The Effects of Freezing and Recovery on the Metabolic Rate of the Spring Field Cricket *Gryllus veletis*

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Abstract

In order to survive in temperate regions of the planet, freeze-tolerant insects have adapted to survive a substantial proportion of their body water being converted to ice. The freezing of body water causes physiological damage that likely requires a substantial amount of energy to repair and regain homeostasis. The mechanisms and processes which allow these species to avoid and recover from this damage are not fully understood. One unexplained process resulting from freezing is a burst of CO_2 that has been observed in the spring field cricket (*Gryllus veletis*) and in a few other freeze tolerant species at the onset of freezing. Crickets that were pharmaceutically killed with the insecticide thiacloprid and cyanide still expelled the same burst while freezing, suggesting that it was not a nervous system or metabolic response. We hypothesize that the expulsion is caused by CO_2 buffered in hemolymph being expelled as the hemolymph freezes, and freezing does not induce active responses from *G. veletis*. We also investigated the recovery of these insects from freezing. In order to understand the energetic cost of recovery from freezing, we measured carbon dioxide (CO_2) production in the spring field cricket (*Gryllus veletis*) as a proxy for metabolic rate 24, 48, and 72 hours after freezing. We then compared the metabolic rates of crickets that froze to the rates of crickets that were chilled but did not freeze. We hypothesized that there was a significant metabolic cost to freezing that persists for several days. The metabolic rates of crickets that froze were significantly higher at 24, 48 and 72 hours of recovery relative to crickets that had been chilled. This elevated metabolic rate was not found in crickets that had frozen that subsequently molted successfully. However, frozen crickets had a much lower survival rate than crickets that had been chilled, and often were unable to move on to the next life stage, suggesting that the cost of freezing affected the ability of these insects to undergo the metabolically costly process of molting. Understanding the mechanisms behind freeze tolerance and their cost to freeze tolerant organisms may be critical in predicting how these species will react to changes to their environment.

Key words: *Gryllus veletis*, freeze tolerance, metabolic rate, molting, respirometry, dissolved CO_2.

Introduction

Many insects in polar and temperate regions encounter temperatures low enough to freeze their body fluids while overwintering. Since they are ectotherms, many insects have no way of contending with the challenges associated with freezing and must avoid it. Some behavioral adaptations, such as migration or burrowing to avoid the cold, allow insects to avoid low temperatures entirely. Supercooling, the process of cooling a liquid (in this case hemolymph and other body fluids) to a temperature below its natural freezing point while remaining liquid, is accomplished by some insects with the help of antifreeze proteins. These proteins bind to ice crystals to prevent other water molecules in the body from binding to the crystal and spreading the growth of ice (Sformo *et al*., 2010). Remarkably, some insects have adapted to survive a substantial proportion of their body water being converted to ice and are termed "freeze-tolerant" (Lee, 2010). Being freeze tolerant allows these insects to survive in polar regions year-round, allowing them to fill niches in ecosystems where other ectotherms would not be able to survive. As it has evolved independently many times, there are many mechanisms that confer freeze tolerance (Toxopeus and Sinclair, 2018). Because of this variation, much is not understood about freeze tolerance and the toll recovery takes on many of these organisms.

Freeze tolerant organisms, while adapted to survive freezing, are not immune to the physiological effects of low temperatures. Low temperatures can cause macromolecules to become less flexible and can become irreversibly damaged, such as proteins denaturing (Dias *et al.,* 2010). This can lead to issues with transcription and translation, as well as disrupting the stability of cells by harming cytoskeletal polymers (Des Marteaux *et al.*, 2018). Membrane fluidity is reduced, affecting processes such as exocytosis, endocytosis, and the functioning of membrane bound proteins (Somero *et al*., 2017). Low temperatures also decrease the binding

affinity of enzymes, which reduces metabolic function (Somero *et al*., 2017). This can lead to a reduction in available ATP, which will not only make the organism less able to maintain homeostasis and more susceptible to predation, but also may cause an increase in anaerobic respiration and a buildup of harmful metabolic by-products (Storey & Storey, 1988). The effects of exposure to low temperatures are detrimental to these insects, and recovery from ice formation is not the only challenge they need to contend with.

Freezing is typically harmful to biological systems because intracellular ice formation destroys cells (Sinclair *et al.,* 2015). Water expansion upon freezing and the sharp structure of ice crystals are two main reasons ice formation in cells is so dangerous. Ice formation can rupture cells and compromise tissues if formed between cells (Izumi *et al*., 2005). However, ice formation is not only dangerous because of its potential for physical harm, but also because water availability is reduced inside the organism (Bradley, 2009). This can lead to increased concentrations of solutes that were dissolved into the haemolymph and cytoplasm as well as cellular dehydration (Lee, 2010). Increases in certain ion concentrations can have harmful effects. For example, an increase in H^+ concentration will lower pH, which can denature proteins in the cell (Harrison, 2001). Cellular dehydration causes cells to lose a lot of their volume, putting undo strain on the cytoskeleton and other structural proteins, as well as cell to cell connections. The failure of these structures will lead to cell death (Li *et al*., 2009). Cells also have to contend with other challenges caused by ice formation during thawing. When the ice within the tissues start to melt, the decreased pressure coupled with rapid rehydration can also damage cells through osmotic shock (Pegg, 2010; Worland *et al*., 2004). We hypothesize freezing to be very metabolically taxing, and this would only be exacerbated by a decrease in enzyme functionality. Buildup of anaerobic end products like lactate and alanine has been

