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Mechanisms of Cold Tolerance in Temperate-Zone Insects

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Abstract

Temperate-zone insects employ various methods in order to physiologically adapt to harsh environments, such as those with extreme cold weather. In my Honors thesis project, I experimentally explored the effects of two molecules, proline and allantoin, that have been shown to be upregulated in response to cold stress in the temperate ant species *Aphaenogaster picea*. I artificially enhanced these molecules in their diet and compared their recovery time following cold shock to those maintained under control diets. To further explore this question, I also studied their effects in the genetic model organism *Drosophila melanogaster,* through both dietary addition of these compounds and eliminating the uric acid pathway, of which allantoin is an intermediate product, with a genetic knock-out. The results indicate that despite allantoin's demonstrated role in protecting against cellular freezing, proline tended to impede recovery in *A. picea* and consistently increased recovery time in *D. melanogaster*, suggesting that it may hinder the ability to recover behaviorally from cold shock. Allantoin had no consistent effect in either species, suggesting it does not play an active role in cold recovery. Moreover, the *rosy* mutant lacking the entire uric acid pathway had a quicker recovery time than the control, suggesting it may in fact be beneficial not to have the pathway activated. One outcome of this work was to highlight the sensitivity of chill recovery assays to even small changes in protocol and laboratory conditions. Further tests with more reliable protocols may be needed in order to determine the impact of these metabolites on cold tolerance in temperate-zone insects.

Introduction

Ectotherms like insects depend on outside sources to maintain their body heat (Huey & Kingsolver, 1989). When organisms suffer from the effects of the cold, it can lead to compromised cells (Bagwell & Ricker, 2007). Leaked solutes and ions move across the cell membrane, leading to the loss of membrane integrity (Koštál et al., 2006). Functioning ion gradients across cell membranes allow a cell to maintain homeostasis, the loss of which can cause osmotic shock and cell dehydration. When an imbalance exists between the active movement and passive movement of ions, it can lead to spreading depolarization in the central nervous system in many organisms (Andersen et al., 2018). This depolarization can lead to the silencing of neurons which then silences the central nervous system, impacting an organism's overall ability to function. Oxidative damage is also a risk under cold stress (Koštál et al., 2006). When antioxidant system functions are overpowered by the production rate of reactive oxygen species (Joanisse & Storey, 1996), they can harm proteins and DNA, leading to loss of functionality and structural changes in cells. Reducing "cold shock" damage could increase animal and plant survival at low environmental temperatures and improve the cryopreservation of their cells (Drobnis et al., 1993).

There are two major ways that insects can counter low temperatures: freeze tolerance and freeze avoidance. Freeze tolerant insects can prevent extracellular freezing while freeze avoidant insects can lower their supercooling point (SCP) to avoid fatal freezing. The basic distinction is dependent on the supercooling point of the insect (Bale, 2002), which is the temperature at which

internal fluids freeze (Pang et al., 2014). Freeze-tolerant insects often possess ice-nucleating agents, polyols and sugars, or anti-freeze proteins, which all have cold-resistance properties (Bale, 2002). Cold tolerant insects accumulate these molecules during the fall and beginning of winter to start the freezing process in the extracellular spaces of their bodies that are less susceptible to damage (Bale, 2002, Fields et al., 1998). Polyols and sugars can reduce the freezing point through colligative methods, which relates to the concentration of solute molecules or ions, to maintain integrity of proteins and cell membranes (Neven et al., 1986). Freeze-tolerant species also eliminate ice nucleating agents from their bodies (Duman et al., 1982). In freeze avoidant organisms, SCPs tend to be lower in comparison to freeze-tolerant organisms (Bale, 2002). There are two main steps that allow freeze-avoidant organisms to survive: reducing body water content while increasing fat content, and the producing polyols and anti-freeze proteins to lower the SCP relative to their melting point.

Insects are able to dynamically adjust their cold tolerance limits through rapid hardening and acclimation (Bale, 2002). Hardening refers to the response to a cold shock, associated with production of damage-response metabolites to counter the harsh effects brought upon by the cold, and allowing them to be better prepared for when they are exposed to future cold shocks (Bale, 2002). Biochemical pathways in their bodies shift to form cryoprotectant compounds such as polyols, sugars and amino acids that can shield or desensitize the insect to the potential lethal effects of the cold shock arising from a sudden change in temperature. Acquiring cryoprotectants can increase cold tolerance because it

lowers the point where the liquids in the hemolymph are able to freeze. Acclimation, on the other hand, is a more gradual process in which organisms prepare for future changes in temperature and environment through the accumulation of protective molecules, ready for when they need to be used (Hahn & Denlinger, 2011). Acclimation normally includes changes in cell membranes, like increasing the amount of unsaturated fatty acids to evade freezing that causes membrane damage (Modlmeier et al., 2012).

Cold response strategies are particularly important for northern temperate species that experience extreme winter conditions. *Aphaenogaster picea* is a temperate ant species found in forests in northeast North America, with a range extending northward to central Maine (Clark & King, 2012). They are important seed dispersers that can perform their job at low temperatures (Warren & Chick, 2013). A previous correlative study in the northern forest ant *Aphaenogaster picea* identified two metabolites, proline and allantoin, that change during the cold response and may be involved in the mechanisms controlling this insect's cold tolerance abilities (J. Cline, unpublished data).

Proline is widely used to provide cold tolerance, with effects demonstrated in taxa as diverse as plants, crustaceans and insects. Proline can help maintain the structure and function of membranes when put through rapid freeze-thaw conditions and prevent the mixing of membranes to maintain bilayer stability to protect undamaged cells (Rudolph and Crowe, 1985; Withers and King, 1979). In insects, proline acts as a cryoprotectant in the hemolymph, or insect bloodstream (Olsson et al., 2016). Many insect species have free amino acids in their

hemolymph, and the most common is proline (Olsson et al., 2016). In *Drosophila melanogaster*, a larval diet spiked with proline led to a successful amplification of their physiological response to the cold, enhancing their freeze-tolerance abilities under an acclimation treatment set to -5°C (Koštál, et al. 2016).

Another metabolite that has been suggested to play a role in cold tolerance is allantoin, which is a by-product of the uric acid pathway (Hilliker et al., 1992). Uric acid is a strong antioxidant that is usually made as a waste product through the elimination of protein. In flies, urate is a vital antioxidant, so those that without the urate enzyme, xanthine dehydrogenase, will be prevented from detoxifying the influx of the reactive oxygen species making them sensitive to hyperoxia. The enzyme, uricase, converts uric acid into a more soluble product known as allantoin (Chambers, Song, & Schneider, 2012). Allantoin can be formed when uric acid is driven by a reactive oxygen reaction (Chambers, Song, & Schneider, 2012), and so allantoin is traditionally considered a marker of oxidative stress (Yardim-Akaydin, Sepici, Özkan, et al., 2006). In plants such as Arabidopsis, knockout mutants with *aln*, a loss-of-function allantoinase allele, increases allantoin (Takagi et al., 2016). The increase activates abscisic acid and jasmonic acid, which work together to activate abiotic stress tolerance responses to drought and osmotic stress. Thus, it is possible that allantoin might play a causal role in responding to stress, and not simply be a by-product of the action of an upstream molecule like uric acid.

