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## The Effect of a Trehalose Diet on Cold Tolerance in *Aphaenogaster Picea*

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Authors	Cline, Julia Clare
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**The effect of a trehalose diet on cold tolerance in**  
*Aphaenogaster picea*

Julia Cline

The University of Vermont

Department of Biology

CAS/HCOL Thesis

*Committee Members*

Dr. Sara Helms Cahan (Advisor)

Dr. Nathan Sanders (Chair)

Dr. Brent Lockwood

## Abstract

Ants living in thermally variable environments need to be able to tolerate both high and low temperature extremes. One way that insects handle cold is through the accumulation of cryoprotectants, such as sugars, that increase cold tolerance by lowering the freezing point of liquids in the hemolymph. The disaccharide sugar molecule trehalose is an important cryoprotectant in many insect species, but its role has not yet been studied in ants. I investigated whether the northern forest ant *Aphaenogaster picea* resists cold by producing trehalose from sugars in their diet. To understand whether dietary trehalose or its monosaccharide, glucose, increases cold tolerance, recovery from cold shocks at 0 °C and -5 °C was compared for ants maintained on trehalose, glucose or no-sugar diets. To determine whether workers produce trehalose in response to cold exposure, whole-organism metabolite concentrations following rapid cold hardening (RCH) or gradual acclimation were compared to room-temperature (RT) controls. While dietary sugar improved baseline cold tolerance, trehalose was not present at detectable levels regardless of condition, suggesting that it is not used as a cryoprotectant in *A. picea*. Other sugars present in the hemolymph may help increase cold tolerance in a cryoprotectant capacity, or they could be used as an energy source. Metabolites associated with scavenging of reactive oxygen species increased under both exposures, and during acclimation the amino acid proline increased, suggesting that it may act as a cryoprotectant to stabilize cell membranes under longer term exposure. Therefore, while trehalose is not involved in increasing cold tolerance, *A. picea* may rely on other molecules to handle the cold.

## Introduction

Ectotherms, including insects, are particularly susceptible to temperature changes because their physiological performance, movement, feeding and social interactions rely on external heat sources to power enzymatic reactions (Huey & Kingsolver, 1989; Sunday, 2014).

In a thermally variable environment, organisms are exposed to predictable cold shocks that are the result of diurnal or seasonal cycles, and also to cold shocks that can occur without warning and be unpredictable in duration and severity (Bale & Hayward, 2010). Cold shocks can cause ice formation, changes in membrane fluidity, or altered protein interactions (Bagwell & Ricker, 2007; Verde, Parisi, & di Prisco, 2006). This could lead to detrimental effects on organisms, such as osmotic shock, or membrane breakdown or leakage (Bagwell & Ricker, 2007).

Species that live in such variable environments have evolved thermal tolerance strategies to function in and survive at cold temperatures (Bale & Hayward, 2010; Stanton-Geddes et al., 2016). The two most widely accepted cold hardiness strategies are freeze-avoidance and freeze-tolerance (Bagwell & Ricker, 2007; Bale, 2002; John G. Duman, 2015; Feng et al., 2016). In cold-tolerance studies, whether an organism is freeze-tolerant or freeze avoidant is often determined by the organism's supercooling point (SCP) (Bale, 2002). The SCP is the temperature at which the entire organism freezes, and organisms have developed ways to lower their SCP below that of the freezing point of water to extend survival (Bagwell & Ricker, 2007). Freeze avoidant organisms tend to have lower SCPs and die once they reach their SCP temperature, while freeze tolerant organisms often have higher SCPs and can generally survive

freezing (Bale, 2002). Freeze-tolerant organisms cryoprotect their tissues and inhibit ice crystallization (Bale, 2002). One way that insects are able to accomplish these strategies is through the accumulation of cryoprotectant compounds, which can increase cold tolerance by lowering the point at which liquids in the hemolymph freeze. While both freeze tolerant and freeze avoiding insects use cryoprotectants, there is evidence that they employ them in different ways and at different times to help lower SCP and avoid mortality (Bale, 2002; Bale & Hayward, 2010; Feng et al., 2016). Insects utilize several different types of cryoprotectants, including sugars, polyols, anti-freeze proteins (AFPs), and amino acids (Bale, 2002; Feng et al., 2016; Košťál & Tollarová-Borovanská, 2009). In high concentrations, sugars and polyols can function through colligative effects, making their potential limited by the ratio of solutes to solvents in the system, so other compounds are often necessary to sufficiently lower the SCP (Duman, 2002; Duman, 2015). Amino acids can also be useful cryoprotectants, and like sugars and polyols can also help protect proteins and stabilize cell membranes (Košťál et al., 2011, 2012; MacMillan et al., 2016). These cold hardiness strategies can be damaging to normal function and can require redirecting of resources, and are thus activated in response to experienced or anticipated cold temperatures (Bale, 2002; Teets & Denlinger, 2013).

On a seasonal basis, organisms may respond to decreasing temperatures by acclimatizing, a physiological process during which they stockpile nutrients and cell membranes change to reduce freezing and increase cold resistance (Modlmeier, Pamminer, Foitzik, & Scharf, 2012). In a lab-setting, this process is referred to as “acclimation.” In addition, many insects acclimatize to prepare for seasonal diapause, whereby they store up energetic resources and then overwinter with a slowed down metabolism to conserve these resources (Hahn & Denlinger, 2011). While insects may downregulate normal metabolic behaviors, they often upregulate the processes that

control cryoprotectant, or even heat-shock protein and other temperature stress protein production (Bale, 2002; Hahn & Denlinger, 2007). These compounds can be produced on a seasonal basis, but also on a much more rapid timescale (Lee, Chen, & Denlinger, 1987).

For a more rapid response, in addition to mobilizing cellular responses to cold damage, many cold-tolerant species can respond to a mild cold shock with rapid cold hardening (RCH), a physiological response that allows them to better withstand future shocks (Michaud & Denlinger, 2007). When RCH occurs and the organism quickly produces compounds, such as glycerol or trehalose, to reduce the negative effects of the initial cold shock, it is then consequently better prepared to handle future cold shocks following initial exposure (Bale, 2002).

Trehalose is a disaccharide sugar, composed of two glucose molecules, that may be important in insect cold tolerance by acting as a cryoprotectant (Feng et al., 2016). Trehalose can quickly be produced from glycogen, the primary carbohydrate storage molecule in insects (Arrese & Soulages, 2010). As a major transport sugar, trehalose can readily be circulated through the hemolymph to where it is needed (Arrese & Soulages, 2010; Hahn & Denlinger, 2011; Jain & Roy, 2009; Schilman & Roces, 2008). The exact mechanisms through which trehalose could contribute to increased cold tolerance are unclear; however, trehalose helps stabilize lipid membranes by delaying state changes that cause them to lose structure, and has been shown to be more effective at protein stabilization than other polyols or sugars (Jain & Roy, 2009; Kaushik & Bhat, 2003; Wen et al., 2016). Water also prefers interactions with disaccharide sugars including trehalose, maltose and sucrose, over interactions with charged salts or even other water molecules (Kaushik & Bhat, 2003). As a result, in cold conditions, water converges on these sugar molecules, and in particular on trehalose, rather than on other water molecules, which helps to prevent ice formation (Jain & Roy, 2009).

While other low molecular weight sugars have also been implicated in insect cryoprotectant systems (Colinet, Larvor, Bical, & Renault, 2013; Fields et al., 1998), trehalose accumulation correlates with increased cold tolerance across many insect species, suggesting that it may act as a cryoprotectant. Trehalose was detected in higher levels than other cryoprotectants in overwintering *Anoplophora glabripennis* beetles (Feng et al., 2016), field-acclimatized *Popilius disjunctus* beetles (Rains & Dimock, Jr., 1978) and diapausing cabbage root flies, *Delia radicum* L. (Košťál & Šimek, 1995). Trehalose also accumulated in lab-acclimated *Dendroides Canadensis* beetles (Wen et al., 2016) and winter acclimatized cabbage armyworms *Mamestra brassicae* (Goto et al., 2001). When the weevil *Sitophilus granarius* and beetle *Cryptolestes ferrugineus* were cold acclimated in laboratory, there were two-fold increases in trehalose concentration that correlated with the most cold-hardy specimens (Fields et al., 1998). For *Cydia pomonella* moth larvae, collected throughout the year, there was a significant relationship between higher trehalose content and lower SCPs, suggesting that the observed threefold increase in trehalose correlates with cold tolerance (Khani et al., 2013).

The ants are an incredibly ecologically successful and species-rich family in the insect order Hymenoptera. Ant species are distributed across habitats that experience a wide range of temperatures, from the Kalahari Desert to the Arctic Circle, and their physiological cold tolerance often determines the cold-edge boundary of their range, making them useful for cold tolerance studies (Bishop, Robertson, Van Rensburg, & Parr, 2017; Modlmeier et al., 2012). Thus far, no studies have been done on cold tolerance and sugar accumulation in ants, and ant hemolymph sugar content has not been extensively investigated (Schilman & Roces, 2008).

*Aphaenogaster picea* is a temperate ant species located throughout North American northeastern forests, along the east coast of the U.S, up through New England and into Canada

(Clark & King, 2012; Ellison, Gotelli, & Farnsworth, 2012). *Aphaenogaster picea* can tolerate (remain upright) temperatures as low as  $-0.5\text{ }^{\circ}\text{C}$  (Warren & Chick, 2013). *Aphaenogaster rudis*, a species that occurs at warmer temperatures and lower elevation, has been replacing *A. picea*, which prefer colder, higher elevations and latitudes, along their southern boundary as climates warm and thermal habitats shift (Warren & Chick, 2013). Both species of ants are involved in a mutualistic seed dispersal relationship called myrmecochory (Ness, Morin, & Giladi, 2009; Stuble et al., 2014; Warren II, Bahn, & Bradford, 2011; Zelikova, 2008). While some plants are flowering earlier in the spring as a result of warming, *A. rudis* are unable to start to forage as early in the spring as *A. picea* due to low temperature constraints (Ness et al., 2009). *A. picea* disperse seeds at temperatures as low as  $4\text{ }^{\circ}\text{C}$ , but *A. rudis* requires at least  $10\text{ }^{\circ}\text{C}$  to forage, which has led to decreases in seed dispersal rates along higher elevational and colder thermal gradients, where *A. rudis* has replaced *A. picea* (Stuble et al., 2014; Warren II et al., 2011). As important seed dispersers in Northeastern forests, changes in *A. picea* range boundaries may negatively affect the surrounding environment, particularly the plants that rely on the ants for seed dispersal (Warren II et al., 2011; Warren & Chick, 2013).

*A. picea* avoids severe cold during the winter by living underground, minimizing activity and relying on built up energy reserves to survive (Lubertazzi, 2011). These energy reserves may include sugars, glycogen or lipids. Sugar stores come from ants ingesting sugar in their diet and either breaking the sugar monomers down into energy or storing them (Boevé & Wäckers, 2003). *Aphaenogaster* species forage primarily for insect protein and dispersing seeds that have lipid-rich (Brew, O'Dowd, & Rae, 1989) elaiosomes (Brew et al., 1989; Clark & King, 2012; Lubertazzi, 2011). As part of the cryoprotectant system in these freeze-tolerant ants, a seasonal buildup of energy resources such as trehalose may increase ant cold tolerance.

