(7), 573-577.

Hadley, N. F. (1994). Ventilatory patterns and respiratory transpiration in adult terrestrial insects. *Physiological Zoology*, *67*(1), 175-189.

Heinrich, B. (1972). Energetics of temperature regulation and foraging in a bumblebee, *Bombus terricola* Kirby. *Journal of Comparative Physiology*, *77*(1), 49-64.

Heinrich, B. (1993). *The hot-blooded insects: strategies and mechanisms of thermoregulation*. Springer Science & Business Media.

Herbst, D. B., Conte, F. P., & Brookes, V. J. (1988). Osmoregulation in an alkaline salt lake insect, *Ephydra* (Hydropyrus) *hians* Say (Diptera: Ephydridae) in relation to water chemistry. *Journal of Insect Physiology*, *34*(10), 903-909.

Heuer B. (1994) Osmoregulatory role of proline in water and salt-stressed plants. In *Handbook of Plant and Crop Stress* (ed. M. Pessarakli), pp. 363–381. Marcel Dekker, New York.

Hoback, W. W., Stanley, D. W., Higley, L. G., & Barnhart, M. C. (1998). Survival of immersion and anoxia by larval tiger beetles, *Cicindela togata*. *The American Midland Naturalist*, *140*(1), 27-33.

Holmstrup M., Bayley M., Pedersen, S. A., Zachariassen, K. E. (2010) Interactions between cold, desiccation and environmental toxins. In: Denlinger DL, Lee RE (eds) Low temperature biology of insects. Cambridge University Press, New York, pp 166–187

Joanisse, D. R., & Storey, K. B. (1998). Oxidative stress and antioxidants in stress and recovery of cold-hardy insects. *Insect Biochemistry and Molecular Biology*, *28*(1), 23-30.

Karthi, S., Sankari, R., & Shivakumar, M. S. (2014). Ultraviolet-B light induced oxidative stress: effects on antioxidant response of *Spodoptera litura*. *Journal of Photochemistry and Photobiology B: Biology*, *135*, 1-6.

Kawarasaki, Y., Teets, N. M., Denlinger, D. L., & Lee, R. E. (2013). The protective effect of

rapid cold-hardening develops more quickly in frozen versus supercooled larvae of the Antarctic midge, *Belgica antarctica*. *Journal of Experimental Biology*, *216*(20), 3937-3945.

Kelty, J. D., Killian, K. A., & Lee, R. E. (1996). Cold shock and rapid cold-hardening of pharate adult flesh flies (*Sarcophaga crassipalpis*): effects on behaviour and neuromuscular function following eclosion. *Physiological Entomology*, *21*(4), 283-288.

Kelty, J. D., & Lee, R. E. (1999). Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. *Journal of Insect Physiology*, *45*(8), 719-726.

Kelty, J. D., & Lee, R. E. (2001). Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophiladae) during ecologically based thermoperiodic cycles. *Journal of Experimental Biology*, *204*(9), 1659-1666.

Kelty, J. D. (2007). Rapid cold-hardening of *Drosophila melanogaster* in a field setting. *Physiological Entomology*, *32*(4), 343-350.

Kennedy, A. D. (1993). Water as a limiting factor in the Antarctic terrestrial environment: a biogeographical synthesis. *Arctic and Alpine Research*, 308-315.

Koštál, V., Zahradníčková, H., & Šimek, P. (2011). Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proceedings of the National Academy of Sciences*, *108*(32), 13041-13046.

Kurkdjian, A., & Guern, J. (1989). Intracellular pH: measurement and importance in cell activity. *Annual Review of Plant Biology*, *40*(1), 271-303.

Lee, R. E., Chen, C. P., & Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science*, *238*(4832), 1415-1417.

Lee, R. E., Elnitsky, M. A., Rinehart, J. P., Hayward, S. A., Sandro, L. H., & Denlinger, D. L. (2006). Rapid cold-hardening increases the freezing tolerance of the Antarctic midge *Belgica antarctica*. *Journal of Experimental Biology*, *209*(3), 399-406.