observed in many species of frozen insects, suggesting that during freezing there is a shift to anaerobic respiration (Storey & Storey, 1985). This shift may occur because gases like oxygen do not diffuse well through ice, so frozen organisms may become hypoxic (Scholander *et al*., 1953). Hypoxia during freezing results can also have dangerous effects during thawing, as there is suddenly a huge increase in oxygen diffusing into tissues again. Oxidative damage has been seen in tissues of insects that repeatedly freeze and thaw, such as *Eurosta solidaginis* (Doelling *et al.,* 2014).

Different insects use different combinations of strategies to combat these affects and achieve freeze tolerance, and some are more successful than others. The lower lethal temperature of a freeze tolerant organism is the lowest temperature at which more than 50% of the population can no longer survive the effects of freezing. The lethal time is the amount of time a species can be held at a certain temperature before 50% of the population dies (Bale, 1996). These vary between species and sometimes within species, depending on how acclimated an organism is and how prepared it is to tolerate freezing (Zachariassen, 1985). These metrics show that the ability to tolerate freezing does not mean that the insect can survive indefinitely at all temperatures, but certain strategies help mitigate the harmful effects of low temperatures and freezing and may allow the insect to survive until they are able to find food and reproduce again.

One hypothesized strategy to achieve freeze tolerance is to control how ice forms and propagates in the body (Horwath *et al.,* 1996; Toxopeus and Sinclair, 2018). This is often accomplished by using ice-nucleating agents (INAs), proteins that promote ice formation (Wilson and Ramlov, 1995). These proteins are distributed in areas of the body that are more resistant to ice-induced damage, so when ice does form it forms in areas where it can do less damage. These proteins promote freezing initiation in extracellular spaces, which avoids cellular

damage and also dehydrates the cell through osmosis, which further reduces the ability of ice to form within the cell (Lee, 2010). INAs also raise the temperature at which ice starts to form (Zachariassen and Kristiansen, 2000). If ice forms at a higher temperature, it forms more slowly, which prevents physical damage of tissue (Salt, 1961). It also allows more time for osmotic dehydration of the cells, keeping ice from forming within the cell membranes (Storey & Storey, 1988). Ice-binding proteins and some lipids also control how ice forms within the organism. These proteins are hypothesized to modify the behavior of individual ice crystals and may prevent sharp edges from forming, thus reducing the likelihood of tissue damage (Walters *et al*., 2011).

It is hypothesized that insects use certain low molecular weight cryoprotectants to increase the osmolality of hemolymph and other sources of body water (Zachariassen, 1985). High solute content reduces the ability for liquids to freeze, so by accumulating non harmful solutes insects can reduce ice content within certain tissues and within their bodies as a whole (Sformo *et al*., 2009). Also, increased concentrations of glycerol have been shown to increase proportions of bound water, water molecules that are "bound" to macromolecules and organelles, thereby reducing the amount of water available for freezing (Lee, 2010). However, ice content reduction as a strategy for freeze tolerance is poorly understood, as some species can survive with large proportions of their body water freezing. While *C. costata* has been shown to accumulate proline while freezing, which reduces ice content, whether or not the individual vitrifies seems to be the best predictor for survival (Rozsypal *et al.,* 2018).

Many freeze tolerant insects accumulate low molecular weight metabolites like proline and arginine not just to limit ice formation, but also to counteract the harmful effects freezing has on macromolecules. Molecular crowding caused by cellular dehydration can result in

accumulation of proteins denatured by low temperatures (Ramløv, 2000). Proline and arginine may combat these aggregations by forming clusters that act as a buffer (Kostál *et al*., 2011). Some freeze tolerant insects alter their cellular structures to make them more resistant to the damage caused by freezing. Higher concentrations of unsaturated phospholipids in cell membranes allow them to maintain fluidity and function at lower temperatures (Kostál *et al*., 2003). Certain protein isoforms that are less likely to denature in the cold are used for cell structure, such as in the cytoskeleton (Li *et al.,* 2009). All of these strategies require time to accumulate the proper materials and are most commonly used by insects that use environmental triggers, such as decreasing photoperiod or temperatures, to kickstart their preparation for freeze tolerance (Philip and Lee, 2010; Marshall *et al*., 2014).

Metabolic suppression is a common strategy for many animals when faced with freezing temperatures (Hahn and Denlinger, 2011; Tattersall *et al*., 2012). Dozens of species of rodents, marsupials, and even some primates hibernate, reducing their metabolic rate and body temperature in order to not use up their fat reserves too quickly (Fishman and Lyman, 1961). Insects have their own version of hibernation, called diapause. Diapause is a period of dormancy characterized by low oxygen consumption and metabolic rate (Saunders, 2020). Many species of insects undergo diapause at predetermined life stages, but some insects use environmental cues, such as shorter light periods and decreasing temperatures, to induce diapause in order to survive months when food may be less available and temperatures become too low for activity (Saunders, 1965). This strategy is used by freeze avoidant insects, but it is also important for freeze tolerant insects (Michaud *et al*., 2008). Since ice formation prevents oxygen from reaching the spiracles of insects, frozen insects often switch to anaerobic respiration. This can cause a buildup of harmful metabolic products like lactate, so decreased metabolism is key to making

sure that concentrations of such products remain at manageable levels (Marshall & Sinclair, 2012).