In this study, I tested the hypothesis that *A. picea* resists cold by producing trehalose, derived from sugars present in their diet. To assess whether dietary trehalose or its monosaccharide building block glucose promote cold tolerance in ants, I manipulated colony diets and assessed whether sugar resources improved chill coma recovery time following either an acute cold shock or rapid cold hardening, as well as its effect on survival during acclimation. To determine whether different types of cold exposure resulted in increased trehalose, individual ants were exposed to no cooling, rapid cold hardening, or gradual cooling to elicit acclimation, and metabolite concentrations were determined using liquid chromatography mass spectrometry.

## **Methods**

### Colony Collections and Maintenance

A total of 11 *A. picea* colonies were collected from the field for experiments. Five adult colonies were collected from Centennial Woods, Burlington VT (44.475219, - 73.188284) in June and July of 2017. Four adult colonies were collected from East Woods, Burlington VT (44.446653, - 73.199550) in October of 2016. Two additional colonies were collected from East Woods in July of 2017. Seven colonies, including the five colonies collected from Centennial Woods between June and July of 2017, and the two colonies collected from East Woods in July of 2017, were used in the diet manipulation study. These colony collections were staggered across three weeks, with 2 to 3 colonies collected per week. The four colonies from East Woods in October of 2016 were used in a separate internal metabolite profiling study.

Colony sites were located by overturning and splitting open rotten logs. Once located, entire colonies, including workers, brood, and queen if possible, were collected with handheld

aspirators and placed in labeled Tupperware or trash bags together with any nest debris containing workers or brood. Colonies were brought back to the lab and were carefully separated out from the forest debris using forceps and a handheld aspirator. Each colony was placed into a labeled 22 x 16 cm Tupperware rearing container. To allow sufficient air circulation, there was 10 x 10 cm section of screen in the lid. Each container had sides coated in Fluon, a white liquid barrier, that once dried prevents insects from escaping. Two nest tubes, standard test tubes 1/3 of the way full of dH<sub>2</sub>O with a folded cotton ball to block the water and a piece of Parafilm partially blocking the entrance, were placed in each container for the ants to nest in. Nest tubes were replaced or added as needed, depending on colony size, if the water level was low, mold was visible, or water was dirty. All colonies were kept in a temperature controlled 18.5 °C room with a 12-hour light/dark cycle.

#### Determining colony condition and ant body size

To determine colony size, whole colonies (from the diet manipulation study described below) were weighed. Whole colonies were placed into a pre-weighed container which had Fluon coated sides to prevent escape and weighed on a Mettler Toledo AX205 to the nearest 0.01 mg. Colonies ranged in mass from 58.84 mg to 600.50 mg. To prepare ants for head width measurements, groups of 10 monomorphic workers were removed from each colony and weighed, frozen, and kept in ethanol. To measure individual head widths, the head was detached from the body at the neck and a TA Leica M60 Modular Stereo Microscope was used to measure to the nearest 0.01 mm with an ocular micrometer. Sizes were calibrated with a calibration slide that resulted in a correction factor of 0.052 for a magnification of 4X. Head width was defined as

the inter-ocular distance, with all measurements taken from the area right below the left eye of the ant where the eye met the edge of the cuticle across to the area right below the right eye.

### Diet Manipulations

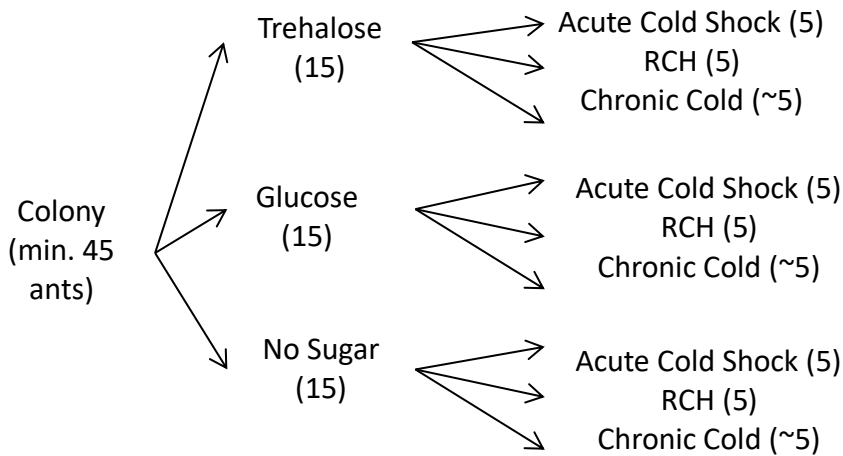


Figure 1. Overview of ant feeding experimental design. Seven colony replicates were used.

For the feeding experiments, for each of the seven colonies, 45 workers were removed from the main colony and separated into three diet treatment groups of 15 to 18 ants each: trehalose, glucose, and no sugar (Fig. 1). When possible, 18 ants were used for each diet treatment group so that even if there were mortalities during the two-week feeding period, there would be at least 15 ants for subsequent cold exposure manipulations. Each group was placed in a new, separate nest container containing a single nesting tube, as well as a tube with a single aqueous diet solution: trehalose [1 M], glucose [1 M], or water only, for 2 weeks. They were not provided with any other food. All diet solutions were provided in a 15 mL tube that laid horizontally in the Tupperware container, partially plugged by a small piece of cotton ball and stabilized with clay. Enough diet solution was provided so that the ants never finished the

solution and so that the cotton ball remained wet throughout the two weeks of experimentation. Ants were observed visiting the solutions.

After two weeks on a specific diet treatment, each diet group was divided into three subgroups for cold exposure: acute cold shock (5 ants), RCH (5 ants), and chronic cold (remaining ants) (Fig. 1). To ensure that measurements of recovery response were not biased by knowledge of the feeding treatment, both the acute cold shock and the RCH treatment groups were placed into randomized numbered test tubes. Each test tube was plugged with cotton pushed halfway down into the tube, trapping the ants in the bottom half of the tube. All tubes were placed in a large test tube rack and sealed with rubber stoppers tightly wrapped in Parafilm.

#### RCH and acute cold shock:

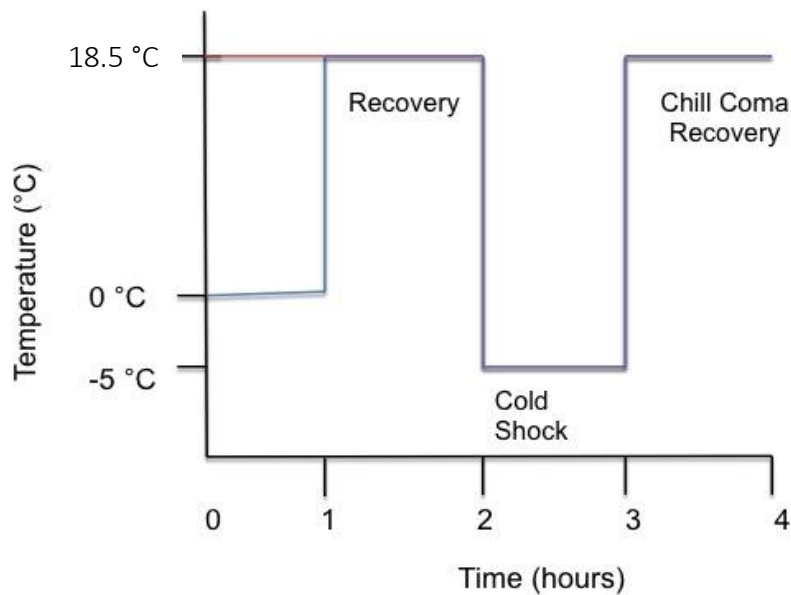


Figure 2. Diagram of rapid cold hardening (RCH) and acute cold shock timeline. At the start of experimentation, RCH ants underwent a 0°C shock for one hour, then recovered at room temperature for one hour. Acute cold shock ants rested at room temperature for this time period.

At the beginning of hour 2, all ants underwent a  $-5^{\circ}\text{C}$  cold shock for one hour, and then CCRT was recorded for individuals once they were removed from the bath.

A 15L PolyScience Refrigerated Circulator water bath was used to manipulate temperature for both RCH and acute cold shock treatments (schematic in Fig. 2). The bath was filled with a 1:1 ethylene glycol :  $\text{H}_2\text{O}$  solution to prevent freezing. An external temperature probe was used to calibrate the temperature settings to achieve the desired water temperature. Each trial included all of the samples (RCH and acute cold shock) from a single colony. To induce the rapid cold-hardening responses, the three RCH tubes were placed into a metal rack that was lowered into a water bath pre-cooled to  $0^{\circ}\text{C}$  for one hour. All tubes were fully submerged. After 1 hour, the rack was removed from the bath and placed on a tray, and the water bath was reset to  $-5^{\circ}\text{C}$ . The rubber stoppers and cotton balls were quickly removed from each test tube, using forceps if necessary, and then the ants were transferred to identically numbered, room-temperature, empty test tubes and plugged with cotton. The ants were allowed to recover at room temperature for 1 hour, which was shown to be sufficient to elicit the hardening response in preliminary tests. Following the recovery period, both the RCH and acute cold shock tubes were submerged in the  $-5^{\circ}\text{C}$  water bath for one hour.

Once exposure was concluded, the test tubes were removed from the cold-water bath and a timer was started to track chill coma recovery time (CCRT). Each test tube was dried with a paper towel so that no liquid obscured the inside of the tube, and then laid horizontally in a clear tray in numerical order. Tubes were continually scanned by a single observer. CCRT was defined for each individual ant as the time until the ant righted itself. If after 15 minutes an ant had not yet recovered, even with slight jostling of the test tube, then it was recorded as a fatality.

Net hardening was calculated at the colony level as the difference between average colony CCRT from acute cold shock and average colony CCRT from cold hardening. Proportional hardening was calculated as arc-sine square-root transformed of the net hardening value divided by acute mean CCRT.

### **Chronic Cold Exposure:**

The chronic cold treatment groups were placed in a new Tupperware nest container devoid of food and placed into a Sanyo incubator set at 4 °C for two weeks to undergo chronic cold exposure. The ants were counted at the start of exposure. After one week, the surviving ants were counted again, and all individuals that had died were removed. At the end of week two, the surviving ants were counted a final time.

### Internal Metabolite Comparison

To test for differences in metabolite production under rapid cold hardening and cold acclimation, four large adult colonies were collected from East Woods in October of 2016 and maintained at 18.5 °C in the laboratory until October of 2017. During this period, several times a week, each colony was provided with ~1 mL of 10 % honey water solution in a small weigh dish with a partial cotton ball, as well as freshly-killed mealworm (*Tenebrio molitor*) prey. Dishes were replaced bi-weekly.

