Effects of freezing on the metabolic rate of the bay mussel, *Mytilus trossulus*

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Abstract:

Mussels that live in intertidal zones of temperate regions risk freezing at every winter low tide as they are exposed to sub-zero temperatures. Therefore, they have evolved the ability to tolerate freezing with a hefty energetic cost, as tissue and cellular damage incurred must be repaired afterward. The metabolic costs of freezing have yet to be investigated in an intertidal species, specifically whether a metabolic cost is associated with crossing the freezing threshold. I hypothesized that animals that freeze will experience relatively more damage than those which do not and will therefore demonstrate a higher metabolic rate associated with repair immediately after freezing. I exposed the intertidal mussel, *Mytilus trossulus*, to -5.5°C for 6 hours and found similar oxygen consumption rates regardless of the outcome. This may indicate that the driver for metabolic shifts is not attributed to the crossing of the freezing threshold but rather the percentage of body water converted to ice. With the predicted increasing frequency of cold snaps due to climate change, we can better predict how mussel populations will respond to these events by understanding the fundamental mechanisms of freeze tolerance.

Introduction:

When winter arrives, the ability to overwinter governs the survival of many animals in areas that experience sub-zero temperatures. This is an extremely difficult task for ectothermic animals who are mostly unable to physiologically regulate their

body temperatures. Surprisingly, there is a small subset of ectotherms that have evolved strategies to survive sub-zero temperatures (Aarset, 1982). One such strategy is freeze tolerance, the ability to survive internal ice formation for a limited period of time. They restrict ice formation to extracellular spaces to maintain a liquid cytoplasm, as ice formation intracellularly is lethal to almost all animals due to excessive cellular damage (Storey & Storey, 1996).

One environment which can regularly experience freezing is the intertidal zones of temperate and polar regions. The intertidal represents the coastal region which is submerged by seawater during the high tide and exposed to the air during the low tide. The ubiquitous presence of water combined with the frequent exposure to the air makes the intertidal zone an environment where ice formation is difficult to avoid when air temperatures are sub-zero. Thus, sessile intertidal invertebrates which can't migrate to the subtidal zone or find refuge against direct exposure to the air must be able to tolerate internal ice formation and its associated physiological and biochemical challenges. For example, the lowering of body temperatures prior to the onset of freezing can cause protein cold denaturation to occur (Privalov, 1990) and ice crystals formed during freezing can cause mechanical damage to tissues such as gills as observed in Kennedy (2022).

Although freezing is expected to incur more damage to an animal, it is unclear whether an additional metabolic cost would be associated with the crossing of the freezing threshold in an intertidal species. One study by Irwin and Lee (2002) has

shown metabolic differences in larvae of goldenrod gall flies when they freeze compared to only experiencing cooling, suggesting the formation of ice crystals imposes an additional cost to the animal. To understand whether there are differences in the metabolic response associated with freezing, I will examine the metabolic costs of freezing via respirometry in the bay mussel, *Mytilus trossulus*. This sessile intertidal bivalve can be found in the intertidal of temperate regions (Hilbish et al., 2000) and is known to use freeze tolerance as a strategy to survive against freezing. I will investigate how the metabolism of the bay mussel will respond after a cold treatment and compare mussels that freeze against those which do not. I will measure metabolism by proxy via oxygen consumption. I hypothesize that there will be a greater increase in metabolic rate immediately after freezing in mussels that freeze relative to mussels that only experience cooling as they experience more damage to be repaired after thawing.

Methods:

Field collection and laboratory acclimation

Mussels were collected from Tower Beach, Vancouver, BC (49°16'26.1"N 123°15'23.7"W). The tides in this area are mixed semi-diurnal, with two low and two high tides daily with different magnitudes. Mussel collections were done under a Scientific Licence, Management of Contaminated Fisheries Regulations from the Department of Fisheries and Oceans Canada (Licence number: XMCFR 34 2021). Site salinity, water temperature, and air temperature were measured with a YSI handheld salinity and temperature meter (Pro 30 series with a PRO 30 COND-T probe) at approximately 25-50 cm water depth on collection days. Mussels were collected from

the same mussel bed located directly in front of the gun tower at Tower Beach from the mid-intertidal zone on each sampling day (Fig. 1)

Mussels with shell lengths of 2-4cm were selected due to logistical constraints. Within 1h of collection, mussels were transported to 20L aquaria, held at 15°C, 20ppt, 12:12 light:dark cycles, and aerated using air stones. Tank water was changed every 48 hours. Seawater was sourced from the Zoology Aquatics Facility and mixed with dechlorinated water to adjust to the desired salinity. The aquaria were placed in incubators (MIR-154, Sanyo, Bensenville, USA). Epibionts were removed and mussels were haphazardly assigned to experimental groups. Mussels are fasted and used 5-8 days after the date of collection. Mussels' shell length were measured anterior to posterior at the longest length using manual calipers and dried before weighing. Environmental measures and mussel dimensions can be found in supplemental table 1. No mortalities were observed within three days after the treatment.

Figure 1. Geographical image of Tower Teach. (A) Star represents the sampling site. Derived from Google Maps. (B) Image of the intertidal zone of Tower Beach

Freezing treatments

The freezing temperature was -5.5°C for mussels in September 2022. Freezing temperatures were referenced from a previous study which found the average temperature to induce freezing (termed the supercooling point) by Kennedy et al., (2022). At this temperature, the chance for ice nucleation is assumed to be equal across mussels in a similar timeframe, and therefore a comparison between mussels based on the outcome (frozen vs supercooled) is possible as the temperature is consistent between groups.