Lee R. E & Denlinger D. L. (eds) (2010). Rapid cold-hardening: ecological significance and underpinning mechanisms. Low temperature biology of insects. Cambridge University Press, New York, pp 35–58.

Lee, R. E., & Denlinger, D. L. (2014). Stress tolerance in a polyextremophile: the southernmost insect. *Canadian Journal of Zoology*, *93*(9), 679-686.

Levis, N. A., Yi, S.-X., & Lee, R. E. (2012). Mild desiccation rapidly increases freeze tolerance of the goldenrod gall fly, *Eurosta solidaginis*: evidence for drought-induced rapid cold-hardening. *Journal of Experimental Biology*, *215*(21), 3768-3773.

Mazza, C. A., Zavala, J., Scopel, A. L., & Ballaré, C. L. (1999). Perception of solar UVB radiation by phytophagous insects: behavioral responses and ecosystem implications. *Proceedings of the National Academy of Sciences*, *96*(3), 980-985.

Meng, J. Y., Zhang, C. Y., Zhu, F., Wang, X. P., & Lei, C. L. (2009). Ultraviolet light-induced oxidative stress: effects on antioxidant response of *Helicoverpa armigera* adults. *Journal of Insect Physiology*, *55*(6), 588-592.

Nicolson, S. W., & Louw, G. N. (1982). Simultaneous measurement of evaporative water loss, oxygen consumption, and thoracic temperature during flight in a carpenter bee. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *222*(3), 287-296.

Nicolson, S. W. (1998). The importance of osmosis in nectar secretion and its consumption by insects. *American Zoologist*, *38*(3), 418-425.

Patil, N. S., Lole, K. S., & Deobagkar, D. N. (1996). Adaptive larval thermotolerance and

induced cross-tolerance to propoxur insecticide in mosquitoes *Anopheles stephensi* and *Aedes aegypti*. *Medical and Veterinary Entomology*, *10*(3), 277-282.

Rajamohan, A., & Sinclair, B. J. (2009). Hardening trumps acclimation in improving cold tolerance of *Drosophila melanogaster* larvae. *Physiological Entomology*, *34*(3), 217-223.

Rockland, L. B. (1960). Saturated salt solutions for static control of relative humidity between 5° and 40° C. *Analytical Chemistry*, *32*(10), 1375-1376.

Scott, R. C., Schuldiner, O., & Neufeld, T. P. (2004). Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Developmental Cell*, *7*(2), 167-178.

Shreve, S. M., Kelty, J. D., & Lee, R. E. (2004). Preservation of reproductive behaviors during modest cooling: rapid cold-hardening fine-tunes organismal response. *Journal of Experimental Biology*, *207*(11), 1797-1802.

Stevenson, R. D. (1985). Body size and limits to the daily range of body temperature in terrestrial ectotherms. *The American Naturalist*, *125*(1), 102-117.

Simpson, S. J., Sword, G. A., Lorch, P. D., & Couzin, I. D. (2006). Cannibal crickets on a forced march for protein and salt. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(11), 4152-4156.

Slessarev, E. W., Lin, Y., Bingham, N. L., Johnson, J. E., Dai, Y., Schimel, J. P., & Chadwick, O. A. (2016). Water balance creates a threshold in soil pH at the global scale. *Nature*, *540*(7634), 567.

Smith, R. L. (1994). Vascular plants as bioindicators of regional warming in Antarctica. *Oecologia*, *99*(3-4), 322-328.

Smith, R. C., Fraser, W. R., & Stammerjohn, S. E. (2003). Climate variability and ecological

response of the marine ecosystem in the western Antarctic Peninsula (WAP) region (pp. 158-173). Oxford University Press, New York.

Spitzer, K., & Danks, H. V. (2006). Insect biodiversity of boreal peat bogs. *Annual Review of Entomology*, *51*.