In this study, I experimentally tested whether these metabolites play a role in driving the cold hardening and acclimation responses in *A. picea* as they tend to

experience more variable environmental changes (Bahrndorff et al., 2009). Because this ant is a non-model species, a more detailed investigation of how these molecules act at the molecular level is challenging to achieve. To circumvent this problem, I tested their role in the fruit fly *Drosophila melanogaster,* which is a well-characterized genetic model species. In addition to testing dietary addition, I exploited the existence of single-gene mutant lines in *D. melanogaster* to test the effects of the loss of allantoin and its precursor, urate, in the *rosy* mutant (lacks the enzyme xanthine dehydrogenase) compared to wild-type *Drosophila* to see whether the loss of these metabolites reduced cold tolerance.

Methods

Aphaenogaster picea Collection

Ten total colonies of *Aphaenogaster picea* were collected from two different colony sites, five from Centennial Woods in June 2018 (44.4758° N, 73.1856° W) and five from a site in Westford, VT in July 2018 (44.588629° N, 73.061560135° W). Colonies were located within rotten logs, and the bulk of the colony was collected by splitting open the logs and placing them in large plastic bags along with other debris to ensure that workers and brood were fully collected. The rest of the individual ants that were not along with the logs or debris were collected using aspirators and placed in Ziploc bags. Collected samples were shaded from direct sunlight to avoid overheating the colonies.

Workers and brood were separated from the debris by placing the material in a large plastic tray and removing individuals with an aspirator or soft forceps.

Aphaenogaster picea **Maintenance**

Each colony was subdivided into six equally sized fragments and maintained in 22 x 16 cm plastic containers. To prevent escape, the sides of the container were coated with Insect-a-Slip (BioQuip Inc). The containers were topped with covers that had screens to allow proper air circulation. Two test tubes partially filled with distilled water were plugged with a cotton ball and provided for *A.picea* to nest in. These nest tubes were replaced as needed when the tubes became dry or if the water became contaminated with mold. The colonies were kept in a temperature-controlled incubator at 20 ^{o} C on an equal 12:12 hour light: dark cycle. More specifically, the colonies collected in June were kept in the incubator at 60% humidity and were provided with a third water tube plugged loosely with cotton to allow ants to drink. For colonies collected in July, the humidity in the incubator was increased to 90% in in order to slow down the evaporation of the moisture from the food, because they were not provided with an extra tube of water in their containers.

Diet manipulation

To investigate the impact of addition of proline and allantoin as well the impact of depleting energy resources on recovery time, I created six separate dietary manipulations for this feeding experiment. These manipulations consisted

of the Control, No Food, No Protein, No Sugar, Spiked Allantoin, and Spiked Proline. Colony fragments were fed a synthetic ant diet (Katie Miller, pers. Communication) consisting of 100 mL of water, 3 grams of dried egg powder, 0.69 grams of whey protein 0.63 grams of calcium caseinate, 7.38 grams of sucrose, 1.30 grams of agar, 0.27 grams of Vanderzant Vitamin mixture, 0.13 grams of methyl- 4- hydroxybenzoate, and 4 grams of the dietary additions (Koštál, Vladimír, et al. 2016). The ant food was a solid agar mixture that was cut up into small squares weighing around 0.15 grams each, and two were placed in every container. The food was replaced every two to three days.

To create this mixture, the ingredients were boiled together in a beaker along with water. The carbohydrate ingredients were mixed together first, followed by the addition of the protein ingredients. Once the mixture was ready, it was poured in the beaker containing the protein ingredients. This was quickly stirred and was poured into separate petri dishes evenly. They were placed under a fume hood in order to cool and solidify.

***Aphaenogaster picea* Cold Shocks**

Chill Coma Recovery Time was assayed in workers with and without a cold-hardening pre-treatment. Sets of three workers from each treatment were held in sealed glass test tubes and submerged in a circulating water bath. The cold shock experiments were performed in a 15L PolyScience Refrigerated Circulator Water Bath which contains an equal amount of ethylene glycol and water to prevent the liquid from freezing.

Acute cold hardening exposure consisted of one hour at -5 °C, while those that were rapid cold-hardened (RCH) were pre-treated at 0 °C for one hour and allowed to recover for one hour prior to the -5 °C. treatment. All workers were placed in tubes at the same time to control for handling time, and all workers from the same colony were tested simultaneously. CCRT was measured by removing the tubes from the water bath and timing the length of time until each worker righted itself. Observers were blind to the treatment condition of the workers in each tube. Following the treatment, ants were frozen in liquid nitrogen and stored at -80 °C.

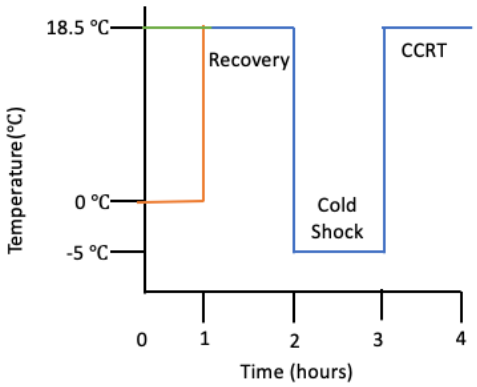

Figure 1. Schematic of rapid cold hardening and acute cold shock procedure.

Acclimation Treatment

To test whether food treatment affected seasonal acclimation, 10 of the workers from each treatment that had not been used to evaluate rapid cold hardening were placed in new test tubes with a water tube but no food. They were placed in an incubator in which the temperature was lowered from 20 to 4 °C at a

rate of one degree/day for three weeks. Acute and cold-hardened CCRT were measured for the acclimated ants as described above.

***Drosophila Canton-S* Diet Manipulation**

To test the effect of allantoin and proline on cold tolerance, larvae were reared on diets spiked with allantoin, proline, or neither as a control. Once they were fully formed adults, the flies reared under allantoin and proline were compared to those under the control diet.

A small batch of *Drosophila* food consisting of 400 vials was prepared with 3L of dH_2O, 27 grams of agar, 140 mL of molasses, 250 grams Torula yeast, 300 grams of cornmeal, 50 mL 95% ethanol, 10 grams of Tegosept, and 20 mL Propanoic Acid (B. Lockwood, pers. Communication). There was 13mL of food (per vial) for each treatment, and those that were assigned augmented diets had 0.04 grams of allantoin and proline added to the vials via an autopipette. Once filled, the vials were left to cool, and once they stopped creating condensation, they were topped with plugs and stored in a refrigerator.