Each colony was divided into three equal groups of at least 50 workers in order to collect sufficient tissue for analysis. Larvae and queens were excluded. The control group was maintained at room temperature until being flash-frozen in liquid nitrogen. The second group underwent a RCH treatment prior to freezing in liquid nitrogen. Following the protocol outlined

above, the ants were subjected to one hour at 0°C followed by 1 hour of recovery at room temperature. The control group and the RCH group were processed on the same day. The third group underwent a gradual cooling regime of 1°C reduction per day from 25°C to 4°C (a total of 22 days) to elicit an acclimation response, beginning on the day that the other two groups were processed. They were kept in standard Tupperware containers with a lid screen for ventilation with nest tubes but no food on a 12-hour light:dark cycle. On day 23, the ants were removed from the 4°C incubator and immediately flash-frozen in liquid nitrogen. All samples were stored at -80°C until metabolomic analysis.

All whole-body samples were sent for analysis, packed in dry ice, to the Southeast Center for Integrated Metabolomics (SECIM) at the University of Florida. Metabolites were quantified using a Thermo Q-Exactive Orbitrap mass spectrometer with Dionex UHPLC and autosampler.

All samples were analyzed in positive and negative heated electrospray ionization with a mass resolution of 35,000 at  $m/z$  200 as separate injections. Separation was achieved on an ACE 18-pfp 100 x 2.1 mm, 2  $\mu$ m column with mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. The flow rate was 350  $\mu$ L/min with a column temperature of 25°C. 4  $\mu$ L was injected for negative ions and 2  $\mu$ L for positive ions. MZmine, a free metabolomics processing program, was used to help identify polar metabolites, and the data was compared against SECIM's internal retention time metabolite library of 1100 compounds.

### Statistical Analyses

All feeding experiment data were analyzed in the statistical program JMP. To determine colony condition, mean ant wet mass (mg) per colony was regressed against mean head width

(mm) per colony; the residuals from this relationship were used as a measure of colony condition (Fig. 3).

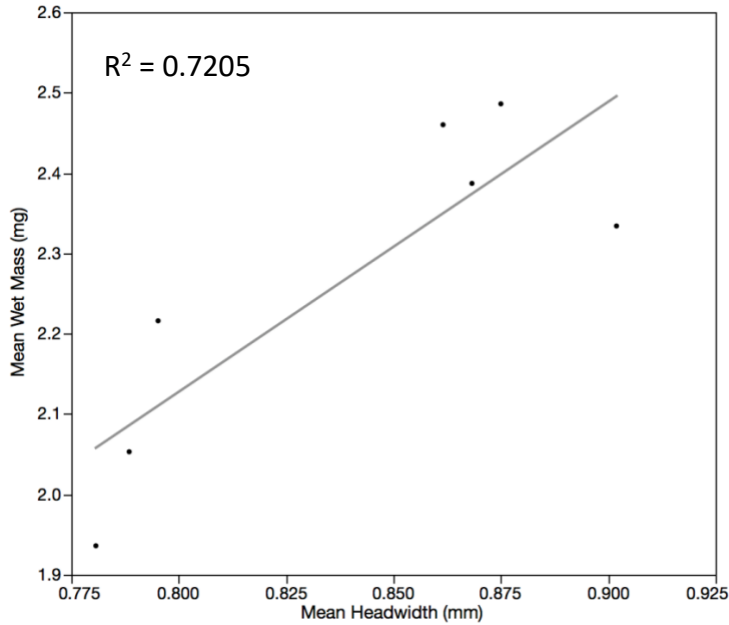


Figure 3. Relationship between mean wet mass per ant (mg) by mean head width (mm) to determine colony condition, the distance that each colony falls from the regression line (n = 7).

To test for the effects of diet and exposure on recovery time while accounting for variation in colony condition, a standard least squares model was run with diet, exposure, diet\*exposure, colony condition\*exposure and head width\*exposure as fixed effects, and mean colony condition and head width as covariates. When significant overall effects were found, pairwise comparisons were conducted with Tukey's HSD tests or Student's t-Tests. Net hardening differences were evaluated with a similar model but excluding exposure and diet\*exposure, and proportional hardening was evaluated with a parametric survival model with diet, mean colony condition and head width as covariates.

To test for differences in ant mortality resulting from cold shock (i.e. not including earlier mortality during feeding) between diet treatments and exposures, an ordinal logistic regression was performed with diet, exposure and their interaction as fixed effects and condition and head width as covariates.

For the chronic cold treatment, because mortality was measured over time, a standard least squares model was used to examine survival by diet over the two-week time period at times 0, 1 week, and 2 weeks. The fixed effects for this model included diet, time point, and the interaction between diet and time point, with head width and condition included as covariates.

For metabolite comparison, univariate ANOVA was performed on positive and negative ion data by SECIM to determine significant metabolites. PLS-DA was used to visualize expression pattern differences between groups and significant compounds. To determine whether detected differences also existed at the level of cold treatment, volcano plots for the RCH and acclimation treatments were used to identify compounds that met a  $p < 0.05$  significance threshold and had at least a two-fold increase or decrease relative to the RT control. Heat maps were used to visualize overall changes in the top 50 most significant metabolites between the three treatments.

## **Results**

### Ant Mortality:

Diet significantly affected mortality, which was recorded during and after exposures to the cold-water bath (Logistic regression, main effect of diet,  $\chi^2_2 = 36.53$ ,  $p < 0.05$ ; Fig. 4).

Mortality increased significantly with ant body size (Logistic regression, main effect of body

size,  $\chi^2_1 = 27.45$ ,  $p < 0.05$ ; Fig. 5), although colony condition had no effect on mortality (Logistic regression, main effect of colony condition,  $\chi^2_1 = 1.740$ ,  $p = 0.19$ ), nor did whether ants were exposed to hardening conditions or an acute cold shock (Logistic regression, main effect of exposure,  $\chi^2_1 = 1.08e^{-9}$ ,  $p = 1.00$ ). There was also a tendency of ants on the no sugar diet to die more often under the acute exposure than ants on the sugar diets, although this trend was ultimately not significant and there was no interaction effect (Logistic regression, main effect of exposure\*diet,  $\chi^2_2 = 4.287$ ,  $p = 0.12$ ; Fig. 4).

During the chronic cold exposure mortality significantly increased over time and percent survival was between 70.7% and 87.3% for all three diets at the halfway point (ANOVA,  $F_{(2,49)} = 8.753$ ,  $p < 0.05$ ; Fig. 6). By the end of two weeks, the glucose and no sugar diets had 65.1% and 66% survival respectively, while survival for ants on the trehalose diet remained high at 85.2%; however, there was no effect of diet on survival (ANOVA,  $F_{(2,49)} = 1.37$ ,  $p = 0.26$ ), nor a significant interaction between diet and time (ANOVA,  $F_{(4,49)} = 1.951$ ,  $p = 0.70$ )

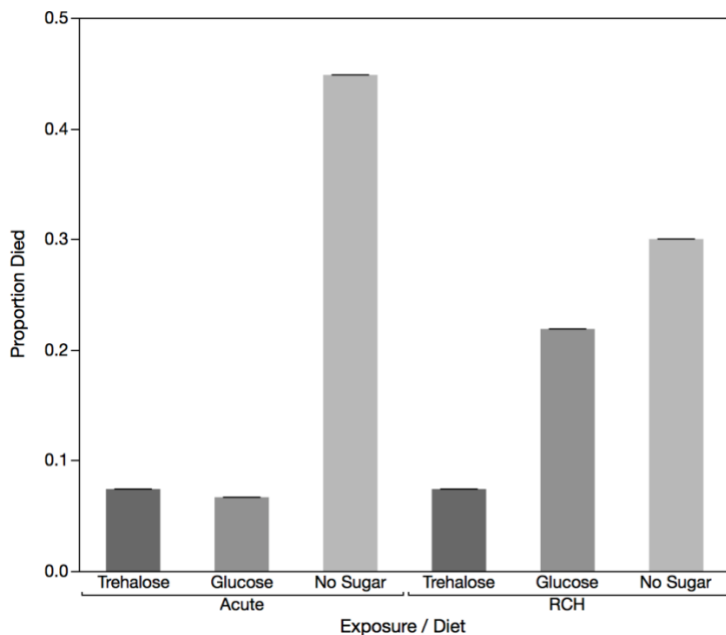


Figure 4. Bar graph showing the proportion of ants that died for each diet and acute and RCH exposures (n = 175). Diet significantly affected mortality (Logistic regression, main effect of diet,  $\chi^2_2 = 36.53$ ,  $p < 0.05$ ) but exposure type had no effect (Logistic regression, main effect of exposure,  $\chi^2_1 = 1.08e^{-9}$ ,  $p = 1.00$ ) and there was no interaction between exposure and diet (Logistic regression, main effect of exposure\*diet,  $\chi^2_2 = 4.287$ ,  $p = 0.12$ ).

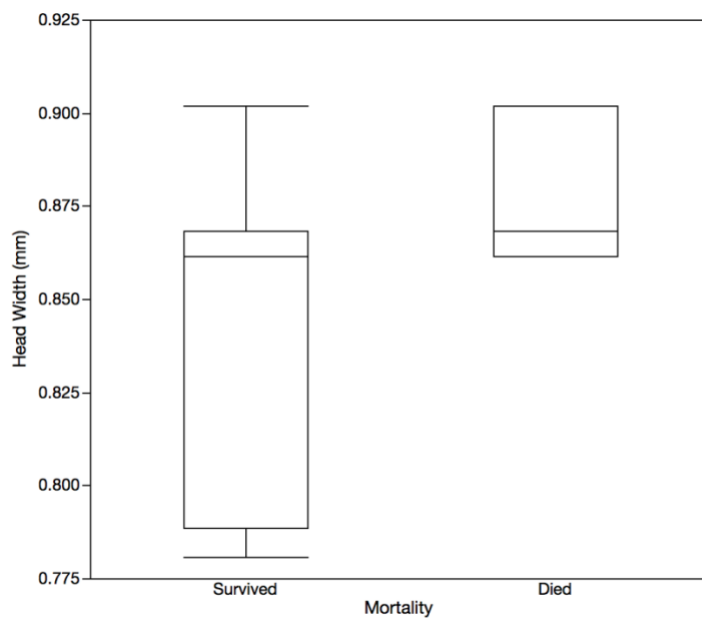


Figure 5. Box plot showing ant mortality during the cold shock procedures of the feeding experiment (n = 180). Larger ants died more often (Logistic regression, main effect of body size,  $\chi^2_1 = 27.45$ ,  $p < 0.05$ ).