Taylor, L. R. (1963). Analysis of the effect of temperature on insects in flight. *The Journal of Animal Ecology*, 99-117.

Teets, N. M., Kawarasaki, Y., Lee, R. E., & Denlinger, D. L. (2011). Survival and energetic costs of repeated cold exposure in the Antarctic midge, *Belgica antarctica*: a comparison between frozen and supercooled larvae. *Journal of Experimental Biology*, *214*(5), 806-814.

Teets, N. M., Kawarasaki, Y., Lee, R. E., & Denlinger, D. L. (2012). Energetic consequences of repeated and prolonged dehydration in the Antarctic midge, *Belgica antarctica*. *Journal of Insect Physiology*, *58*(4), 498-505.

Teets, N. M., Kawarasaki, Y., Lee, R. E., & Denlinger, D. L. (2013). Expression of genes involved in energy mobilization and osmoprotectant synthesis during thermal and dehydration stress in the Antarctic midge, *Belgica antarctica*. *Journal of Comparative Physiology B*, *183*(2), 189-201.

Teets, N. M., & Denlinger, D. L. (2013). Autophagy in Antarctica: combating dehydration stress in the world's southernmost insect. *Autophagy*, *9*(4), 629-631.

Vaughan, D. G., Marshall, G. J., Connolley, W. M., Parkinson, C., Mulvaney, R., Hodgson, D. A., King, J. C., Pudsey, C. J., & Turner, J. (2003). Recent rapid regional climate warming on the Antarctic Peninsula. *Climatic change*, *60*(3), 243-274.

Winterbourn, M. J. (1998). Insect faunas of acidic coal mine drainages in Westland, New

Zealand. *New Zealand Entomologist*, *21*(1), 65-72.

Worland, M. R., & Block, W. (2003). Desiccation stress at sub-zero temperatures in polar terrestrial arthropods. *Journal of Insect Physiology*, *49*(3), 193-203.

Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology*, *208*(15), 2819-2830.

Yi, S.-X., & Lee, R. E. (2003). Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes. *Journal of Insect Physiology*, *49*(11), 999-1004.

Yi, S.-X., Gantz, J. D., & Lee, R. E. (2017). Desiccation enhances rapid cold-hardening in the flesh fly *Sarcophaga bullata*: evidence for cross tolerance between rapid physiological responses. *Journal of Comparative Physiology B*, *187*(1), 79-86.

Yoder, J. A., Benoit, J. B., Denlinger, D. L., & Rivers, D. B. (2006). Stress-induced accumulation of glycerol in the flesh fly, *Sarcophaga bullata*: evidence indicating anti-desiccant and cryoprotectant functions of this polyol and a role for the brain in coordinating the response. *Journal of Insect Physiology*, *52*(2), 202-214.

Figure legends

Figure 1. Brief exposure to diverse abiotic stresses enhanced organismal freeze tolerance. **A.** Low temperature and dehydration cues, but not high temperature, enhanced freeze tolerance relative to starved control. Frozen controls are shown for reference, though were not significantly different from starved. * denotes significance relative to starved treatment only ($p < 0.01$). **B.** Exposure to osmotic perturbation in ultrapure water and seawater enhanced freezing relative to iso-osmotic control ($p < 0.01$). * denotes significance relative to iso-osmotic control. **C.** Exposure to UV irradiation enhanced freeze tolerance relative to UV control ($p < 0.05$).

Figure 2. Starvation reduced cellular damage. Ratio of surviving midgut and fat body tissues, relative to frozen control. * denotes significance at $p < 0.05$.

Figure 3. High and low temperature perturbation reduced cellular damage in midgut and fat body cells. Ratio of surviving midgut and fat body tissues relative to starved control. Treatments not sharing a letter were significantly different ($p<0.05$).

Figure 4. Dehydration reduced cellular damage in midgut and fat body tissues. Ratio of surviving midgut and fat body tissues, relative to starved control. * denotes significance at $p < 0.01$.