***Drosophila* Rearing**

To collect embryos, 300 three-day old flies were placed in screen-covered plastic holding vials with a grape-jelly agar plate to encourage egg-laying. Every 24 hours for two days, the agar plate was replaced with an empty plate to promote a clean environment and to replenish the food source, which was a yeast paste made of active dry yeast and water. This was done to acclimate the flies to the

environment. The last agar plate, containing embryos that were an hour old, was divided into six pieces so that each section had the same number of eggs. Then each piece was placed in the appropriate food vial treatments containing allantoin-spiked food, proline-spiked food or control food vial and kept at 25°C, 12:12 hour light/dark cycle with 60% humidity. Embryos were checked daily for adult eclosion (around 7-11 days). Once adults emerged, they were collected and subsequently prepared for cold shocks. There was no specific age of flies utilized for the assays, so there was a wide age range from 0 to 14 days depending on the number of flies available.

Drosophila **Cold Shock Treatment**

CCRT was assayed in the Canton-S strain of *D. melanogaster* with and without a cold-hardening pre-treatment. Approximately 30-40 flies were held in sealed 16 X 150mm glass test tubes and submerged in a circulating water bath. Acute cold hardening exposure consisted of one hour at 0 °C, while those that were rapid cold-hardened were pre-treated at 4 °C for one hour and allowed to recover for one hour prior to the 0 °C treatment (Figure 2). All flies were placed in tubes at the same time to control for handling time, and all flies of the various treatments were tested simultaneously.

CCRT was measured by individually placing 48 flies from each treatment from the test tube into a 96 well plate that was divided in order to get an equal number of RCH and Acute flies onto the plate. Activity was recorded with a camcorder to identify their recovery time for 30 minutes. A fly was considered to

have recovered when it was no longer on its back and was instead upright on legs. Assays were conducted on two separate days at approximately the same time of day.

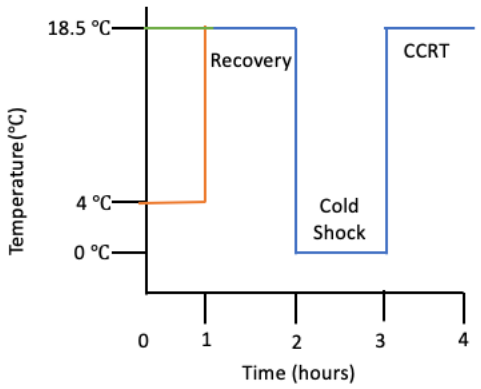

Figure 2. Schematic for rapid cold hardening and acute cold hardening protocol for *D. melanogaster*.

***Drosophila Canton-S* and *Rosy* (ry^{506}) Diet Manipulation**

In addition to the wild-type, I utilized the *rosy* mutant, a strain of the wild-type *Canton-S* that possesses the mutant allele ry^{506} that is located on the third chromosome (Lucchesi and Manning, 1988). This mutant has a deletion of coding sequences, so it does not generate an identifiable transcript of xanthine dehydrogenase.

A small batch of *Drosophila* food, consisting of 400 vials, the Lockwood lab *Fly Food Recipe* protocol was prepared and adjusted accordingly to account for the new diet treatments (B. Lockwood, pers. Communication). Along with the

previously used Allantoin and Proline, an additional treatment of 0.04 g Uric Acid was utilized.

About 30-40 flies were added to the treatment vials, and they were allowed to mate and lay eggs for 3-5 days, using the adults to assay. The age range of adults varied from 0 to 14 days old. Assays were conducted on three separate days, at approximately the same time of day.

***Drosophila Canton-S* and *Rosy* Cold Shock Treatment**

Preparation

To be able to simultaneously test multiple treatments, a 96-well polystyrene round-bottom plate was prepared by designating which diet treatments and strain of fly would be in each row. The plate was then covered by two layers of clear sealing plastic, and a sharp, thin blade cut was used to cut X's into the plastic above each well so that flies could be individually aspirated into the well while simultaneously preventing them from escaping.

Experiment

Only acute CCRT was assayed in *Drosophila Canton-S* and *Rosy*. Flies were aspirated one treatment at a time by filling one row consisting of twelve wells. Once all the flies were placed in their appropriate wells, the plates were covered with a lid and then wrapped with parafilm to make sure the plate was sealed. The plate was then submerged for an hour at 0ºC (Figure 3). Plates were placed in a

20°C incubator with the light on for recovery. A camcorder was set up with a stopwatch placed in sight to record recovery times of the flies.

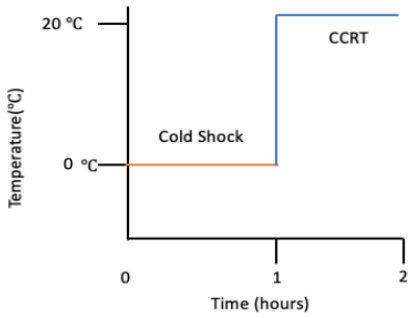

Figure 3. Schematic of acute cold hardening protocol in the Canton-S and *rosy* assay.

Statistical Analysis

All feeding experiment CCRT data for *A. picea* were analyzed using a mixed model in the statistical program JMP. Fixed effects included diet, cold shocks, set, and interactions between factors, while the colony in the *A. picea* experiment was analyzed with a random effect. Data regarding *Drosophila* was analyzed with a standard least squares model, which, depending on the experiments, included shock type, diet, strain, round, and interactions between factors. When significant main effects were found, pairwise post-hoc Tukey's HSD tests were run to determine significant differences among treatments.

Results

Aphaenogaster picea

Although two sets of colonies were collected in June and July, the colonies collected in the second set showed signs of poor condition, with excess mortality during rearing and nearly double the CCRT values of the first set, a highly significant increase (ANOVA, $F_{(1,7.7)} = 15.36$, $p < 0.0048$; Figure 4). As a result, the second set of colonies was excluded from further analysis and all the results below will include just the first five colonies.