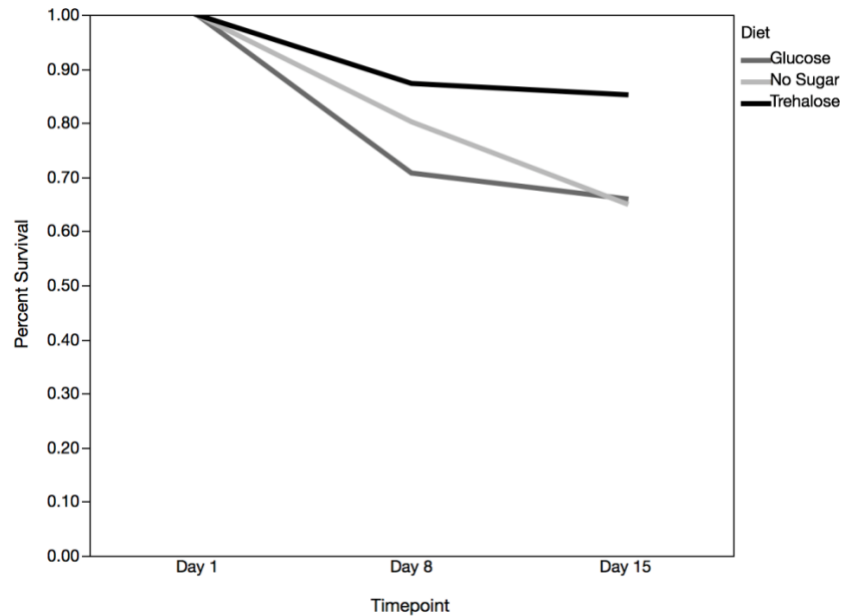


Figure 6. Ant survival during chronic cold exposure as a function of diet. Ant survival was recorded at the start of the two-week chronic cold treatment (Day 1; 4 °C), halfway through treatment (Day 8; 4 °C), and at the end of treatment (Day 15; 4 °C). Diet had no effect on chronic cold survival (ANOVA,  $F_{(2,49)} = 1.37$ ,  $p = 0.26$ ); data analyzed with a standard least squares model.

#### Factors Affecting Recovery Time (CCRT):

Diet significantly affected CCRT (ANOVA,  $F_{(2,123)} = 5.188$ ,  $p < 0.05$ ; Fig. 7). Ants on the trehalose and glucose diets recovered 16% and 12.6% faster respectively than ants on the no sugar diet (post-hoc Tukey HSD tests, both  $p < 0.05$ ). Exposure type also directly affected CCRT, with RCH ants on average recovering 30.2%, more than two minutes, faster than acute cold shock ants (ANOVA,  $F_{(1,123)} = 69.93$ ,  $p < 0.05$ ; Fig. 8). There was no significant interaction effect between diet and exposure type on recovery time (ANOVA,  $F_{(2,123)} = 0.6320$ ,  $p = 0.53$ ; Fig. 9). There was a weakly positive relationship between body size and recovery time

(ANOVA,  $F_{(1,123)} = 3.681$ ,  $p = 0.0573$ ; Fig. 10) but colony condition had no main effect on recovery time (ANOVA,  $F_{(1,123)} = 2.651$ ,  $p = 0.10$ ; Fig. 11). There was no significant interaction effect between body size and exposure type (ANOVA,  $F_{(1,123)} = 1.025$ ,  $p = 0.31$ ), however there was an interaction between colony condition and exposure type (ANOVA,  $F_{(1,123)} = 5.797$ ,  $p < 0.05$ ; Fig. 11), where colonies in better condition recovered faster under acute cold shock but slower under RCH.

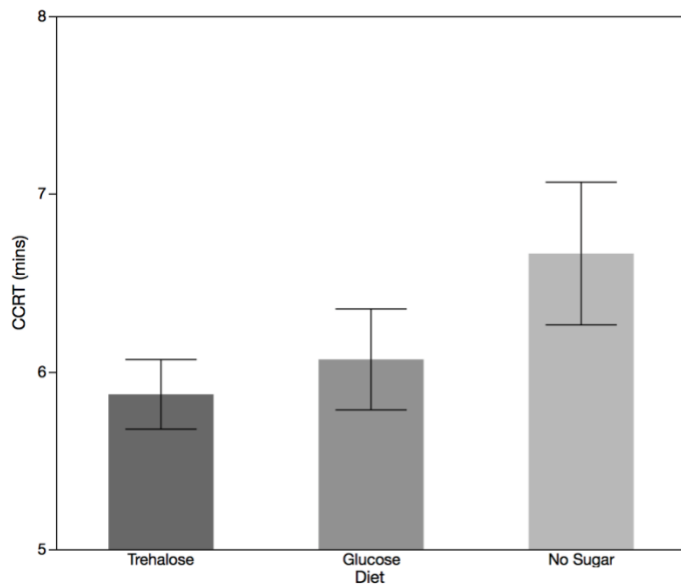


Figure 7. Bar graph (mean  $\pm$  SEM) of recovery time by diet ( $n = 180$ ). Ants on the sugar diets recovered quicker than ants on the no sugar diet (post-hoc Tukey HSD tests, both  $p < 0.05$ ). Data analyzed with a standard least squares model.

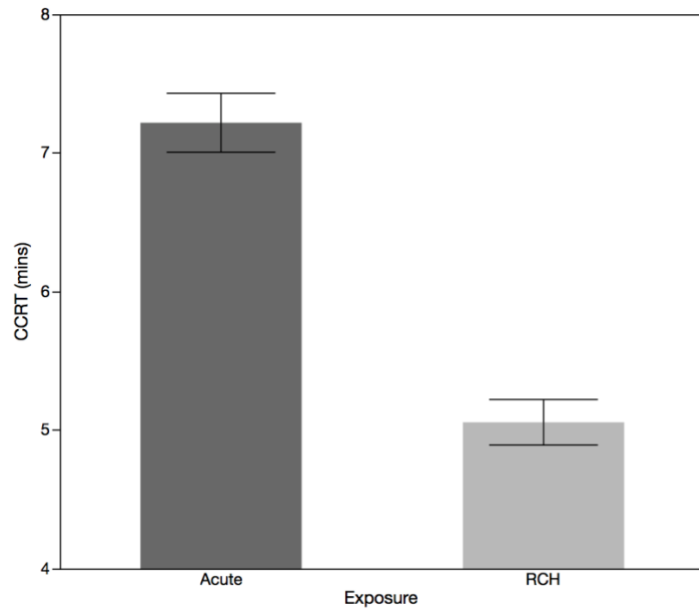


Figure 8. Bar graph (mean  $\pm$  SEM) showing the effects of acute cold shock and RCH on recovery time ( $n = 180$ ). Ants hardened successfully (ANOVA,  $F_{(1,123)} = 69.93$ ,  $p < 0.05$ ). Data analyzed with a standard least squares model.

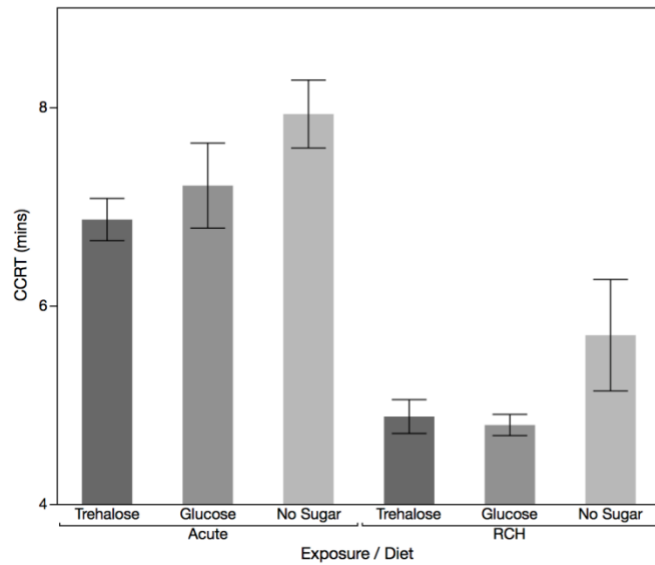


Figure 9. Bar graph (mean +/- SEM) showing the combined effects of exposure and diet on recovery time (n = 180). Sugar did not enhance hardening response (ANOVA,  $F_{(2,123)} = 0.6320$ ,  $p = 0.53$ ). Data analyzed with a standard least squares model.

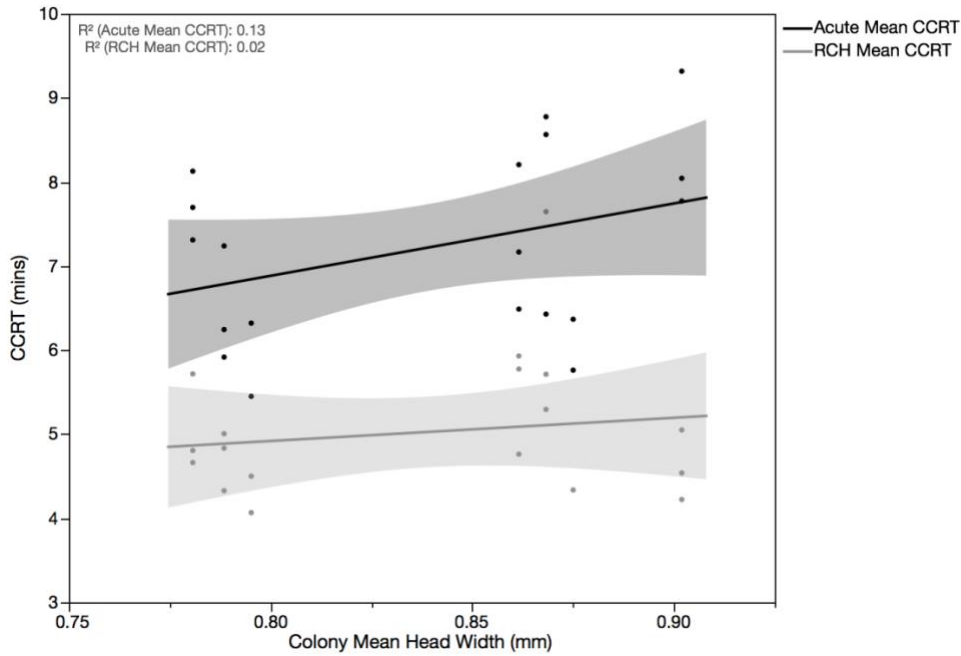


Figure 10. Lines of best fit for colony head width (mm) and CCRT (mins) by exposure type (n = 19). There was a weakly positive relationship between body size and recovery time (ANOVA,  $F_{(1,123)} = 3.681$ ,  $p = 0.0573$ ). Data analyzed with a standard least squares model.

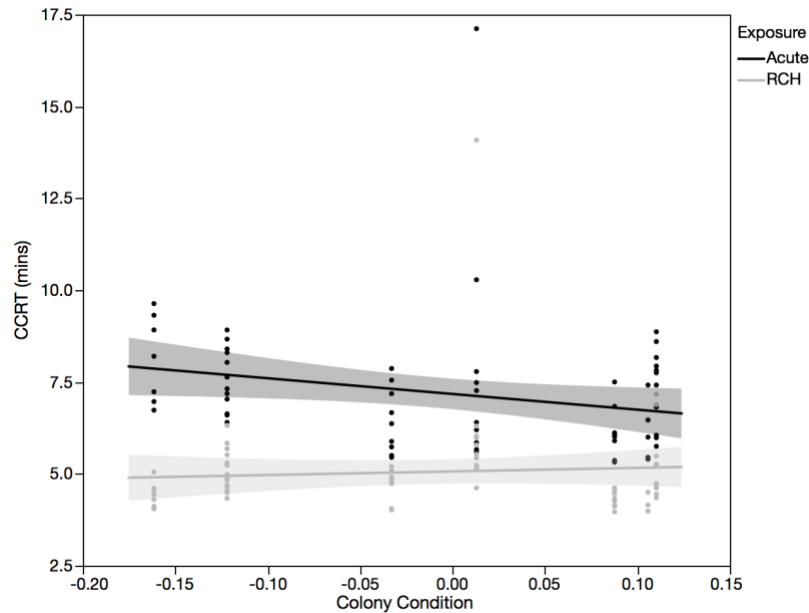


Figure 11. Lines of best fit for colony condition and recovery time (mins) by exposure type ( $n = 19$ ). Colony condition had no effect on recovery time (ANOVA,  $F_{(1,123)} = 2.651$ ,  $p = 0.10$ ), however colony condition did affect recovery time between exposure types (ANOVA,  $F_{(1,123)} = 5.797$ ,  $p < 0.05$ ). Data was analyzed with a standard least squares model.