Figure 5. Osmotic perturbation in ultrapure water and seawater reduced cellular damage in midgut and fat body tissues. Ratio of surviving midgut and fat body cells relative to iso-osmotic control. Treatments not sharing a letter were significantly different ($p<0.05$).

Figure 6. Exposure to high and low pH reduced cellular damage in midgut and fat body tissues. Ratio of surviving midgut and fat body cells relative to iso-osmotic control. * denotes significance at $p < 0.05$.

Figure 7. Exposure to solar radiation reduced cellular damage in fat body tissue. Ratio of surviving midgut and fat body cells relative to UV control. * denotes significance at $p < 0.05$.

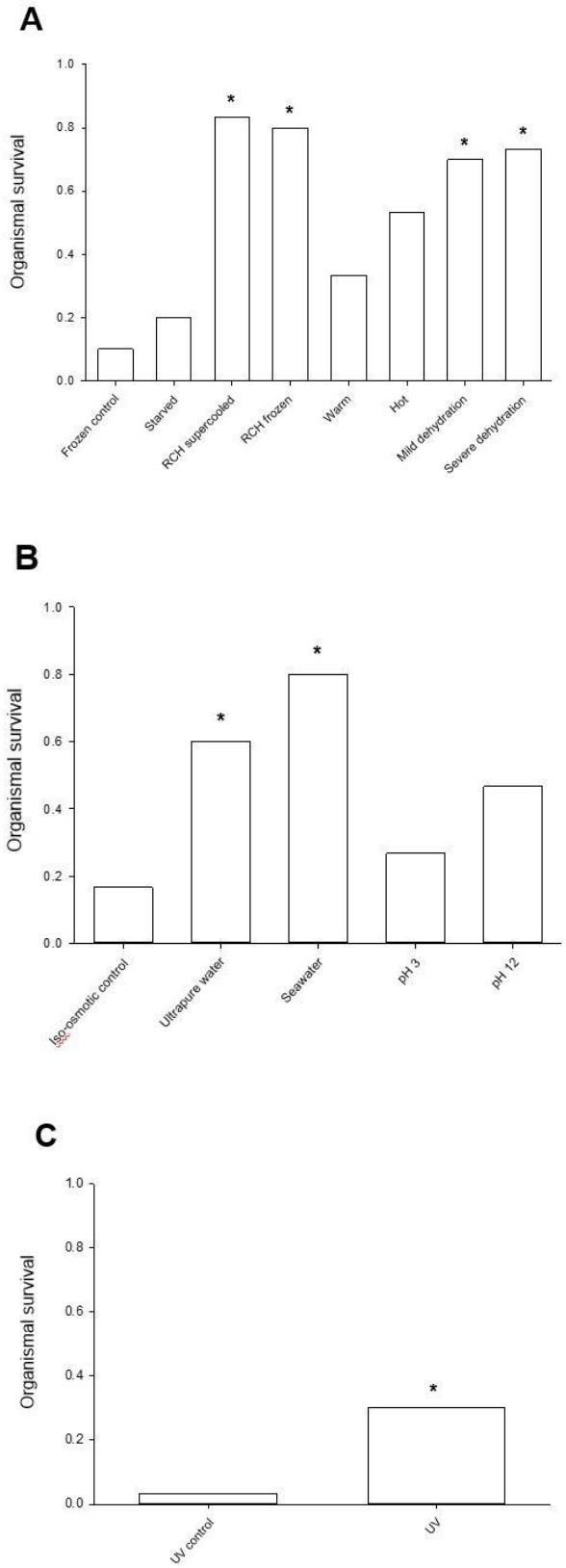
A
Organismal survival
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
Frozen control
Starved
RCH supercooled
RCH frozen
Warm
Hot
Mild dehydration
Severe dehydration
B
Organismal survival
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Iso-osmotic control
Ultrapure water
Seawater
pH 3
pH 12
C
Organismal survival
1.0
0.8
0.6
0.4
0.2
0.0
*
UV control
UV