Recovery from freezing introduces a new set of challenges that freeze tolerant insects must overcome. Despite metabolic suppression during freezing, there may still be an accumulation of potentially harmful anaerobic metabolites, and recovery would require that these end products are removed and oxidative damage repaired (Storey & Storey, 2013). Cell dehydration can result in high concentrations of other solutes which must be dealt with post-thaw. For example, large amounts of Ca^{2+}, which can disrupt cell signaling, can only be mitigated by accumulating ion chelators (Storey and Storey, 2010). Ion and water balance are disrupted during freezing, requiring ATP-fueled transport proteins to re-establish ion equilibrium across cell membranes (Storey and Storey, 2013). Freezing may also cause mechanical damage via expansion during crystallization that may need to be recognized and repaired through cell proliferation and apoptosis during recovery (Marshall and Sinclair, 2011). These additional challenges may underlie the long recovery times in insects that have been frozen, as many species take hours and even days to recover full function after freezing (Kostál *et al.,* 2011). All of these recovery processes are hypothesized to require an increase in ATP production and metabolic rate. However, thawing may not initiate active responses in insects, as larvae of *P. marioni* do not increase metabolic rate during thawing (Sinclair *et al.,* 2004), and there is no change in adenylate charge during thawing in *Eurosta solidaginis* (Storey and Storey, 1985).

There is debate on whether freezing induces active or passive responses in certain freeze tolerant organisms. The process of freezing appears to initiate active metabolic and transcriptional responses in some freeze tolerant animals. For instance, the wood frog, *Lithobates sylvaticus* increases metabolic rate and the expression of stress response pathways when ice

formation begins (Storey & Storey, 1984). In addition, both the wood frog and enchytraeid worms (Pedersen and Holmstrup, 2003) mobilize glucose at the onset of freezing. However, similar active responses to freezing may be absent in freeze tolerant insects. The larvae of *Pringleophaga marioni* do not increase CO_2 production prior to freezing (Sinclair *et al.,* 2004), suggesting that freezing is passive and is driven solely by the crystallization of water (Kristiansen & Zachariassen, 2001). However, there is a burst of CO_2 at the onset of freezing in larvae of *P. marioni*, which could represent an active metabolic response (Sinclair et al., 2004). This burst of CO_2 production has been viewed in other species as well and is an example of one of the many aspects of freezing we still do not understand. Another issue is that most research has focused on short-term recovery from freezing, and there is very little information on the long-term effects of freezing on metabolic rate and reproductive success (Toxopeus and Sinclair, 2018). Further research is needed cataloging how insects recover from freezing over time, and whether the effects of freezing are still present in future life stages.

Although all these mechanisms are hypothesized to confer freeze tolerance, a lot is still unknown about how many species are able to survive and recover from such a taxing process. Most of the molecules that have been shown to be correlated with freeze tolerance have been identified through targeted studies. There have not been enough untargeted studies that might identify unexpected molecules as vital to freeze tolerance (Toxopeus and Sinclair, 2018). There is still much we do not understand about how freeze tolerance works in different species, as there are a lot of different strategies and contradictions. In most species, intracellular ice formation is immediately lethal. However, for other species it does not seem to be an issue, but the mechanism for surviving it is unknown (Toxopeus and Sinclair, 2018). Because there is no one

molecule that confers freeze tolerance, more studies into the mechanisms and costs of freezing in different freeze tolerant insects are needed to gain a clearer understanding of this strategy.

The spring field cricket (*Gryllus veletis*) is a tractable model to explore some of the unknowns in freeze tolerance. This species is native across temperate North America (Alexander and Bigelow, 1960). This species spends most of its life as a nymph, as adults are only active from May to July. Nymphs begin to hatch in August and September, and as temperatures begin to drop and photoperiods begin to shorten, late instar nymphs acclimate and become freeze tolerant (Toxopeus *et al*., 2019). Acclimated nymphs freeze at approximately -6 °C, survive temperatures as low as -12 °C, and survive for seven days frozen at -8 °C (Toxopeus *et al.,* 2019). The burst of CO_2 seen at the onset of freezing in some other freeze tolerant organisms is also observed in *G. veletis*.

We examined the potential causes of the unexplained burst of CO_2 and whether freezing induces active metabolic responses and if recovery from freezing is metabolically costly. First, we hypothesized that (1) the inability of hemolymph to buffer CO_2 when it freezes, rather than an increase in metabolism, drives a burst of CO_2 release from G. veletis at the onset of freezing. We predicted that pharmaceutically killed crickets would still exhibit the burst, and that the amount of CO_2 observed in the hemolymph would correlate to the amount that is expelled at the onset of freezing Second, we hypothesized that (2) recovery from freezing has a significant metabolic cost that persists for several days. We predicted that crickets that froze would have a higher metabolic rate 24, 48, and 72 hours after freezing, and that this raised metabolic rate would still be visible after the crickets molted. This research will help us gain insight into some of the mechanisms of freeze tolerance and the metabolic cost of short-term and long-term recovery from freezing.