### Cold Hardening Results:

Colony condition was the only factor that significantly affected net hardening (ANOVA,  $F_{(1,14)} = 15.36$ ,  $p < 0.05$ ) and proportional hardening (ANOVA,  $F_{(1,4)} = 7.871$ ,  $p < 0.05$ ). Colonies in better condition hardened less (Fig. 12). There was no correlation between ant size and proportional hardening (ANOVA,  $F_{(1,4)} = 1.266$ ,  $p = 0.26$ ; Fig. 13). Overall, there was no effect of diet on net hardening ability (ANOVA,  $F_{(2,14)} = 1.576$ ,  $p = 0.24$ ) or proportional hardening (ANOVA,  $F_{(2,4)} = 0.955$ ,  $p = 0.62$ ). While the no sugar ants did show the greatest absolute improvement in recovery time, there were no significant differences in either proportional hardening or net hardening between diets (Fig. 14, 15).

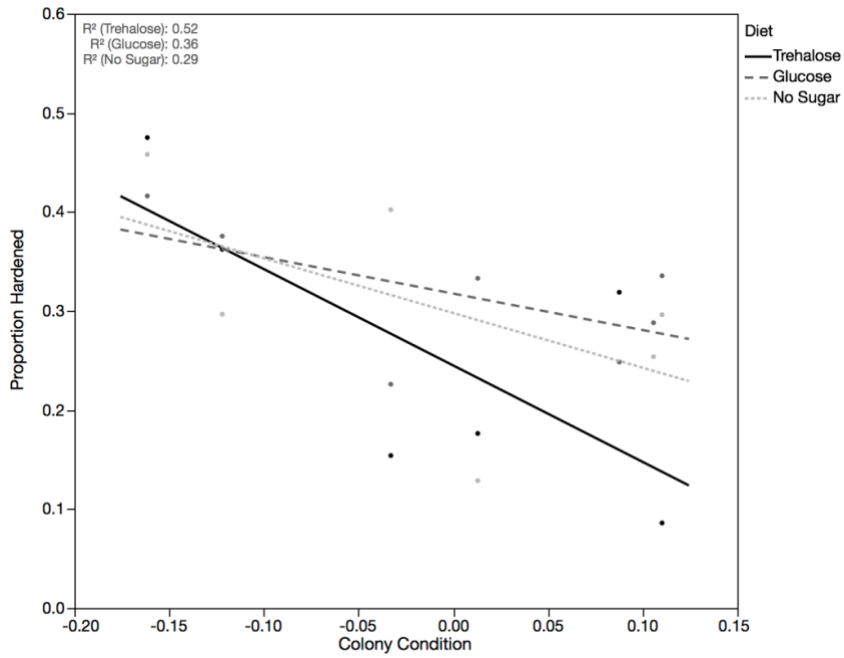


Figure 12. Trend line for proportion hardened as a function of colony condition ( $n = 19$ ). Each line represents one of the diet treatments; colonies in better condition hardened less (ANOVA,  $F_{(1,4)} = 7.871$ ,  $p < 0.05$ ). Data analyzed with a parametric survival model.

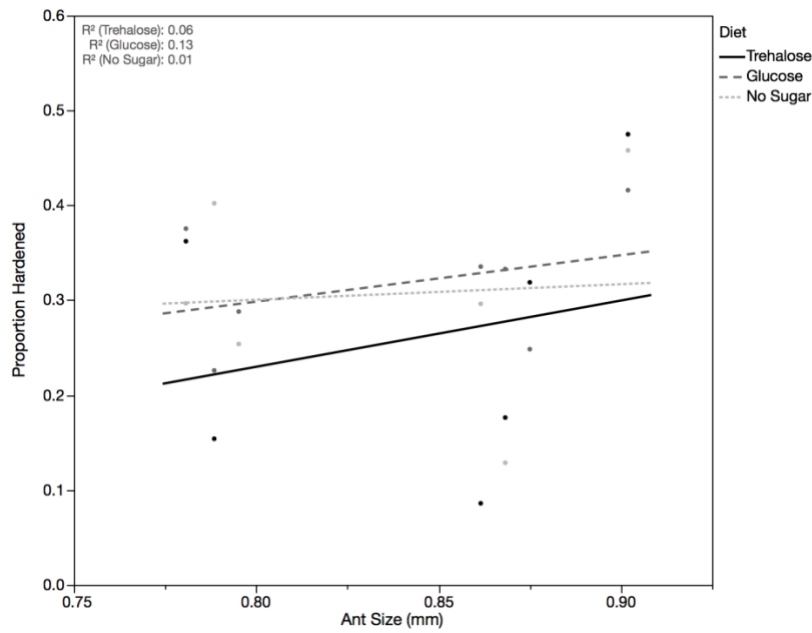


Figure 13. Trend lines for proportional hardening as a function of mean ant size (n = 19). Each line represents one of the diet treatments; ant size had no effect on hardening ability (ANOVA,  $F_{(1,4)} = 1.266$ ,  $p = 0.26$ ). Data analyzed with a parametric survival model.

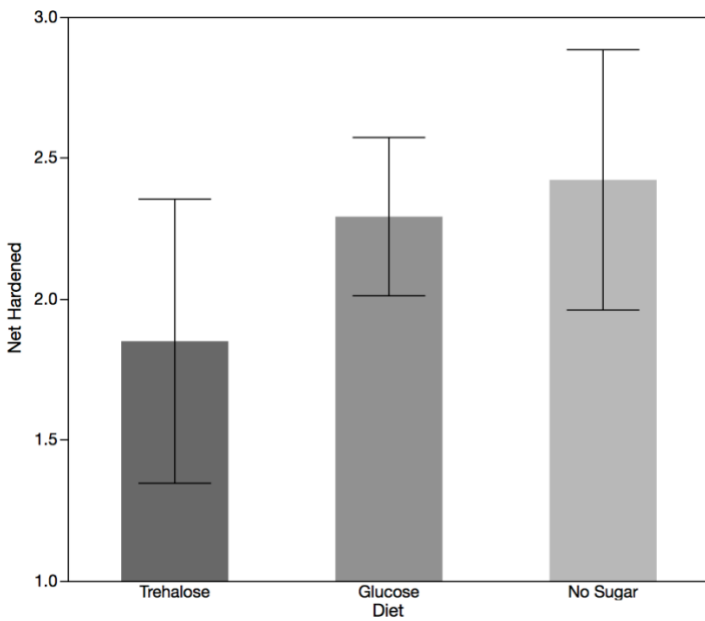


Figure 14. Bar graph (+/- SEM) of net hardening by diet (n = 19). There were no significant differences in net hardening ability between diets (ANOVA,  $F_{(2,14)} = 1.576$ ,  $p = 0.24$ ). Data analyzed with a standard least squares model.

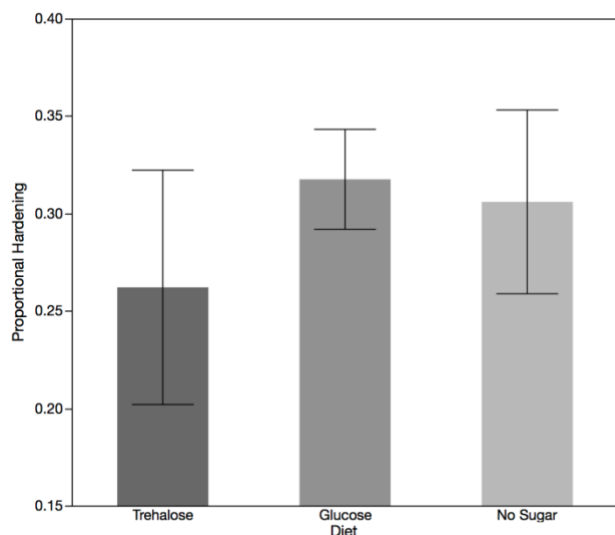


Figure 15. Bar graph (+/- SEM) of proportional hardening by diet (n = 19). There were no significant differences in proportional hardening ability between diets (ANOVA,  $F_{(2,4)} = 0.955$ ,  $p = 0.62$ ). Data analyzed with a parametric survival model.

#### Internal Metabolite Comparison:

When considering the entire metabolomic profile, samples from similar treatments clustered together, and between treatments, the RT and RCH samples were more similar to each other than to the acclimation treatment samples (Fig. 16).

Metabolites clustered into three groups with similar abundance profiles (Fig. 16). The first group, which contained 13 metabolites, was found in higher amounts during acclimation, and found in lower amounts during RCH and RT. The second group, which contained three metabolites, was found in higher amounts during RCH and lower amounts during acclimation and RT. The third group was found in higher amounts during RCH and RT and lower amounts during acclimation. Ants that underwent acclimation had lower than average amounts of metabolites compared to RCH and RT. This includes smaller amounts of stachyose and 2-aminoethyl dihydrogen phosphate, which appeared in larger than average amounts under both

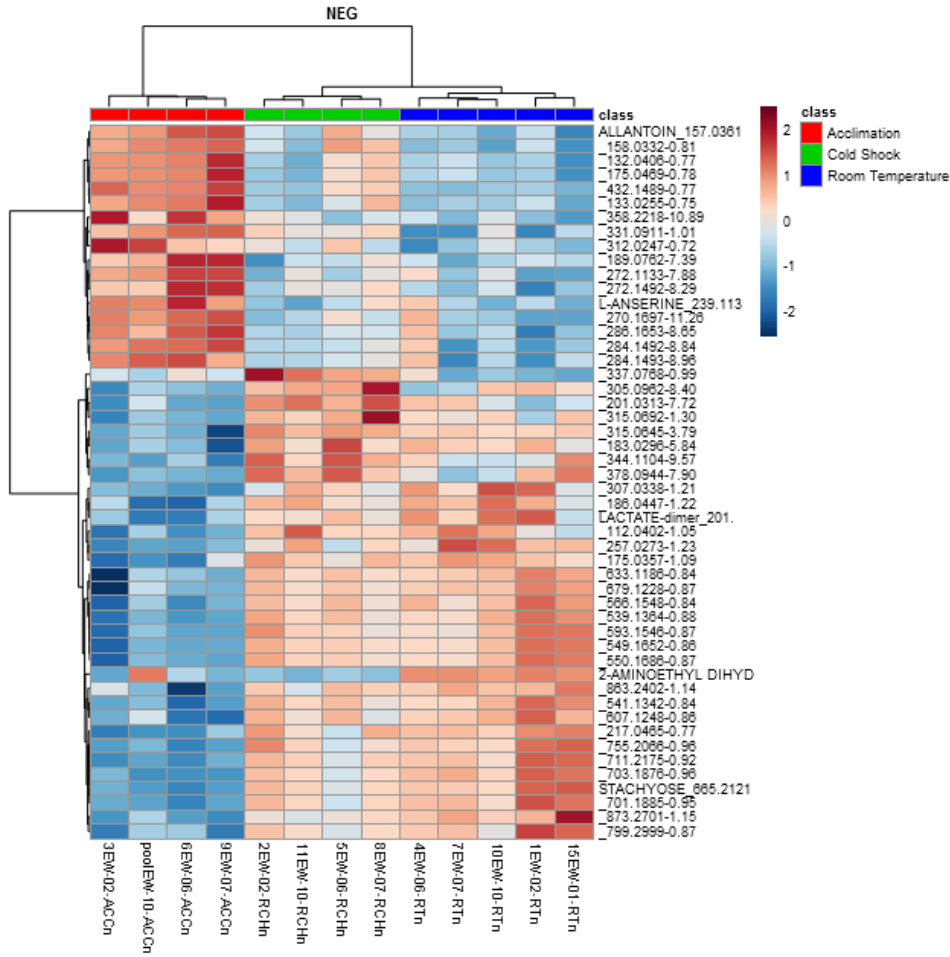
RCH and RT. The opposite was true for Allantoin and L-Anserine, which were found relatively more in the acclimation treatment and less in both the RT and RCH treatments.