Figure 1

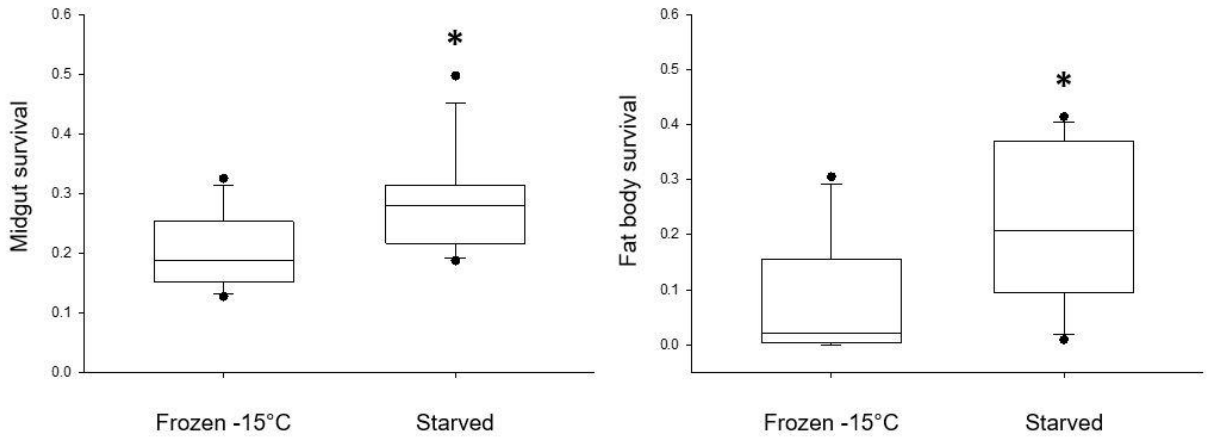
Midgut survival
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Frozen -15°C
Starved
Fat body survival
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Frozen -15°C
Starved

Figure 2

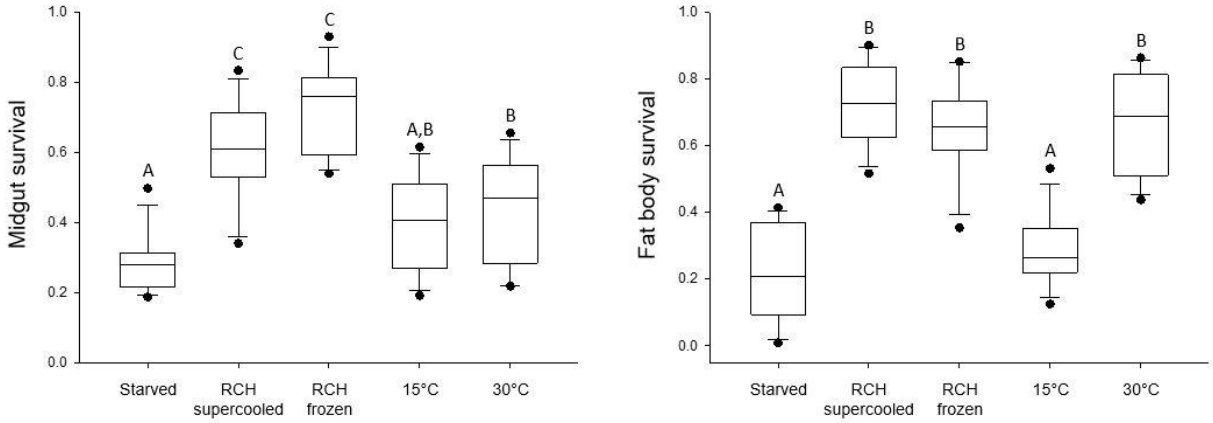
Midgut survival
1.0
0.8
0.6
0.4
0.2
0.0
A
C
C
A,B
B
Starved
RCH supercooled
RCH frozen
15°C
30°C
Fat body survival
1.0
0.8
0.6
0.4
0.2
0.0
A
B
B
A
B
Starved
RCH supercooled
RCH frozen
15°C
30°C