Methods

Insect rearing and acclimation

We selected *G. veletis* nymphs from a colony reared at Western University that originated from individuals collected in 2010 from a wild population in Lethbridge, Alberta (Coello Alvarado *et al.,* 2015). We exposed fifth instar nymphs of both sexes to a declining photoperiod and temperature regime in a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, IL, USA) over six weeks (Toxopeus *et al.,* 2019). This regime mimicked the natural climatic change that induces freeze tolerance in *G. veletis.* The photoperiod began at 11.5:12.5 L:D and daylength decreased by 36 min each week, and daily maximum and minimum temperatures began at 16 °C and 12 °C and decreased over six weeks to 1 °C and 0 °C. While recovering from freezing, crickets were kept in Sanyo MIR 154 incubator at 15 °C and were provided food and water *ad libitum*.

Cyanide and thiacloprid injections

To determine if the burst of CO_2 expelled at the onset of freezing was an active response, we measured CO_2 production in pharmaceutically killed crickets using flow-through respirometry. We used thiacloprid to determine if metabolic activity during freezing and thawing is the result of a nervous system response, and cyanide to determine if metabolic activity is the result of aerobic respiration. We injected crickets in the arthrodial membrane near the anterior edge of the pronotum with either 4 µL of 0.6 mM thiacloprid (Sigma-Aldrich, St Louis, MO, USA) solution or 4 µL of 1 mM hydrogen cyanide (Carbonyl cyanide 3-chlorophenylhydrazone, Sigma-Aldrich, St Louis, MO, USA) solution (HCN) using a 5 µL Hamilton syringe (32 gauge; Hamilton Company; Reno, NV, USA). We placed these crickets in the respirometry chamber 30 min after injection and continuously measured CO_2 production during freezing and thawing. CO_2

and water were removed from the air, and a pump separated the air into two tubes, one sample line and one baseline. Air in the sample line flowed through a chamber containing an individual cricket [controlled by mass flow valves (Sierra Instruments) and a mass flow controller (MFC2; SSI)]. A Li7000 CO_2 analyzer (LiCor; Lincoln, NE, USA) measured CO_2 in the sample line and compared it to the baseline, giving us the amount of CO_2 produced by the cricket in $\mu mol/mol^{-1}$. We included baseline measurements of dry, CO_2-free air before and after each measurement to correct for instrument drift. Data were recorded continuously and converted to µL CO_2 on Expedata software (SSI).

Total hemolymph CO_2

We measured total hemolymph CO_2 (TCO_2) with a custom-built sparging column and flow-through respirometry system (Lee *et al.,* 2018). We collected 2.5 µL of hemolymph from acclimated crickets with a Hamilton Syringe from beneath the pronotal membrane. Hemolymph samples were injected into a custom-built gas sparging column (4.7 mL internal volume) containing 250 µL of H_2O, 0.25 µL of antifoam, and 0.25 µL of HCl. This solution forces CO_2 out of the hemolymph through elimination reactions. Dry, CO_2-free air was passed through the column [controlled by mass flow valves (Sierra Instruments) and a mass flow controller (MFC2; SSI)] at 10 ml/min, pushing gaseous CO_2 from the column into a two-channel LI-7000 infra-red CO_2 gas analyzer (LI-COR, Lincoln, NE, USA). The analyzer measured CO_2 released by hemolymph into excurrent air. Five sodium bicarbonate standards were also injected into the sparging column before and after hemolymph samples (10, 15, 20, 25 and 30 mmol L^{-1}). The syringe was primed between all injections by submerging in CO_2 free distilled water and repeatedly pumping the plunger until all air was expelled. These injections were used to

standardize the amount of CO_2 we measured from the hemolymph, as we knew the exact amount of CO_2 in each standard.

Metabolic cost of recovery from chilling and freezing

In order to measure how metabolic rates of these insects were affected by freezing we had to first freeze them. We cooled crickets from 15 °C to -8 °C at 0.25 °C min^{-1} and held them at -8 °C for 1.5 h. A 36 AWG type T thermocouple placed on the abdomen of each cricket recorded the temperature using PicoLog software (Pico Technology, Cambridge, UK). After 1.5 h at -8 °C, we removed crickets from the block and placed them into individual 180 mL plastic cups in a Sanyo MIR 154 incubator at 15 °C to recover. At this temperature and time, approximately half the crickets would freeze and half would remain unfrozen, allowing us to see if freezing or just chilling to a certain temperature would effect metabolic rate. We used stop-flow respirometry to measure the metabolic rates of these crickets at 24, 48 and 72 h after being exposed to -8 °C during recovery at 15 °C. We used a multiplexer (RM8; SSI) to sequentially flush three chambers containing crickets with CO_2-free dry air for 5 min at 200 mL min^{-1}, then sealed each chamber for 10 min before being passing this air through a LI-COR Li7000 CO_2 analyzer (LiCor; Lincoln, NE, USA). This procedure was repeated three times for each individual cricket. Baseline measurements were included before and after each measurement to control for instrument drift. Data were recorded using Expedata software (SSI). For the stop-flow measurements, we integrated beneath peaks of $\dot{V}CO_2$ (volume of CO_2 mL/min^{-1}) to obtain the total volume of CO_2 produced. We used the absolute difference sum of activity to quantify the activity of each individual, and the nadir function in Expedata to find the 300 s interval with the lowest amount of activity. For each cricket, we used this approach to quantify the amount of time

spent moving during each chamber seal period. We selected the recordings with the least amount of time spent moving for analysis from the three recordings taken.