Out of the 507 metabolites that showed up in significantly different amounts between groups (ANOVA,  $p < 0.05$ ; Table 1), 19 were identified. Table 2 includes the compound name,  $p$  value and false discovery rate correction for the  $p$  value of these 19 metabolites (ANOVA,  $p < 0.05$ ). After the FDR correction, only two metabolites, stachyose and allantoin, were still significant (Table 2). The VIP scores from the PLS-DA showed that allantoin was highly expressed in the acclimation ants with low expression in the room temperature (RT) ants, while stachyose was the opposite, with low expression in acclimation ants and high expression in RT ants (Fig. 17), although with multiple permutations the overall PLS-DA model was not significant.

At the level of cold treatment, metabolites that changed significantly in comparison to control with at least a 2-fold difference and a  $p$ -value  $< 0.05$ , included five metabolites that increased in the acclimation treatment and two metabolites that increased in the RCH treatment (Table 3, Fig. 18). This includes allantoin, which was the only metabolite to increase under both RCH and acclimation (Table 3). Other metabolites that increased under acclimation include L-anserine, 4-guanidinobutanoate, 4-imidazoleacetic acid, and proline, while L-cystathionine was the only other metabolite to increase under RCH (Table 3).

In contrast, 10 metabolites decreased under acclimation, including stachyose, raffinose, and glycerol 3-phosphate, while only one metabolite, 2-Aminoethyl Dihydrogen Phosphate,

decreased under RCH (Table 4; Fig. 19)



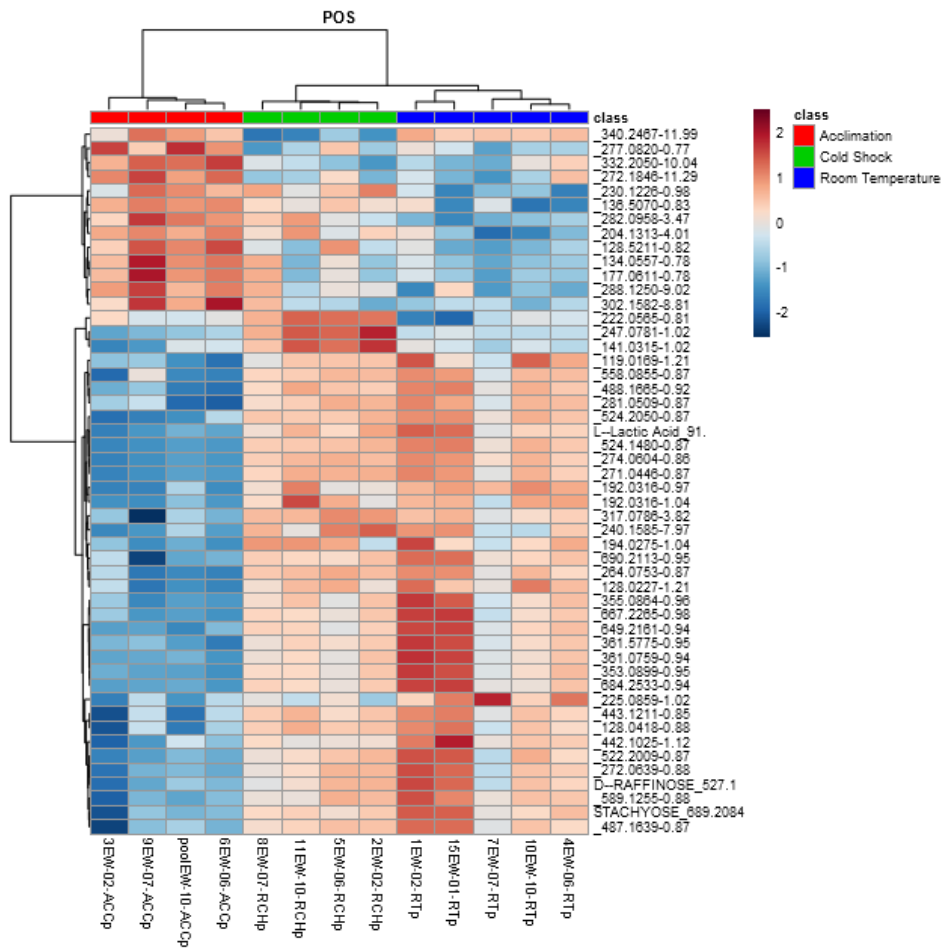


Figure 16. Heat maps from the top 50 from ANOVA for both positive and negative modes showing amounts of metabolites relative to the mean. Samples from similar treatments clustered together, and between treatments, the RT and RCH samples were more similar to each other than to the acclimation treatment samples.

Table 1. Total numbers of identified and unknown significant metabolites, with p value < 0.05 analyzed by ANOVA.

Data Set	Significant metabolites (p-value <0.05)		
	Identified Metabolites	Unknown Metabolites	Total metabolites
Positive Mode	4	155	159
Negative Mode	15	333	348

Table 2. Significant known metabolites from ANOVA in the positive and negative mode data sets. Significant p values are in bold.

Identity of Metabolite (Positive Mode)	False Discovery Rate Correction (Corrected P value)	P value
L--lactic acid	0.07239	<b>0.00019937</b>
Stachyose	0.15329	<b>0.00084706</b>
D--raffinose	0.22416	<b>0.0028398</b>
Allantoin	0.33815	<b>0.0054014</b>

<b>Identity of Metabolite (negative mode)</b>	<b>FDR</b>	<b>P value</b>
Stachyose	<b>0.015078</b>	<b>5.0153E-05</b>
Allantoin	<b>0.045813</b>	<b>0.00046648</b>
L-anserine	0.080644	<b>0.0015146</b>
LACTATE-dimer	0.087381	<b>0.0020505</b>
2-aminoethyl dihydrogen phosphate	0.087381	<b>0.0022178</b>
C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> -disaccharide- 6C/6C glc-glc/glc-frc/gal- glc_	0.14436	<b>0.006595</b>
D-ribose 5- phosphate/ribulose 5- phosphate	0.14851	<b>0.0069589</b>
Homocysteine	0.15109	<b>0.007776</b>
Proline	0.17269	<b>0.010524</b>
C5-sugar alcohol	0.17269	<b>0.010694</b>
4-guanidinobutanoate	0.19327	<b>0.014892</b>
Urate	0.21797	<b>0.02058</b>
L-cystathionine	0.22964	<b>0.02334</b>
Mannitol	0.25295	<b>0.033166</b>
Tartaric acid	0.26005	<b>0.040044</b>

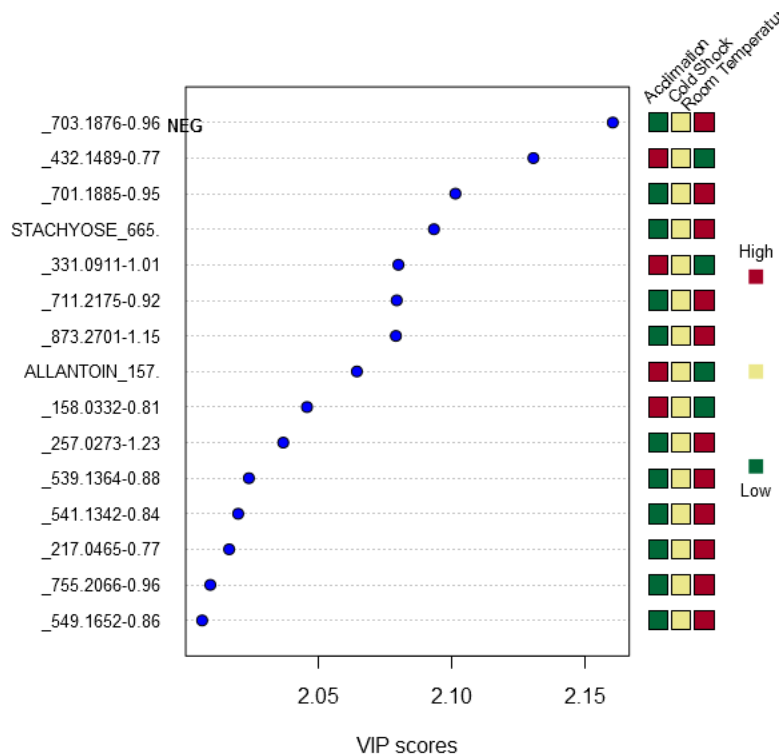


Figure 17. The top 15 features from PLS-DA. Typically, a VIP of 2 or greater is highly significant. Red indicates high expression and green indicates low expression.

Table 3. Significant metabolites in the acclimation and RCH treatments that had positive fold changes. These metabolites, from the volcano plots for the acclimation and RCH treatments, met a  $p < 0.05$  significance threshold with at least a two-fold increase compared to room temperature (T Test,  $p < 0.05$ ).

Acclimation	Fold change	P Value
L-anserine	3.768	0.0254
Allantoin	2.407	0.0123

Allantoin	2.145	0.0138
4-guanidinobutanoate	1.371	0.0311
4-imidazoleacetic acid	1.367	0.0337
4-guanidinobutanoate	1.114	0.0396
Proline	1.076	0.0253
<b>Rapid Cold Hardening</b>	<b>Fold change</b>	<b>P Value</b>
L-Cystathionine	1.360	0.0373
Allantoin	1.120	0.0399

Table 4. Significant metabolites in the acclimation and RCH treatments that had negative fold changes. These metabolites, from the volcano plots for the acclimation and RCH treatments, met a  $p < 0.05$  significance threshold with at least a two-fold decrease compared to room temperature (T Test,  $p < 0.05$ ).