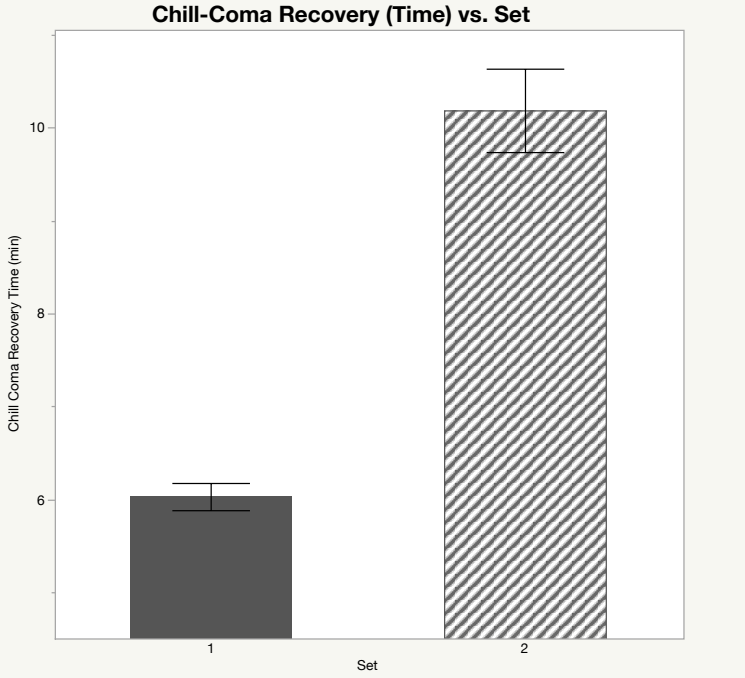

Figure 4. Mean (+/- SEM) CCRT of *A. picea* workers from the first and second collection set. There was a significant difference between the two sets in their ability to recover (ANOVA, $F_{(1,7.7)} = 15.36$, $p < 0.0048$).

Hardening improved cold recovery (Figure 5) with RCH having a general quicker recovery time. Food treatment did not significantly affect baseline cold

tolerance (ANOVA, $F_{(1, 162)} = 0.92$, $p>0.4716$). Post-hoc tests determined that there is not a significant difference among any food treatments and the recovery time. Although the difference was statistically insignificant, allantoin-fed ants tended to have a better baseline recovery, similar to hardened values. In contrast, proline-fed ants showed typical baseline values but then showed a trend toward less hardening ability.

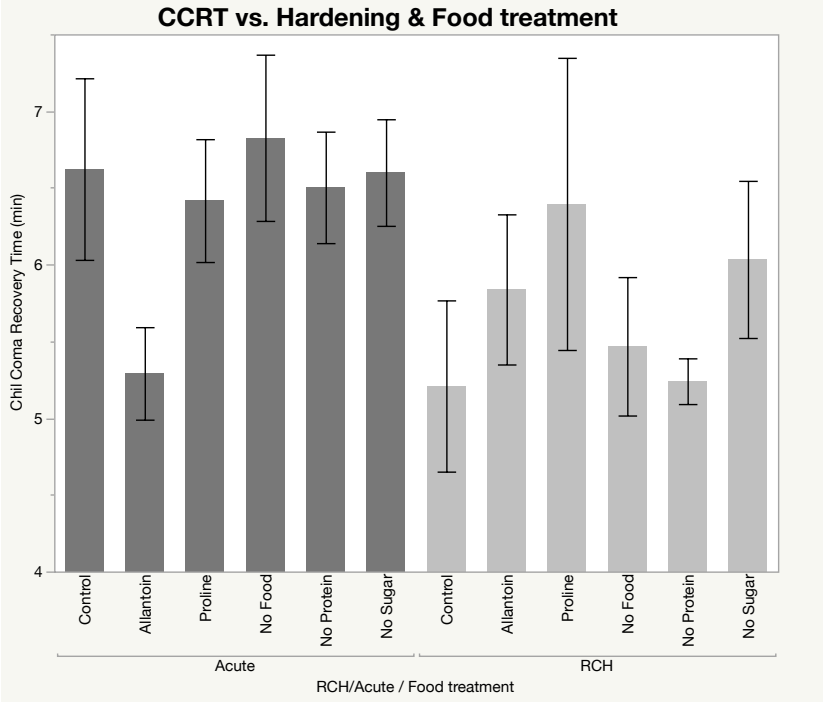

Figure 5. Mean (+/- SEM) effect of food treatment and hardening on CCRT. There were no significant trends of the effects of food treatment, but hardened ants recovered significantly faster (ANOVA, $F_{(1, 162)} = 7.19$, $p<0.01$).

Acclimation Treatment

In the cold-acclimation assay, food treatment had a significant impact on recovery time (ANOVA, $F_{(1, 153.7)} = 2.61$, $p<0.0268$). Post-hoc Tukey HSD tests,

designated by the letters, represent the difference between the dietary additions on the chill-coma recovery time; the no food treatment and no protein treatment were significantly different from each other but did not differ from the control (Figure 6). There were no marginally insignificant trends seen between proline and allantoin to the control group on the average recovery time. In addition, hardening significantly improved cold recovery with RCH having a general quicker recovery time (ANOVA, $F_{(1, 153)} = 7.12$, $p<0.0084$).

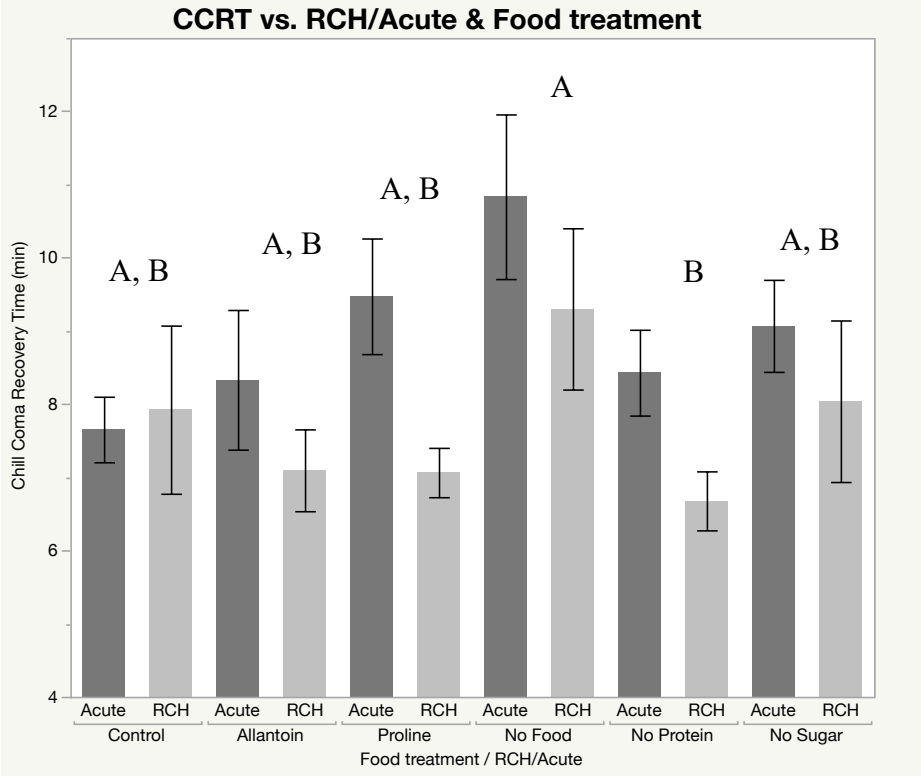

Figure 6. Mean (+/- SEM) effect of food treatment and hardening on CCRT in cold-acclimated flies. Post-hoc Tukey's HSD tests, designated by the letters, show that there is a significant difference between food treatments, specifically between no food and no protein on recovery. There were no other significant differences of the

effects of food treatment on recovery. Hardened ants recovered significantly faster (ANOVA, $F_{(1, 153)} = 7.12$, $p<0.0084$).