Developmental performance after chilling and freezing

We tracked the developmental performance of subsets of late-instar nymphs that did not freeze (i.e. unfrozen) and the subsets that froze (i.e. frozen) after cooling (from 15 °C to -8 °C at 0.25 °C min^{-1}). We also tracked the development of a group of acclimated, but uncooled crickets. After cooling, we placed these crickets in 180 mL plastic cups at 15 °C and provided rabbit food and water *ad libitum*. At 48 h post-freeze or post-chill, we removed food from crickets. At 72 h post-freeze or post-chill, we measured CO_2 production and oxygen consumption using stop-flow respirometry and recorded the mass by zeroing the weight of a microcentrifuge tube and then weighing the cricket in the tube (179.7 ± 42g). We also measured the head capsule width (3.9 ± .4mm) and femur length (7.3 ± .7mm) of each cricket using a millimeter caliper. All measurements were done by the same person using the same equipment, thus limiting variability. Crickets were returned to food and a 15 ^{o}C incubator after respirometry measurements. After initial metabolic rate was measured, we checked daily for evidence of molt or mortality. If a cricket did not move in response to gentle prodding with a paintbrush, we labeled it as unresponsive. We considered crickets to be dead when they were unresponsive for five consecutive days. At molt, we measured the CO_2 production and oxygen consumption of each cricket using stop flow respirometry. Crickets were transferred to 22 °C under a long day photoperiod (14:10; L:D) one week after molt.

Data analysis

We analyzed data in R (Ver. 3.6.2). We used a two-way, repeated-measures ANCOVA with body mass as a covariate to compare mean metabolic rate between frozen, unfrozen, and control

treatment groups 24, 48, and 72 hours after freezing. We used the same test to compare the mean metabolic rates of these groups after molting. We checked assumptions of ANCOVA according to Zuur *et al.,* (2010). To compare the amount of buffered CO_2 in hemolymph to the mean amount seen in the burst at the onset of freezing, we first extrapolated the amount of CO_2 we measured in 5 μL of hemolymph to the estimated mean total amount of hemolymph in the crickets (40.8 μL) by multiplying the values by 8.16 (MacMillan and Sinclair, 2010). We then used a two sample T-test of unequal variance to compare the amount of CO_2 buffered in the hemolymph to the amount measured during the burst at the onset of freezing. We also used the same test to compare the amount of CO_2 expelled at the onset of freezing between live crickets and crickets that had been pharmaceutically killed using thiacloprid.

Results

Crickets that were pharmaceutically killed using injections of thiacloprid and cyanide still exhibited a burst of CO_2 at the onset of freezing (Figure 1A-B). This burst was positively correlated with initial body mass of the crickets ($r=0.51$, $p < 0.05$). This burst was not exhibited by crickets killed with cyanide that did not freeze, indicating that the expulsion of CO_2 in these cases was not a result of the injections (Figure 1C). The mean amount of CO_2 expelled at the onset of freezing by crickets who were not pharmaceutically killed with these chemicals was not significantly different to the crickets that were killed ($p=0.6$). The mean amount of CO_2 observed in the hemolymph (8.97 ± 3.7 μL) and the mean amount observed in the burst at the onset of freezing (9.3 ± 4.3 μL) were also not significantly different ($p=.87$).