<b>Acclimation</b>	<b>Fold change</b>	<b>P Value</b>
Stachyose	-3.448	0.0217
Stachyose	-2.914	0.0469
D-(+)-Raffinose	-2.629	0.0153
Lactate-dimer	-1.714	0.0351
Sn-Glycerol 3-Phosphate	-1.633	0.0043
Gluconic Acid/D-Gulonic acid Gama-Lactone	-1.527	0.0422
Lactose+K	-1.349	0.0110

Glycerol 2-Phosphate/SN-Glycerol 3-Phosphate	-1.341	0.0210
Disaccharide (6c/6c)/2-alpha-D-Glucosyl-D-Glucose	-1.236	0.0144
C5-sugar alcohol	-1.232	0.0113
Lactate	-1.087	0.0284
L-2-Phosphoglyceric acid	-1.100	0.0468
<b>Rapid Cold Hardening</b>	<b>Fold change</b>	<b>P Value</b>
2-Aminoethyl Dihydrogen Phosphate	-3.629	0.0004

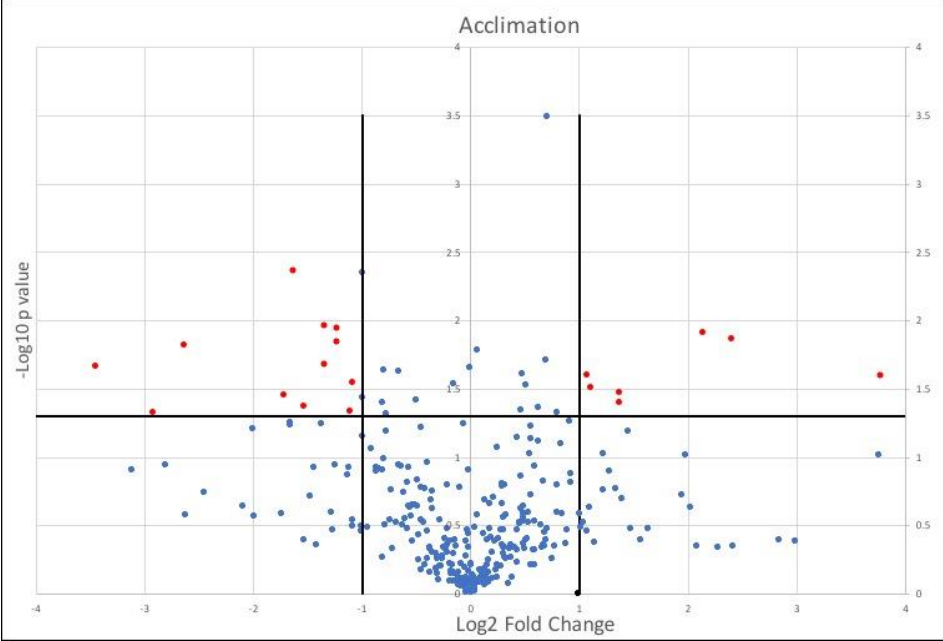


Figure 18. Volcano plot of  $-\text{Log}_{10}$  p value by  $\text{Log}_2$  fold change for the acclimation treatment. Student's T-Test used for  $p < 0.05$  significance threshold (horizontal line); fold change of at least 2 represented by vertical lines. Red points are significant.

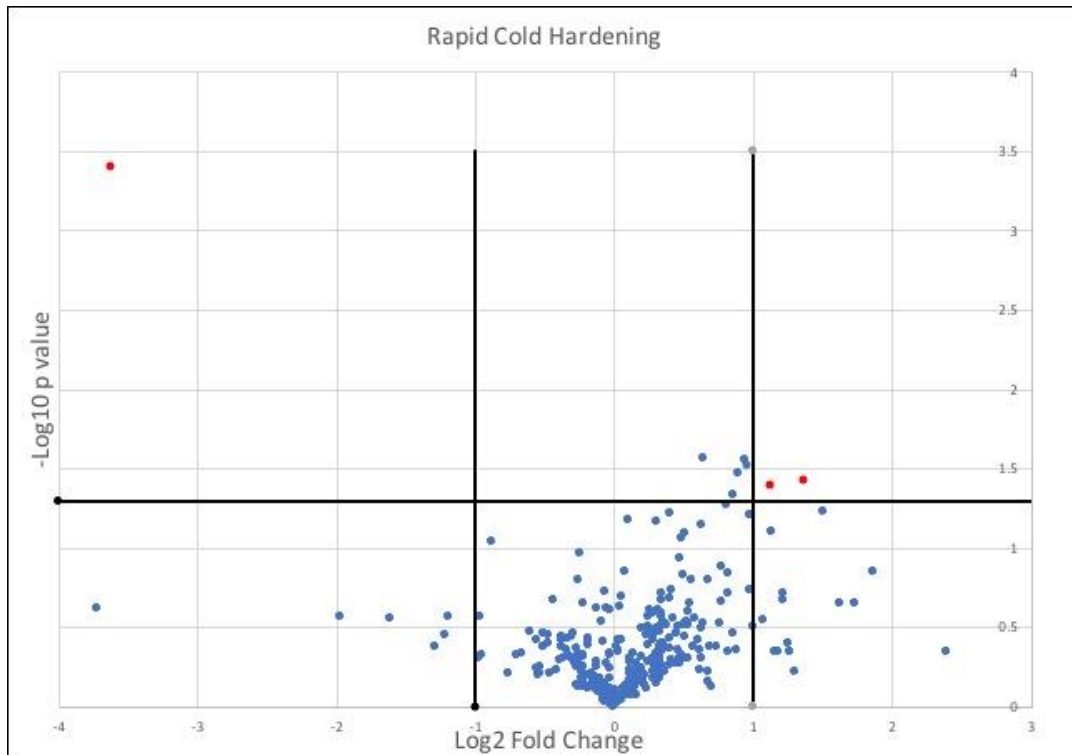


Figure 19. Volcano plot of  $-\text{Log}_{10}$  p value by  $\text{Log}_2$  fold change for the RCH treatment. Student's T-Test used for  $p < 0.05$  significance threshold (horizontal line); fold change of at least 2 represented by vertical lines. Red points are significant.

## Discussion

Ants living in temperate habitats such as northeastern forests experience a wide range of temperatures (Modlmeier, Pamminer, Foitzik, & Scharf, 2012). I studied whether *A. picea* resists cold by producing trehalose, derived from sugars present in their diet. The results of this study indicate that although having access to dietary sugars enhances baseline cold tolerance, trehalose is not used as a cryoprotectant in *A. picea*. It is possible that other forms of sugar circulating in the hemolymph are used to confer baseline cryoprotection, or they may enhance tolerance through their use as an energy source to maintain homeostasis (Rockstein, 2012).

Instead, workers appear to use two overlapping strategies to overcome seasonal cooling and acute cold shocks. During acclimation, the metabolomics profile indicated that the amino acid proline may serve as a cryoprotectant. Under both rapid cold hardening and acclimation, there was also an increase in metabolites associated with scavenging of damaging reactive oxygen species, including allantoin, an intermediate in uric acid metabolism known to confer stress resistance in plants (Chambers, Song, & Schneider, 2012; Meng et al., 2017; Watanabe et al., 2014).

It is well supported in the literature that low molecular weight sugar alcohols and disaccharides, and even oligosaccharides, can act as cryoprotectants and/or are correlated with cold tolerance in a variety of insect and plant species (Sengupta, Mukherjee, Basak, & Majumder, 2015; Teets & Denlinger, 2013). Many insects, including the ant *Solenopsis invicta*, use trehalose as the main circulating blood sugar instead of glucose because of its higher energy potential and lower chemical reactivity (Schilman & Roces, 2008), and trehalose also functions as a cryoprotectant in a number of insect taxa. Contrary to expectation, however, trehalose did not appear in the metabolomics profile under any conditions, suggesting that this species uses another sugar, such as glucose, as its primary circulatory molecule, and thus trehalose would be unavailable to act as a baseline cryoprotectant. Moreover, neither the sugars nor sugar alcohols detected in these ants increased in response to the cold exposures used in this study, suggesting that they may not utilize sugars for induced cryoprotection during cold hardening or long-term seasonal cold exposure.. Sugars that might have been expected to increase and act in a cryoprotectant capacity, including an unidentified disaccharide, stachyose, D-raffinose and a C5-sugar alcohol, actually decreased during the acclimation treatment. The decrease of these

metabolites without a concomitant increase in the amounts of their monomers suggests that they may be broken down to be used as an energy source. Energy resources could be especially important for preventing direct chilling injury because ants on the no sugar diet, which had fewer resources to repurpose for responding to the cold, had higher mortality during the RCH and acute cold shock. This does not necessarily exclude sugar from passively playing a protective role during cold shock conditions, however. Workers provided with sugar resources, either glucose or trehalose, displayed increased baseline cold tolerance but no increase in the amount of cold hardening. This could be directly caused by cryoprotective effects provided by higher baseline concentrations of circulating sugars derived from the diet, such as by directly stabilizing and protecting cell membranes or by reducing extracellular ice formation (Denlinger & Lee, Jr., 2010; Duman, 2002; Olsson et al., 2016). Sugars could also provide a non-colligative benefit; more cold-tolerant species tend to have higher levels of baseline osmolytes, and research on *Drosophila* has shown that this can help to increase cold tolerance because it can help to protect membranes and also assists in maintaining osmotic balance across membranes when impaired Na<sup>+</sup>/K<sup>+</sup> ion pump function under cold conditions leads to ion leakage (Olsson et al., 2016).

While ants with access to sugar resources had better recovery times, ants in better condition had lower hardening, suggesting that it was the sugar itself providing a benefit, and not its use as a potential fuel source. Ants in better condition would presumably have had plenty of resources to utilize as fuel, but these resources were in the form of glycogen or lipids rather than free sugars. The interaction effect between colony condition and exposure type, whereby colonies in better condition had improved recovery times under acute cold shock but not under RCH, suggests that when these stored resources (glycogen or lipids) are used, for example for

fuel, they are more beneficial during an acute exposure than for use during RCH. To examine whether or not sugars are providing some cryoprotective benefit to RCH ants, a hemolymph ice nucleating activity test (Li, 2016) could be performed to compare the freezing point of extracts from the hemolymphs of RCH, acclimatized and control ants to determine if the levels of sugar present in the RCH are sufficient to lower the freezing point of the fluids. To examine more generally whether or not sugars are providing some protective benefits to *A. picea*, hemolymph osmolyte levels between *A. picea* and a less cold tolerant species such as *A. rudis* could be compared before and after cold shocks, with the expectation that the more cold tolerant *A. picea* would have higher levels of non-ion osmolytes initially and also a higher relative increase in osmolality after cold shock due to less dependence on  $[Na^+]$  to maintain hemolymph osmolality (Olsson et al., 2016).