Effect of allantoin and proline addition on *Drosophila melanogaster*

There was a significant effect of diet on the recovery time of *D. melanogaster* (Figure 7). Proline-fed flies took significantly longer to recover than those in the control treatment ($p < 0.05$); however, allantoin was not significantly different from that of the control.

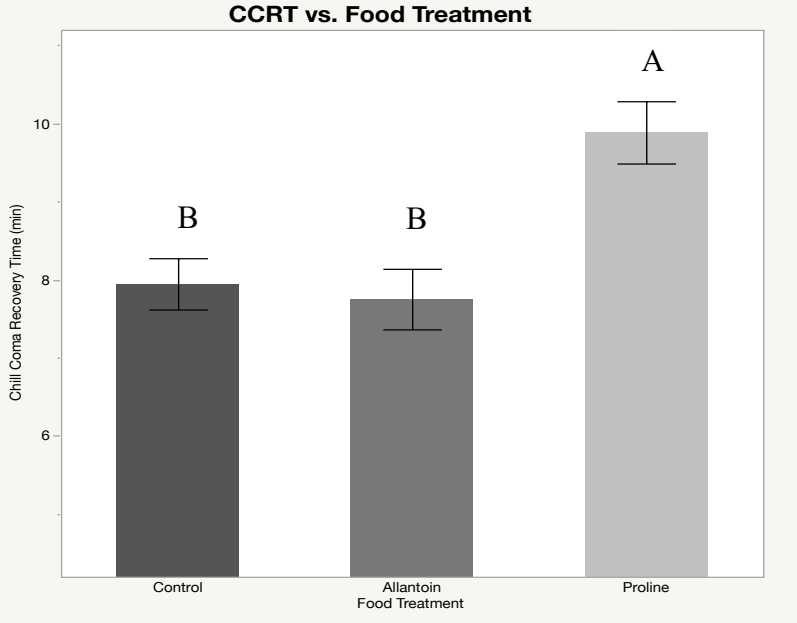

Figure 7. Bar graph (mean +/- SEM) showing the difference in recovery time based on the dietary additions (ANOVA, $F_{(1, 202)} = 14.34$, $p<0.001$). Flies treated with a proline diet exhibited significant increase in recovery time to the control. Letters indicate significantly different treatments based on Tukey's HSD pairwise comparisons.

Although overall patterns were consistent, there was a significant interaction effect between the food treatment and round recovery time (ANOVA, $F_{(1, 204)} = 6.82$, $p<0.0014$). Although allantoin did not differ from the controls in either round, post-hoc tests determined that average recovery time for those that were fed the allantoin diet across the two rounds significantly differed from each other, with significantly faster recovery in the second round that was not matched by faster recovery in the round 2 controls (Figure 8). Similarly, proline tended to have higher CCRT value in both rounds, but only the second round was significantly higher than the control.

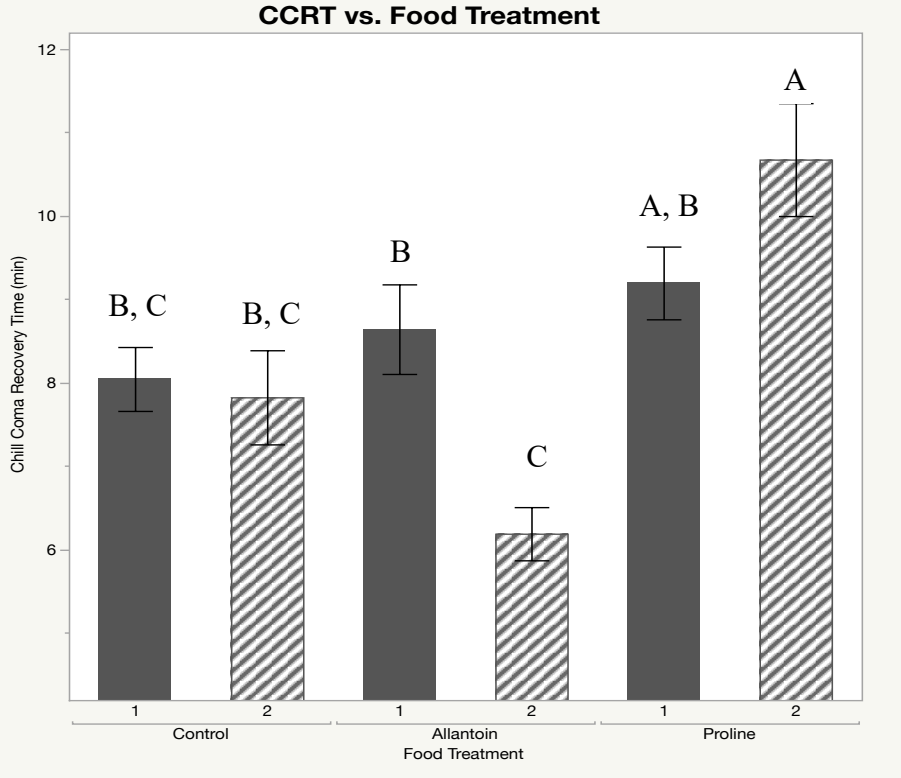

Figure 8. Mean (+/- SEM) CCRT for each food treatment across the two rounds. There was a significant interaction effect between diet and round (ANOVA, $F_{(1,}$

$_{204)}$ = 6.82, p<0.0014). Letters indicate significantly different treatment combinations from Tukey's HSD pairwise comparisons.

Effect of dietary additions in wild-type and *Rosy* mutant *D. melanogaster*

CCRT for the Canton-S wild-type flies was significantly longer in comparison to the *Rosy* flies (ANOVA, $F_{(1, 249)} = 28.69$, p<0.0001; Figure 9), a pattern that was consistent in sign across three rounds of testing although the magnitude varied significantly (interaction effect between strain and round, $F_{(3, 249)} = 5.37$, p<0.0013; Figure 10). The average CCRT over the three rounds for the *Canton-S* flies increased by 30% in comparison to the *Rosy* flies.