Figure 3

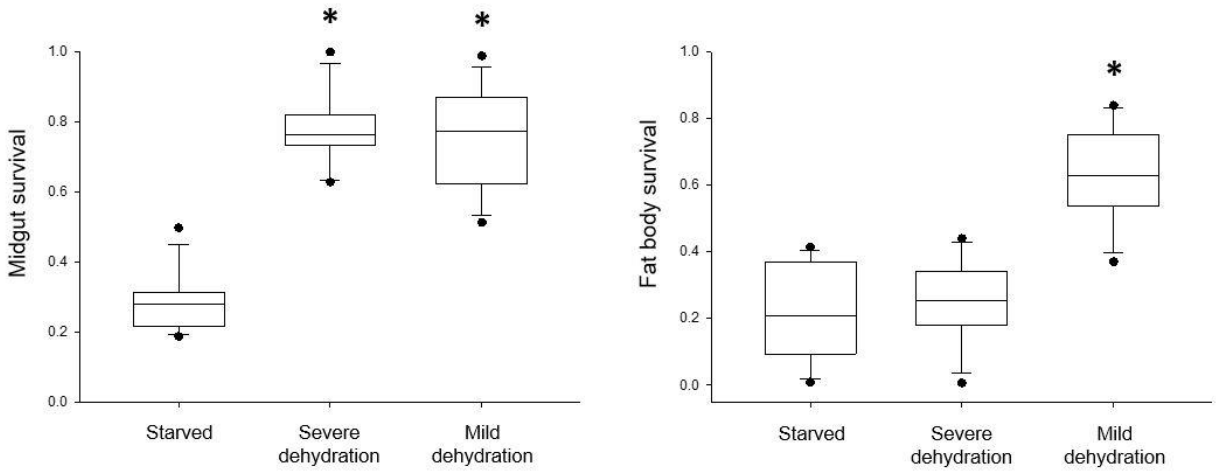
Midgut survival
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Starved
Severe dehydration
Mild dehydration
Fat body survival
1.0
0.8
0.6
0.4
0.2
0.0
*
Starved
Severe dehydration
Mild dehydration

Figure 4

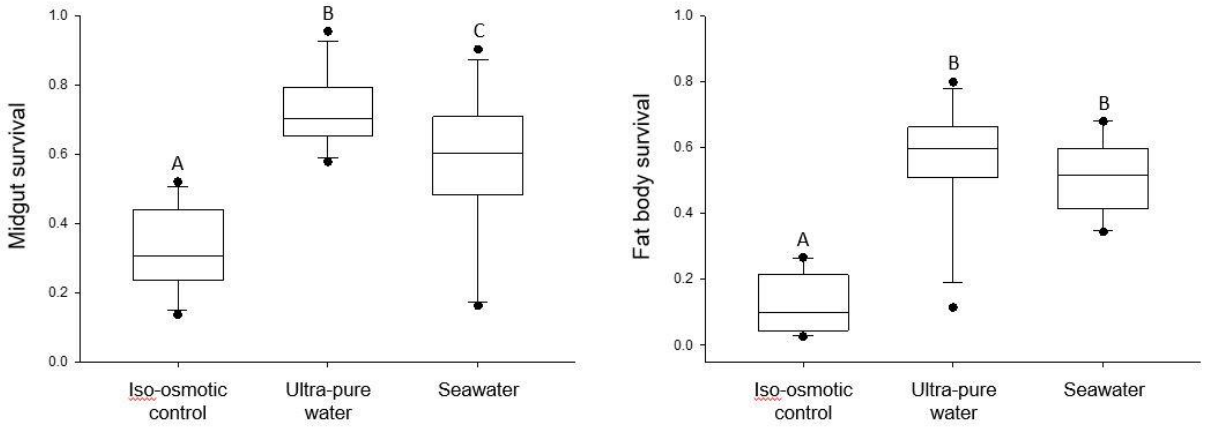
Midgut survival
1.0
0.8
0.6
0.4
0.2
0.0
A
B
C
Iso-osmotic control
Ultra-pure water
Seawater
Fat body survival
1.0
0.8
0.6
0.4
0.2
0.0
A
B
B
Iso-osmotic control
Ultra-pure water
Seawater