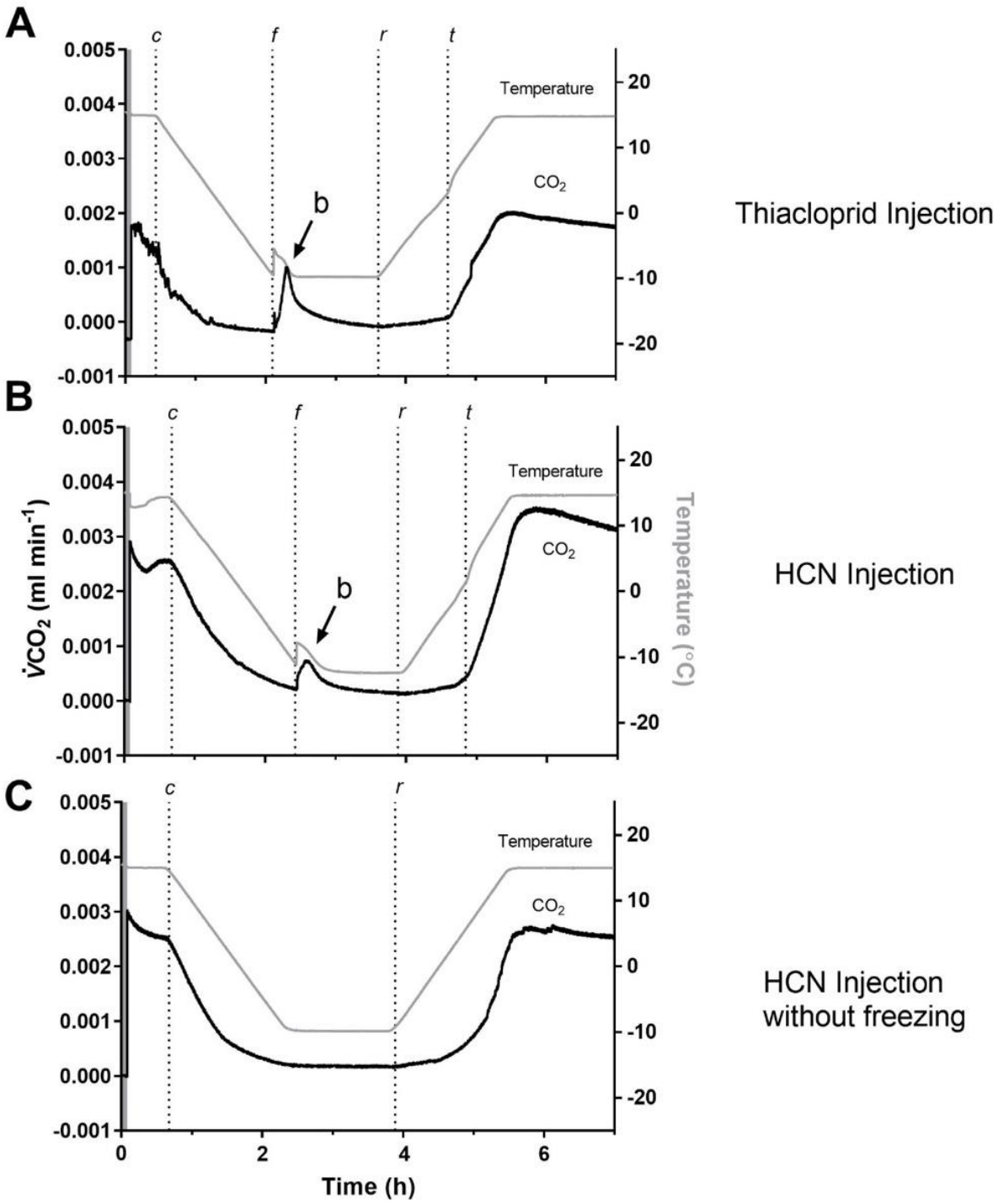

Fig 1. Volume of CO_2 produced during cooling, freezing, and thawing. Fig 1A shows the CO_2 production of crickets killed with a thiacloprid injection. Fig 1B shows the CO_2 production of crickets killed with a cyanide injection. Fig 1C shows the CO_2 production of crickets killed with a cyanide injection that did not freeze.

Frozen crickets exhibited a mean VCO_2 about 40% higher than the mean VCO_2 of crickets that were chilled to -8 °C but did not freeze (unfrozen) and crickets that were acclimated to freezing but kept at 15 °C (control) ($p = .03$). This difference in mean VCO_2 was found at 24, 48, and 72 hours after being exposed to freezing temperatures (Figure 2). Although the control crickets had higher metabolic rates overall compared to the unfrozen crickets, the difference was not significant. Between 24 and 72 hours of recovery, there were no significant changes in mean VCO_2 in any of the groups of crickets. There was no significant difference in mean VCO_2 between male and female crickets.

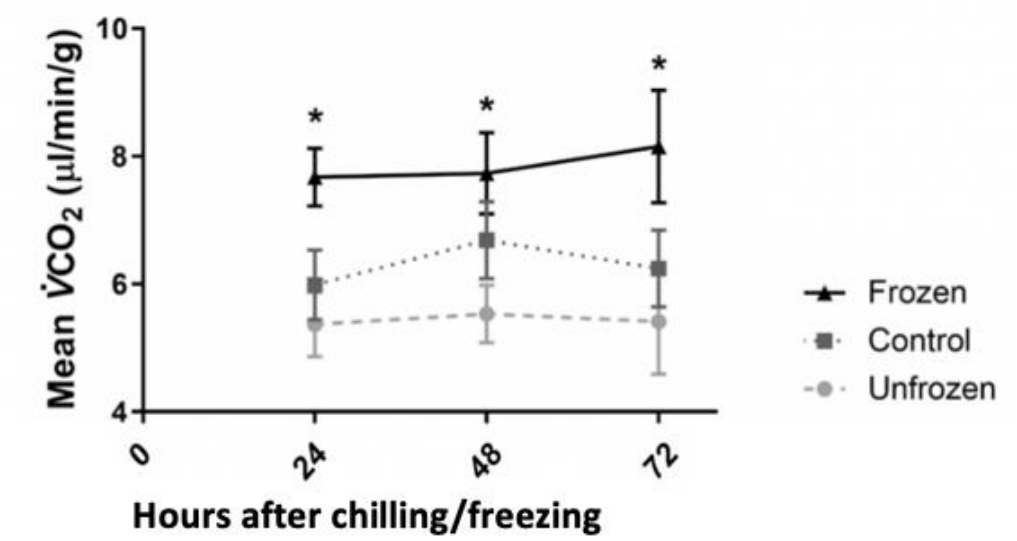

Fig 2. Mass-corrected rates of CO_2 production ($\dot{V}CO_2$) of *G. veletis* at 24, 48, and 72 hours after freezing and chilling. Asterisks indicate times at which there was a significant difference between the metabolic rates of the frozen and control groups.