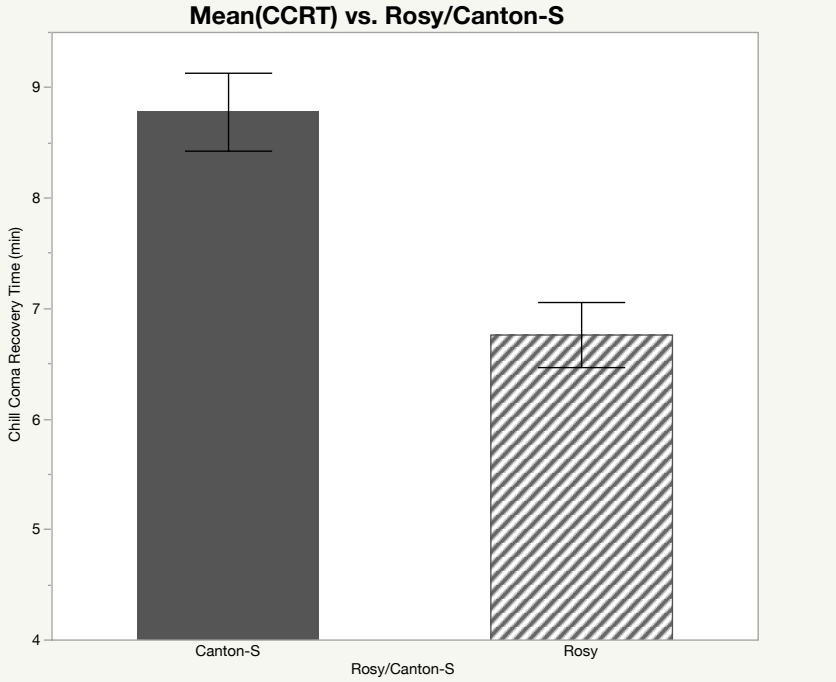

Figure 9. Mean (+/- SEM) CCRT of wild-type *D. melanogaster* vs. *Rosy* mutants. *Rosy* flies recovered significantly faster than *Canton-S* flies (ANOVA, $F_{(1, 249)} = 28.69$, p<0.0001).

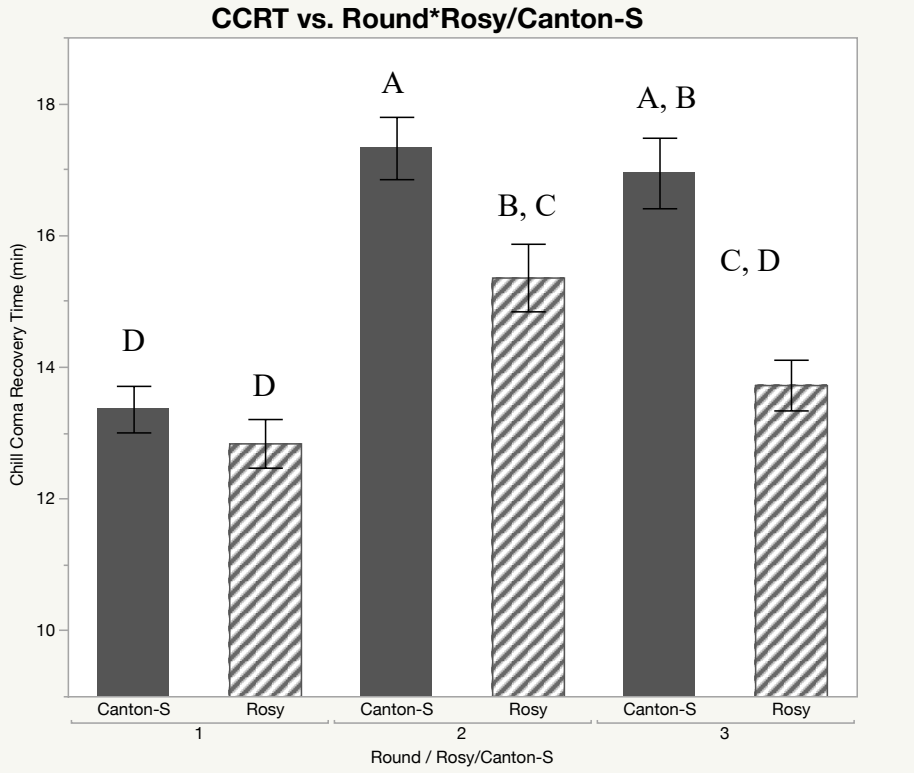

Figure 10. Bar graph (mean +/- SEM) displaying the interaction effect between Round and *Canton-S/Rosy* on the recovery time (ANOVA, $F_{(2,\ 249)} = 5.05$, ($p<0.0071$). Post- hoc Tukey HSD tests determined the variability among the rounds of cold shocks, with the first round recovering significantly faster.

Diet had a significant main effect on CCRT ($F_{(3,\ 249)} = 5.37$, $p<0.0013$; Figure 11). Post-hoc tests, shown by the letters, determined that none of the diet additions, including proline, allantoin, and uric acid diets differed significantly from the control. Rather, proline and allantoin were both significantly higher than uric acid. However, a marginally insignificant trend exists in that both proline and allantoin additions to diet appeared to have a longer recovery time in both types of flies than in control flies. On the other hand, the addition of uric acid tended to show faster recovery time overall in both types of flies in comparison to the

baseline control. Even though there was a main effect of diet on CCRT, there was no interaction effect between the diet and the strain of fly.

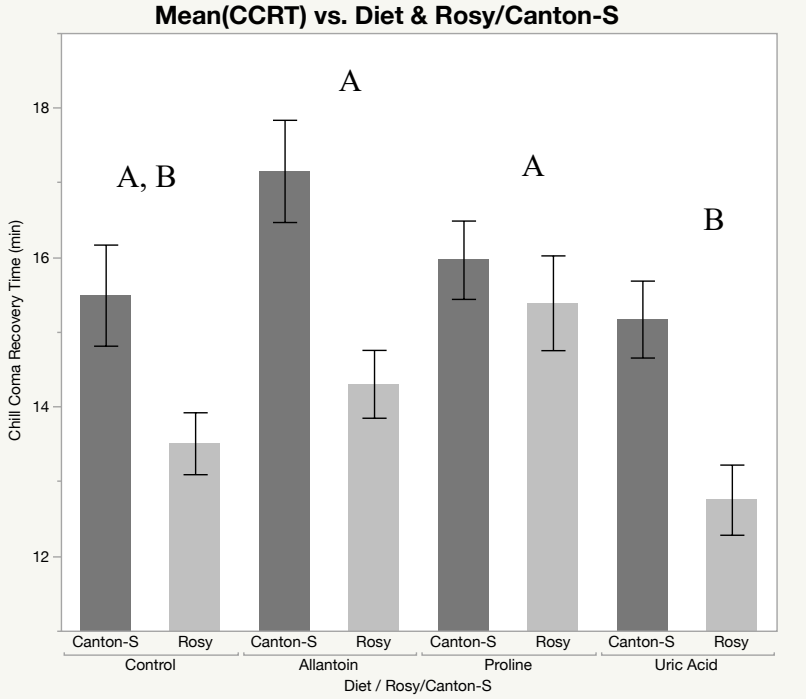

Figure 11. Mean (+/- SEM) CCRT of flies on different food diets. Letters indicate significantly different dietary treatments based on Tukey's HSD pairwise comparisons.