Figure 5

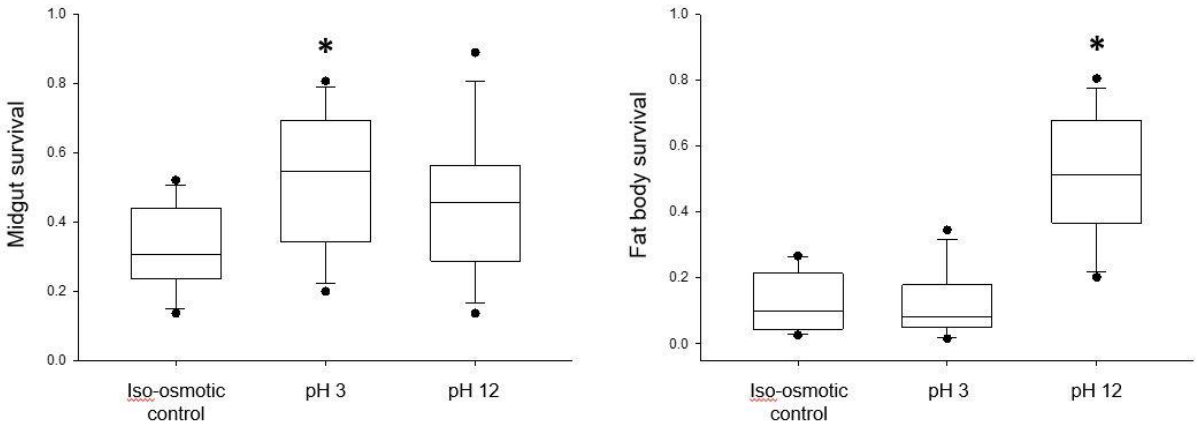
Midgut survival
1.0
0.8
0.6
0.4
0.2
0.0
*
Iso-osmotic control
pH 3
pH 12
Fat body survival
1.0
0.8
0.6
0.4
0.2
0.0
*
Iso-osmotic control
pH 3
pH 12

Figure 6

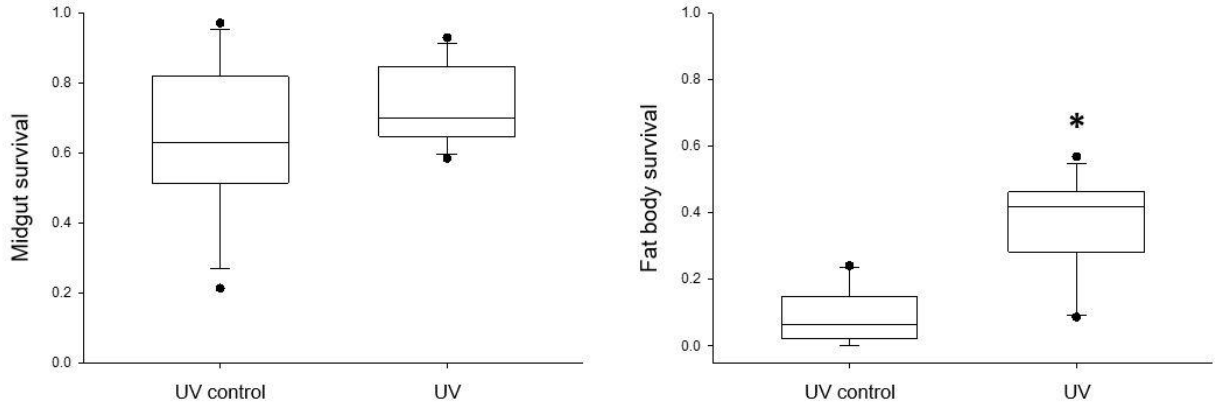
Midgut survival
1.0
0.8
0.6
0.4
0.2
0.0
UV control
UV
Fat body survival
1.0
0.8
0.6
0.4
0.2
0.0
*
UV control
UV