Frozen crickets used to study long term recovery did not have significantly higher mean $\dot{V}CO_2$ after 72 h of recovery from freezing, nor was their mean $\dot{V}CO_2$ significantly higher after molting than unfrozen and control crickets ($p = .42$). However, crickets that froze did seem to

have more trouble completing the molting process than crickets that did not freeze, but the difference in molting success was not statistically significant. After 3 months of monitoring, 0 out of 12 crickets that froze made it to adulthood (survival rate = 0%), compared to 7 out of 14 crickets that were cooled but didn't freeze (survival rate = 50%), and 5 out of 7 that were only acclimated (survival rate = 71.4%).

Discussion

These experiments with fifth instar *G. veletis* allowed us to investigate many questions we had about freeze tolerance. We hypothesized that the burst of CO_2 observed in *G. veletis* and other freeze tolerant species was the result of the passive degasification of the hemolymph as it froze. This hypothesis is supported by the presence of the burst in pharmaceutically killed crickets and the fact that a similar amount of CO_2 is buffered in the hemolymph to the amount that is expelled at the onset of freezing. We also hypothesized that recovery from freezing has a metabolic cost that can last a long time after freezing and may affect the individuals transition to the next life stage. We observed elevated metabolic rates in frozen crickets up to 72 hours after freezing, and the survival rate of frozen crickets was much lower than the other treatment groups.

The burst of CO_2 observed at the onset of freezing in *G. veletis* indicated that there was a response to freezing in this species with an unknown mechanism. This burst was still observed in all crickets that were pharmaceutically killed with thiacloprid and frozen. Because thiacloprid binds to acetylcholine receptors on neurons, preventing those neurons from firing, the fact that the burst was still observed indicated that the burst was not the result of a nervous system response. The burst was also observed in crickets pharmaceutically killed with cyanide. Cyanide prevents the production of ATP, so the fact that the burst was still observed suggested that it was

not the result of aerobic respiration. Both of these experiments showed that the burst of CO_2 was likely not an active response to freezing.

The high p value of .87 shows that the amount of CO_2 expelled at the onset of freezing is not significantly different to the amount buffered in the hemolymph. In fact, this value suggests that the amounts were very similar, and that a likely cause of the burst was CO_2 being forced out of the hemolymph as it froze. However, the total amount of hemolymph in the late instar male *G. veletis* used in the experiment was estimated using the mean hemolymph volume of adult female *G. pennsylvanicus*, a similar species (MacMillan and Sinclair, 2010). Adult male *G. veletis* have been found to have similar body weights to adult female *G. pennsylvanicus*, suggesting that they likely have similar total volumes of hemolymph as well (French and Cade, 1987; Carriére *et al.*, 1996). Even though late instar nymphs are close to the size of adult *G. veletis*, they do have a lower average body weight, suggesting that the total hemolymph estimate of 40.8 µL may be an overestimation. However, even when dropping the total hemolymph estimate by 10 µL, the amounts of CO_2 observed in the burst and in the hemolymph are not significantly different. The data we have now suggest that the observed burst is a result of hemolymph freezing, but in order to solidify that claim research into the true total amount of hemolymph in late instar *G. veletis* nymphs needs to be done.

As the hemolymph converts from a liquid to a solid, the dissolved CO_2 would be forced into a smaller and smaller amount of liquid hemolymph. When all the CO_2 is forced into the portion of hemolymph that remained unfrozen, the oversaturation forces much of the dissolved CO_2 to become gaseous and be expelled. In certain other freeze tolerant organisms, like wood frogs, CO_2 production increases during temperature decrease and near the onset of freezing (Sinclair *et al.*, 2013). This is believed to indicate an active response to prepare for freezing.

Active responses to freezing are not only limited to vertebrates. Enchytraeid worms are observed to mobilize glucose stores at the onset of freezing (Pederson & Holmstrup, 2003). However, in *G. veletis*, the increase in CO_2 production upon freezing is likely a passive response to the normal chemical process of degasification, suggesting that this may be the case for other freeze tolerant insects. However, more studies on other species that exhibit this burst are needed before that can be asserted. This experiment also demonstrated that the method described in the section *total hemolymph CO_2* can be used to ascertain the amount of dissolved CO_2 in hemolymph. This method was adapted from Lee *et al.*, 2018, and should be used to further investigate if increased CO_2 production during freezing is the result of buffered CO_2 in the hemolymph being expelled. Investigating the strange nuances of freeze tolerance like this burst of CO_2 is important as there is still much we do not understand about the differing mechanisms that these species use to be freeze tolerant.