Discussion

As temperature is a major factor regulating the survival and success of a species (Bale, 2002), I attempted to study the effects of various metabolites such as proline, allantoin on cold tolerance in the northern forest ant *A.* picea and the model genetic organism *D. melanogaster.* The results indicated that despite its protectant role in come contexts, proline actually impedes chill coma recovery, and thus may pose a tradeoff for insects facing stressful winter temperatures.

Allantoin showed inconsistent effects, suggesting that this metabolite may not play an active role in cold recovery in these insects. Resolving whether there are species and contexts in which it is important will depend on developing more consistent assays to test this metabolite. Surprisingly, *Rosy* mutants lacking the entire uric acid pathway possessed a significantly better recovery time, hinting that antioxidant responses common under temperature stress could in fact make it harder to recover neuronal function under cold conditions.

Proline

In most cases, proline acts as a cryoprotectant in order to enhance an organism's ability to handle harsher environments. Cryoprotectants allow insects to have the ability to evade freezing damage in order to survive harsh cold (Rudolph and Crowe, 1985; Withers and King, 1979). Organisms that are exposed to prolonged cold conditions can lose their freeze tolerant abilities, however, it is well supported by the literature that there is a significant, positive relationship between increased levels of intracellular proline to improved resistance to freeze stress (Terao et al., 2003). Proline has also been shown to allow organisms to stabilize their cell membranes in low-temperature environments, which may allow for the improvement in cold tolerance (Fields et al., 1998).

Despite the benefits of proline in those contexts, the data collected in this study showed that dietary proline decreased the ability to recover from stress. Proline never improved performance in this study, and it actually increased chill coma recovery time across most experiments (Figures 7, 8, 10). Thus, it is

possible that proline can have toxic effects, particularly at high dosages. Literature has shown that exogenous proline application can create an imbalance in organic ions in plants (Hayat et al., 2012). The possibility of providing *Drosophila* with too much proline suggests that the benefit it could have had on recovery was outweighed by the toxicity of the high proline dosage. When proline was provided at low concentrations, it was demonstrated that there were no adverse effects on growth. However, higher concentrations hindered the efficiency of plant growth. With the diets of *Drosophila* along with *Aphaenogaster* being artificially enhanced by proline, it is possible that there was an excess amount of this amino acid in their systems, which amplified its toxic effects. Although each organism was provided the same amount of food and identical amounts of the food additives in their diet, it might be beneficial to see how much proline these organisms accrue as a result of their augmented diet throughout their lifespan. To investigate a more in-depth effect of proline, one could rear *Drosophila* on a proline diet based on a dose-response curve and test the concentrations of proline present in the hemolymph and tissues (Kostal et al., 2012). To do this, the *Drosophila* would be provided with a range of different concentrations of proline, and then placed under cold shocks would aid in determining what concentration improved CCRT the most, as well as seeing the point at which flies begin to show a decline or inability to recover. After performing cold shocks, the concentrations in these *Drosophila* would be tested to see how much of proline they have after cold stress. This methodology could be used to gauge how differing levels of proline in *Drosophila* cause varying toxic

effects as well as identifying the optimal concentration of proline to improve recovery.

Although it is a possibility that the addition of proline could have negative effects on cold tolerance, proline itself might be highlighting the existence of potential tradeoffs creating both benefits and costs that could impact the ability to recover in response to the cold. It could be that proline helps insects avoid freezing damage, but it actually impedes their cold tolerance. There is some evidence in the literature that proline may act in opposing directions depending on the aspect of cold tolerance considered. In flies, during cold and warm acclimation, the proline and arginine metabolism pathways along with the glutathione metabolism pathway can be altered. Flies accumulated higher levels of proline and arginine when acclimated to the cold in comparison to those that acclimated to warm conditions, along with increased levels of glutamate (Macmillan et al., 2016). This presents an idea that polar and charged amino acids, like proline, could be vital for an organism's chill tolerant abilities. Flies that had been selected to undergo chill coma recovery, however, displayed downregulation of proline metabolism. Therefore, these flies had lower amounts of proline in their systems, suggesting that proline plays differing roles in chill survival versus chill coma. This potential selection for cold tolerance can prompt trade-offs with surrounding pathways that may be linked to dealing with oxidative stress, suggesting that an increase in proline could lead to protection against oxidative stress by working to maintain other pathways like glutathione's. This would occur in addition to aiding these insects to evade freeze damage to their

cells. In turn, it would not directly be impacting their ability to recover after putting them through cold shocks.

Allantoin and Uric Acid Pathway

In addition to proline, allantoin was the only other metabolite to consistently increase with cold stress in *A. picea,* following both hardening and seasonal acclimation (J. Cline, unpublished data). Allantoin is considered a marker of oxidative stress (Yardim-Akaydin, Sepici, Özkan, et al., 2006) as its production from uric acid is driven by a reactive oxygen reaction (Chambers, Song, & Schneider, 2012). In addition to being a direct intermediate of uric acid, allantoin is also a more soluble catabolite than uric acid, which may provide an upper hand when dealing with oxidative stress (Gus'kov et al., 2001). This suggests that allantoin possesses the ability to protect DNA from damage produced by reactive oxygen species.

The results in this study indicate that there is no statistically significant impact of allantoin on the ability to recover in *A. picea* (Figure 5). Under the acute treatment however, there seems to be a marginally insignificant trend in that ants provided with allantoin showed a slight improved resistance to cold (Figure 5). The effect size under acute hardening was much larger than rapid cold hardening where the opposite effect occurred. If there had been more replicates of the cold shock assays along with a larger sample size, this trend could have potentially presented as statistically significant rather than appearing to occur by chance. In many cases, an organism under a condition where they deal with cold

stress fare better when they have previously been exposed to a similar condition (Powell and Bale, 2004). The trend in this study suggests that allantoin poses a tradeoff between rapid cold hardening and cold stress. If the assays were replicated with a larger sample size, and this pattern remained the same such that there was a significant reduction in CCRT in acute ants fed allantoin that everyone showed only after hardening shows that allantoin's role in combating oxidative stress is not sustainable in organisms undergoing multiple cold shocks.

In the experiments with *Drosophila Canton-S*, the addition of an allantoin diet did not significantly improve chill coma recovery time after cold-stress (Figure 7, 8). There appears to be a very minute improvement under shock (Figure 7), but the two round of cold shocks with *Canton-S* produced very different results from each other with the first round of an allantoin diet worsening recovery with the second round seemingly improve recovery (Figure 8). Therefore, further tests with *Drosophila* were needed to understand the impact of allantoin on recovery.