While many metabolites extracted from these ants remain unidentified, out of the identified metabolites, the amino acid proline appears to be the most likely candidate for acting as a major cryoprotectant molecule in *A. picea* during acclimatization. Proline increased under the acclimation treatment, and there is correlative (Feng et al., 2016; Fields et al., 1998; Wang, Kong, Sun, & Xu, 2012) and experimental evidence (Košťál, Šimek, Zahradníčková, Cimlová, & Štětina, 2012) that it acts as a cryoprotectant in both insects and plants. Proline is part of the arginine pathway, as is 4-guanidinobutanoate, which also increased in these ants during acclimatization. Under cold conditions, proline is thought to act as a cryoprotectant by stabilizing proteins and cell membranes, or by being used for metabolism in mitochondria (Košťál et al., 2011, 2012; MacMillan et al., 2016).

The extent of the cold response depends on the severity of the cold exposure, which has direct impacts on organism function. A major consequence of cold exposure is the loss of membrane functions, which can result in the membrane breaking apart and leakage of ions from the intra- to extra cellular space and vice versa (Overgaard, 2007). Without ion homeostasis, the potential for injury is increased and membrane excitability is impaired, causing chill coma (MacMillan & Sinclair, 2011; Olsson et al., 2016). To prevent these types of injury, insects must compensate according to the type of cold stress that they are exposed to. It has previously been noted in *Drosophila* that the changes occurring during the hardening response are less extreme than those made during acclimation (Overgaard, 2007), which could be the result of mechanical constraints on gene expression or be due to different priorities of response under different cold conditions. During RCH, preventing direct-chilling injury in the short term is the priority, while under acclimation preventing harmful cellular changes over a longer time period is the focus (Denlinger & Lee, Jr., 2010). RCH relies on rapid modifications, such as increased metabolism and in some cases cryoprotectant accumulation, to prevent immediate injury and are favored over longer-term solutions (Denlinger & Lee, Jr., 2010; Teets & Denlinger, 2013). Unlike during acclimation, however, where major changes in gene expression have been observed, the timescale that RCH occurs on is also often too short for large changes in gene expression to occur, which constrains the types of mechanisms possible (Teets & Denlinger, 2013). This may explain why proline increased only under acclimation and not during RCH; changes in proline metabolism may require changes in gene expression of rate-limiting enzymes (MacMillan et al., 2016). An experiment on *Drosophila* has also showed that proline only accumulates in acclimated, rather than hardened, individuals (Košťál et al., 2011). RCH and acclimation both involve a response to oxidative stress, however, during acclimation the downregulation of

metabolism results in the production of fewer damaging reactive oxygen species overall (Teets & Denlinger, 2013). This, combined with the extended time period, may have allowed energy resources in acclimating ants to be directed towards preventing further freezing injury, such as through the increased synthesis of proline.

In addition to nutritional status, cold tolerance of *A. picea* workers was size-dependent, with larger workers more likely to die and displaying slower recovery time from cold shock (Fig. 10). Some studies have found that ant body size, specifically in *Leptothorax acervorum*, follows Bergmann's Rule, where larger organisms are found at higher latitude and altitude in cold environments (Bernadou, Romermann, Gratiashvili, & Heinze, 2016; Chown & Gaston, 2010), while others have found contradictory results that in ant species in general there is no relationship between ant body size or colony size and latitude or altitude (Geraghty, Dunn, & Sanders, 2007). Collections across a latitudinal transect indicate that there is no relationship between body size and latitude in *A. picea* (K. Miller, unpublished data), even though larger size is hypothesized to provide starvation resistance (Blackburn, Gaston, & Loder, 2008). Larger ant size may actually be detrimental, because a study on *Solenopsis invicta* fire ants showed that larger individuals had higher super cooling points than smaller individuals (Hahn, Martin & Porter, 2008), which could explain the longer recovery times observed in this experiment, and which might also increase mortality. Our results support this hypothesis. If the biophysical relationship between size and cold tolerance is a general feature of insects, it suggests a trade-off for insects following Bergmann's Rule, which may have improved starvation resistance during cold seasons yet are less capable of recovering from severe cold stress.

Changes in metabolism and energy conservation have been associated with cold stress, since energy stores may be consumed to maintain homeostasis or help prevent or repair chilling injuries (Bale & Hayward, 2010; Churchill & Storey, 1989; Denlinger & Lee, Jr., 2010; Jozefczuk et al., 2010). There have been sometimes contradictory reports about the effects of cold exposure on energy reserves in insects, because the physiological response is often both species and temperature specific (Marshall & Sinclair, 2012). RCH and acclimation can work both independently and in concert and they are likely driven by different mechanisms (Michaud & Denlinger, 2007; Overgaard, 2007). In this species, the acclimation response appears to involve down-regulating metabolism over a longer time period to maintain homeostasis and cell membrane integrity, while RCH is a short term increase in freeze tolerance that did not affect as many metabolites (Denlinger & Lee, Jr., 2010; MacMillan et al., 2016; Michaud & Denlinger, 2007)(Table 3, 4). In the acclimation treatment, ants may have prioritized breaking down larger sugar molecules and lactate during the acclimation treatment to maintain glucose levels since they did not have access to food resources (Rockstein, 2012). The decreases in intermediate metabolites of glycolysis with no overall change in glucose levels, including glycerol-3-phosphate, gluconic acid, and phosphoglyceric acid, indicate that ants were either directing fewer resources towards glycolysis, that the glycolysis intermediates may have been used in other pathways, or some combination thereof. For example, one possible reason for the decrease in lactic acid is that the acetyl CoA produced in glycolysis might be directed towards fatty acid synthesis rather than continuation of the glycolysis pathway (Rockstein, 2012). Acclimation has previously been associated with lowered Krebs cycle activity in *Drosophila* (Teets & Denlinger, 2013). While acetyl CoA could be used for other processes within the organism, cell membrane integrity is an essential part of cold resistance, and changes in glycerophospholipid synthesis

have been observed in other acclimating species, such as the carp *Cyprinus carpio* (Brooks et al., 2002). An increase in unsaturated fatty acids would cause a subsequent increase in membrane fluidity, which would allow organisms to counteract the negative effects of cold on their cell membranes (Denlinger & Lee, Jr., 2010). Another possible explanation may be that the ants were using the fats they produced from this process directly for energy (Arrese & Soulages, 2010). L-2-phosphoglyceric acid is also involved in the production of diglycerides and the storage of lipids in the insect fat body, further supporting the idea that lipid synthesis is increased as a result of acclimation (Arrese & Soulages, 2010).

Oxidative stress, when there is an excess of reactive oxygen species (ROS) relative to antioxidants, may be a consequence of cold exposure and can cause damage to cells and DNA (Joanisse & Storey, 1996). One marker of oxidative stress in many organisms, including humans and *Drosophila*, is an increase in the metabolite allantoin, which is the product of a reaction between uric acid and ROS (Chambers et al., 2012; Yardim-Akaydin, Sepici, Özkan, Şimşek, & Sepici, 2006). Increases in allantoin, a heterocyclic nitrogen compound that is an intermediate component of purine metabolism, have also been associated with cold response in both plants and animals (Desautels & Himms-Hagen, 1979; Meng et al., 2017; Watanabe et al., 2014). Allantoin increased under both RCH and acclimation in this experiment, suggesting that ants may induce production of antioxidant molecules in order to deal with oxidative stress. While some studies on plants have suggested that allantoin itself may act as an antioxidant (Brychkova, Alikulov, Fluhr, & Sagi, 2008) and quench the ROS, other studies argue that allantoin does not have antioxidant properties (Takagi et al., 2016; Wang et al., 2012; Watanabe et al., 2014). Allantoin's direct role in stress tolerance, and by extension cold tolerance, has not yet been

studied in insects, but recent studies in mice (da Silva et al., 2018) and Arabidopsis seedlings (Irani & Todd, 2018) have found that it might contribute to stress resistance by protecting aspects of antioxidant pathways or by inducing gene expression in those pathways, suggesting that it could directly contribute to stress tolerance in ants. To determine whether it is uric acid, allantoin, or some combination thereof that is performing the ROS quenching in insects, it could be useful to investigate how altering this biochemical pathway would affect insect ability to handle cold stress. In addition to allantoin, anserine, a molecule which protects DNA from damage and is another marker of oxidative stress, had the largest relative increase overall during acclimation, suggesting that metabolites that play a protective role are important for ants experiencing longer term cold stress (Leinsoo, Abe, & Boldyrev, 2006). While the exact mechanisms have yet to be determined, cystathionine, which increased in these ants under RCH and increased in flesh flies under RCH as well (Michaud & Denlinger, 2007), may also be involved in an antioxidant replenishing pathway, or even in the production of fatty acids that may be involved in lipid metabolism or membrane structure (Arrese & Soulages, 2010; D'Alessandro, Nemkov, Bogren, Martin, & Hansen, 2017).

While metabolomics provides a glimpse into what biochemical changes are occurring as a result of different cold treatments in these ants, the locations of the metabolites within the fluids and tissues of the ants are not known, and the direction of the pathways that metabolites are detected in may also be difficult to determine (Denlinger & Lee, Jr., 2010). For example, a metabolite could change in amounts due to cold temperatures deactivating enzymes up or downstream in the pathway, and a metabolomics profile does not tell why changes are occurring (Denlinger & Lee, Jr., 2010). One limitation of the metabolomics portion of this experiment was

small sample size, which made it difficult to detect differences; in particular, a larger sample size would be more representative of each group and could show clearer differences between metabolites, especially those that were near significance. It is important to take into consideration that many studies have shown fluctuating levels of cryoprotectants, including sugars, polyols and amino acids, on a species by species basis and also under acclimation periods of differing lengths and intensities, or between acclimation and RCH treatments (Bemani, Izadi, Mahdian, Amin samih, & Khani, 2012; Feng et al., 2016; Fields et al., 1998). As such, under a longer-term exposure, exposure at a different temperature range, or exposure mimicking the overwintering period, levels of metabolites, including sugars, may differ.

It is unclear how well these results on the biology of cold tolerance in *A. picea* can be generalized to other ant species or insects due to significant differences in metabolites and cold tolerance among species (Teets & Denlinger, 2013). For example, *A. rudis* has been replacing the more cold-adapted *A. picea* along their southern range, but minimum temperatures determine the range boundary limits of these ants, suggesting that there are significant differences in cold tolerance ability between these two species (Warren & Chick, 2013). Further investigation into these differences, in particular under a range of fluctuating temperatures that would more accurately represent the complex temperature ranges experienced by these ants in nature, could help elucidate what the differences are, and why *A. picea* are biologically better suited to resist cold.

In summary, while there is no evidence that trehalose is directly involved in increasing cold tolerance, *A. picea* may rely on other metabolically-expensive molecules, such as proline, to

handle the cold. The extent and severity of cold stress affect the potential for chill injuries, so different mechanisms are used depending on the cold situation, with less extreme physiological changes occurring during RCH than acclimation. Ants under longer term exposure down-regulate metabolism, break down sugars, and increase ROS scavenging. Ants undergoing shorter term cold shocks, including RCH, rely on initial energy stores in the form of sugar, as well as reducing oxidative stress, and colonies in worse initial condition benefited more from hardening. Larger ants in general could not survive or recover from cold shocks as well as smaller ants. These results provide a basis for understanding how *A. picea* has persisted in Northeastern forests and how different types of cold stress may impact their ability to do so.

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