The elevated metabolic rate observed in frozen crickets 24, 48, and 72 hours after freezing indicates that the metabolic cost of freezing is high and persists for many days. An increase in metabolic rate suggests that more energy is required by the organism, likely to repair the physiological damage caused by freezing and to regain normal cell function (Toxopeus & Sinclair, 2018). Because of this higher metabolism, after thawing these crickets are likely more susceptible to dangers like starvation and predation, since they may not have enough stored energy reserves to meet this metabolic demand. This same phenomenon has been observed in other freeze tolerant insects that evolved this strategy independently, such as the beetles *Eleodes blanchardi* (Zachariassen *et al.*, 1979) and *Perimylops antarcticus* (Block *et al*., 1998). However, other species, such as the caterpillar *Pringleophaga marioni*, have been shown not to have an increase in metabolic rate after freezing (Sinclair *et al*., 2004). This may be because this species

metabolic rate was only taken between 2 and 5 hours after freezing. Metabolic suppression is a common strategy for freeze tolerant and cold tolerant species (Irwin & Lee, 2002; Hahn & Delinger, 2011). It reduces the number of harmful metabolites that can build up during freezing, and prevents the insect from using up all of its energy reserves. The metabolic cost of freezing may not be shown fully in the time directly after freezing, as the insect may still be depressing its metabolic rate artificially. Although some studies do show certain insects do not have an increased metabolic rate many hours after freezing (Marshall & Sinclair, 2011), there may be other factors that allow those insects to better deal with the harmful effects of freezing. Further long-term studies are needed comparing the metabolic rates of freeze tolerant insects that froze and ones that did not freeze in the hours and days after freezing, as unrelated species exhibiting the same elevated metabolic rate suggests that this energetic cost may be present in many other freeze tolerant species. Understanding how insects meet this increased metabolic demand may help us predict how these species will fare in varying climatic and environmental conditions.

The long-term recovery of these crickets further illustrates how costly freezing can be. Crickets that froze were much less likely to reach adulthood than crickets that did not freeze. Even though most of the crickets that froze appeared fine directly after freezing, their long-term survival was likely much lower because of damage suffered during freezing that made it impossible to complete the molting process. Many of the frozen crickets died either during the molting process or around the time they would have been expected to molt. Molting is a metabolically costly process in insects, as creating a new exoskeleton takes a lot of energy allocation and is physiologically stressful (Soluk, 1990). Insects still recovering from freezing may not have the metabolic ability to repair tissues damaged from freezing and deal with the energetic demand of molting. It has been shown that in some species cold stress has prevented

insects from progressing to the next life stage (Stetina *et al*., 2018). Although the metabolic rates of frozen crickets after molting was not significantly higher than the metabolic rates of chilled crickets and control crickets, this may be because the sample size was much smaller, as many of the frozen crickets had already expired by that point in their life cycle. Even if metabolic rate does not remain increased for that long after freezing, there may be other physiological damage caused by freezing that affects the molting process. Even though the survival rate of frozen crickets was 0%, this is obviously impossible as late instar *G. veletis* nymphs spend months frozen in the fall, winter, and spring, and enough of them survive to perpetuate the species (Alexander and Bigelow, 1960). However, the contrast of their survival rate to the survival rates of the chilled and control crickets does provide evidence that the frozen crickets were disadvantaged in the conditions we gave them to recover in. A repeat of the long term monitoring we did with this species is needed with a much larger sample size. A study like this may be able to ascertain more accurate survival rates of this species after being frozen. Also, the amount of crickets that seemed to have recovered from freezing in the first few days after freezing only to die in the next few weeks suggests that more studies need to be performed that examine the long-term survival rate of freeze tolerant species. Most studies on freeze tolerant species up to this point have focused on survival directly after or in the few days after freezing, and very few studies investigate the survival rate of these organisms in the weeks and months after thawing (Toxopeus & Sinclair, 2018). Some species that seem to have recovered immediately after freezing may not be able to survive until adulthood.

Freeze tolerant organisms are not immune to the effects of freezing and need time and resources to recover from its harmful effects. In normal, fairly predictable climatic conditions, recovery is still a challenge, but as the climate changes, it may become even more of one. As

human-caused climate change continues to become more severe, variations in temperature and extreme climatic events are becoming more common (Easterling *et al.,* 2000; Salinger *et al*., 2005). In polar and temperate regions, where most freeze tolerant species live, these effects are felt more strongly due to polar amplification (Holland and Bitz, 2003). Polar amplification is the phenomenon that the greenhouse effect produces larger change in temperature in polar regions compared to the planetary average. The freeze tolerance ability of *G. veletis* is dependent on consistent lowering of temperature along with the shortening of photoperiod (Toxopeus *et al*., 2019). If temperatures no longer lower at the rate adapted to, *G. veletis* nymphs will not properly prepare for freezing, and when temperatures do eventually drop below freezing, they won't be able to survive. Acclimation is not only important for *G. veletis*, as other species, such as *Chymomyza costata*, also need specific climatic cues to increase their freeze tolerance ability (Kostál *et al*., 2003). All these species may experience lower survival rates due to climate variability. As global temperatures increase, polar winters will be warmer and days above the freezing temperature of water will increase (Easterling *et al*., 2000). This increases the likelihood that insects that normally stay frozen throughout the winter may thaw prematurely. As discussed above, many freeze tolerant species have an increased metabolic demand in the days after thawing, and thawing too early in the season may limit their ability to find enough nutrients to meet that demand. Also, freezing and thawing repeatedly has been shown to cause more oxidative damage in certain species like *Eurosta solidaginis* (Doelling *et al*., 2014). If metabolic rate in other freeze tolerant species is elevated after freezing like in *G. veletis,* not being able to rely on a semi-predictable climate may severely impact the populations of freeze tolerant species around the globe.

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