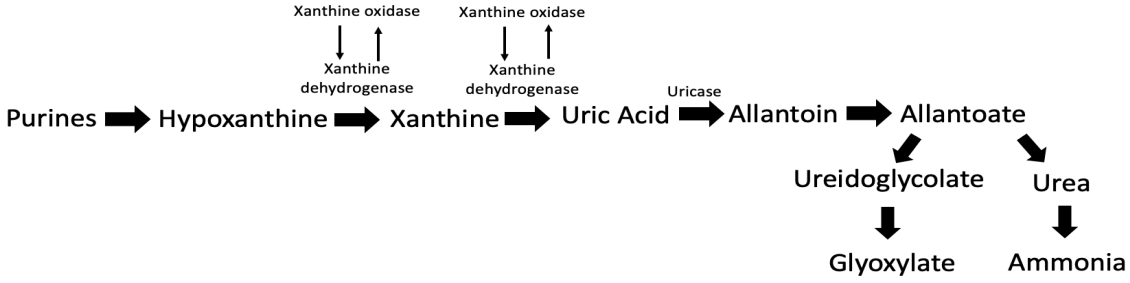

Figure 12. This is a visual representation of the uric acid pathway, and how it leads to the production of allantoin. In the *rosy* mutant, the enzyme xanthine dehydrogenase is knocked out.

Allantoin is a part of the uric acid pathway, and thus changes in its production, such as using the *Rosy* mutant which lacks the pathway, could implicate other members of the pathway in the chill-coma recovery process. Incorporating the mutant into my experiments and looking at chill coma recovery time when compared to the wild type of *Canton-S* allowed for a deeper investigation on how the lack of the pathway as well as attempting to restore the pathway can affect cold tolerance. Overall, all experiments with these two types of flies showed that the *Rosy* mutants had a lower CCRT than the *Canton-S* (Figures 9, 11), implying that the lack of a uric acid pathway can in fact be beneficial for insects and improve their stress tolerance. It may be that uric acid works similarly to proline by possibly sharing a cryoprotectant role that moderates signaling along with metabolic adjustments to help insects deal with harsh cold (Boardman et al., 2018).

In addition to the *rosy* trend, providing the flies with uric acid through their diet in order to restore the missing pathway was not statistically significant (Figure 10). However, in comparison to the other dietary conditions, the addition of uric acid appeared to lead to a faster recovery relative to the control (Figure 10). This is surprising as many studies show the benefits of possessing the uric acid pathway. However, *Drosophila* that were knocked down of the *Uro* gene that codes for uricase, has previously shown to increase levels of uric acid, since there is nothing to stimulate its breakdown (Lang et al., 2018). This mutant has an insulin-like signaling pathway that can affect the production of nutrients like uric acid. Manipulating this pathway via suppression decreased the production of uric

acid and ultimately enhanced the longevity and survival of *Drosophila*. It has been shown that in *Drosophila*, that systems involving thioredoxin reductase work as antioxidant systems in *Drosophila* (Missirlis et al., 2001). More specifically, it has been shown that thioredoxin reductase can work in conjunction with the superoxide dismutase and catalase system as well as potentially compensating for each other to combat oxidative stress. It may be a possibility that *rosy* mutants have compensatory mechanisms that provide protection against oxidative stress, which would allow for improvement in *Drosophila*'s ability to tolerate the harsh cold.

On the other hand, it is possible that the cold provides protection under hypoxic stress by reducing metabolic rate and obtaining certain products that result from anaerobic metabolism (Boardman et al., 2018). It may be that oxidative stress could actually be less of a problem under cold conditions, so activating antioxidants may be less helpful and might actually interfere with other cold responses. Literature shows that when *Drosophila* underwent both heat shock and cold shocks, the cold shocked flies experienced lower rates of mortality (Boardman et al., 2018). Cold stressed flies also have different transcripts that are upregulated and there is documentation regarding the upregulation of urate oxidase in flies under cold shocks (Boardman et al., 2018). In addition, the *rosy* mutant is considered to be temperature sensitive lethal, which means that it is hypersensitive to increased temperatures (Hilliker et al., 1992). This poses the idea that oxidative stress may produce a much larger problem with an increase in temperature along with an increase in metabolic rate. It would be beneficial to

explore role of various temperature changes in *Canton-S* and *Rosy* to determine the impacts on oxidative stress. It would also be important to focus more on the uric acid pathway and its intermediates to see what their impact is on cold tolerance, as well potentially surrounding pathways that could be affected when there is a nonfunctional uric acid pathway.

Overall, there is a great amount of variability associated with the allantoin diet on recovery with *Drosophila* (Figures 7, 8, 10). Due to this fact, the cold shocks involving the allantoin diet may not be the most reliable set to draw conclusions about its mechanism in cold tolerance. Some possible reasons for the variability could be due to surrounding environmental temperature and humidity of the room that affect the of recovery the organisms. In addition to these factors, it is important to note variability in the change of the protocol regarding performing cold shocks. The assays were first performed in tests tubes and then later moved to polystyrene round-bottom plates in an attempt to standardize the methodology and keep everything as consistent as possible. For future studies regarding testing chill coma recovery time, it would be beneficial to maintain a similar and more effective protocol among all experiments to ensure the reliability of the test results. It would also be important establish the metabolic status regarding the concentration of the specific metabolites being tested prior to the cold shock as well as after the cold shock in order to compare the effects of these dietary additions (Olsson et al., 2016). In addition, it would be important to age and sex flies as previous studies showed that cold tolerance can be affected by these factors (MacDonald et al., 2004). For the actual assay, it would be best to

place only about 6-10 flies in a single test tube per treatment. In the assays performed in this study, there were many flies placed under cold shock, and then once they were removed, flies were chosen at random. The idea here is similar to that of the cold shocks done with *A. picea* where all ants that were put through hardening (3 per food treatment) were observed. However, it would be necessary to have a larger sample size and a greater number of testing rounds in order to accurately compare the treatments. Hopefully, adjustments such as these would prevent the extreme variability that was observed in the experiments conducted for this study.

Conclusion

To summarize, this study displayed that high concentrations of proline might hinder an insect's ability to recover from the cold even though many studies confirm its role as a cryoprotectant. The data implicates a potential tradeoff with proline in that proline helps insects to avoid freeze damage, but actually impairs their cold tolerant abilities. In addition, there is no conclusive evidence of allantoin's role in cold tolerance in insects, but future adjustments regarding chill coma assays may be able to significantly replicate some of the trends that were seen in the experimental assays in this study. *Rosy* flies may pose an interesting question as to whether the lack of a major pathway, the uric acid pathway, is truly beneficial to insects as they recovered significantly better than the wild-type flies. Overall, there is great variability among several different experiments with *A. picea* as well as *Drosophila melanogaster Canton-S* and

Rosy, but these results provide a framework to understand how temperate-zone insects are able to survive harsh, cold conditions.

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