Figure 7

Chapter 5

Concluding remarks

Small ectotherms experience precipitous changes in their internal conditions as a result of ambient temperature and humidity, microhabitat effects, and physical activity (Stevenson, 1985; Heinrich, 1993). Rapid acclimatory responses are critical adaptations to counter the deleterious effects of these fluctuations (Lee and Denlinger, 2010). Our understanding of rapid acclimation has changed significantly in recent years, as it has become clear that many ecologically relevant abiotic stresses trigger these responses. Even so, their effects and underpinning mechanisms remain poorly characterized, particularly in responses triggered by cues other than chilling. This dissertation explored the physiological mechanisms and ecological importance of rapid acclimation when triggered by diverse environmental cues.

Though the effects of cold- and drought-induced RCH are similar on an organismal level, these responses appear to operate via disparate mechanisms. These mechanistic differences may be important when insects are exposed to multiple stresses simultaneously, as the interactions among multiple physiological responses may enhance stress tolerance more than one can separately (Yi et al., 2017). Further, these differences suggest that there are multiple paths to rapidly enhance stress tolerance, many of which remain poorly understood.

Rapid acclimation also has a measurable effect on higher-order processes in insects. Flight and reproduction require the coordination of multiple organs, and homeostatic dysregulation in any number of biochemical processes may disrupt the ability to fly (Candy et al., 1997). By enhancing flight performance and fecundity, cold- and drought-induced RCH seem to help maintain homeostasis in stressful conditions. These effects may be critical for insects' success in temperate and polar environments, where ambient conditions are often unfavorable and can change dramatically.

Further, rapid acclimation is triggered by numerous abiotic stresses in a polar extremophile. The diversity of cues that triggered a protective response is shocking, even including starvation for only 2 h, and exposure to sunlight. There is also a remarkable degree of

cross tolerance among these responses, where exposure to one stress enhances the tolerance of another. These results suggest that insects are exquisitely attuned to their environment, and they use diverse environmental cues to predict the onset of abiotic stresses and adjust their physiological state accordingly.

The results from this dissertation describe a series of rapid acclimatory responses that are triggered by multiple abiotic stresses. Their effects enhance stress tolerance at multiple levels, from measures of direct, structural damage during freezing to highly-integrative behaviors such as flight. Thus, these generalized rapid acclimatory responses are likely ecologically important and indicate that the physiological adjustments made during brief stress exposure are more complicated that we have previously appreciated.

References

Candy, D. J., Becker, A., & Wegener, G. (1997). Coordination and integration of metabolism in insect flight. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *117*(4), 497-512.

Heinrich, B. (1993) The Hot-Blooded Insects: Strategies and Mechanisms of Thermoregulation (Harvard Univ. Press, Cambridge, MA), p. 1.

Lee, R. E., Denlinger, D. L. (2010). Rapid cold-hardening: ecological significance and underpinning mechanisms. In: Denlinger, D.L., Lee, R.E. (Eds.), Low Temperature Biology of Insects. Cambridge University Press, Cambridge, pp. 35–58.

Stevenson, R. D. (1985). Body size and limits to the daily range of body temperature in terrestrial ectotherms. *American Naturalist*, 102-117.

Yi, S.-X., Gantz, J. D., & Lee, R. E. (2017). Desiccation enhances rapid cold-hardening in the flesh fly *Sarcophaga bullata*: evidence for cross tolerance between rapid physiological responses. *Journal of Comparative Physiology B*, *187*(1), 79-86.