To induce freezing, mussels were removed from the aquarium and placed individually in 25 mm Drosophila tubes. Then a copper-constantan type-T thermocouple (OMEGA Engineering, St-Eustache, Quebec) was attached to the shell of each mussel and secured using cotton or styrofoam. Thermocouples were connected to computers using PicoLog 6 beta software for Windows through Picolog TC-08 interfaces (Pico Technology, Cambridge, UK) throughout the freeze exposure to track mussel body temperatures and determine freezing events (Fig. 2). This was demonstrated as a rapid release of heat and dramatic rise in body temperature immediately prior to ice formation (i.e. the supercooling point) (Lee, 2010). Mussels were placed in a cooling bath with a methanol and water mixture (60:40, v/v) and circulated by a circulator (Thermofisher, PC200 Model). The temperature was set at the acclimation temperature of 15°C and cooled at -1.25°C/min until -5.5°C where they were held for 6 hours. Each mussel was only used once.

To test the effects of freezing against cooling, mussels were subjected to -5.5°C. The first trial was conducted on September 13th, 2022 and 3/7 (43%) mussels froze, indicating this was a reasonable temperature. Due to logistical constraints, only 7 mussels were able to be tested in a given trial. Thus to increase sample sizes, a second trial was conducted on September 30th, 2022, where 2/7 (29%) mussels froze. Altogether, 5/14 (36%) froze. One mussel from the second trial was excluded from the subsequent analysis due to the absence of activity post-freeze.

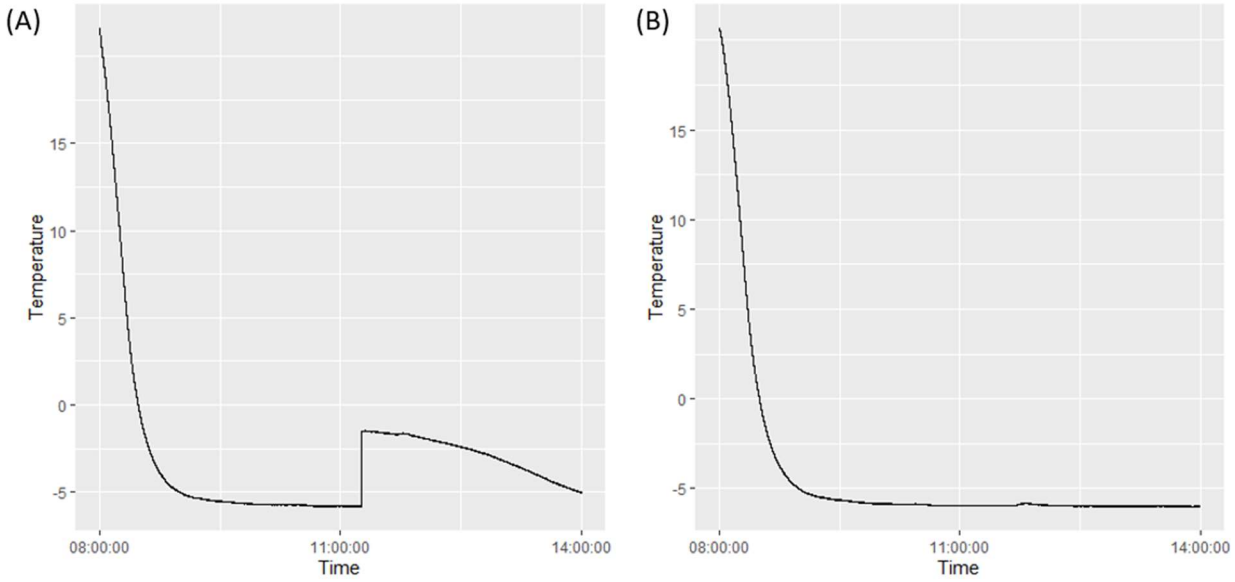

Figure 2. Example temperature trace of mussels exposed to -5.5°C for 6h (A) temperature trace of a mussel which froze as evidenced by the sharp increase in temperature representing the supercooling point. (B) Temperature trace of a mussel which did not experience freezing.

Closed Respirometry

Mussels were placed into individual 80mL glass chambers sealed with rubber stoppers. Temperature was maintained at 15°C during the trials with incubators (MIR-154, Sanyo, Bensenville, USA). Chambers were mixed using a magnetic stir bar sitting on top of a stir plate, and mussels are elevated to prevent contact with the stir bar. With

the PreSens Measurement Studio 2.0 Software, oxygen partial pressure was taken with Oxy-4 SMA trace (G3), Polymer Optical Fibers, and Oxygen Sensor Spot SP-PSt3-NAU and the water temperature was taken using a Pt100 Temperature Sensor (PreSens Precision Sensing, Regensburg, Germany). Atmospheric pressure was also recorded by the Oxy-4 SMA trace (G3). Oxygen sensors were calibrated to 0% oxygen saturation with continuous bubbling of nitrogen gas and 100% oxygen with fully aerated water. The sampling frequency is 0.33 Hz. Background oxygen consumption was measured in a separate empty chamber and adjusted for in the analysis. Mussels were measured for 70 mins prior to the freezing treatment and also for 70 mins within 10 mins after the cold treatment.

Statistical Analysis

Statistical analyses were performed using R (v. 4.2.1; R Development Core Team, 2022). Mussels were subset based on their outcome after exposure to -5.5°C. Respirometry data were subset in 10 minute increments for the first 40 minutes after the first moment of significant respiration (defined by significant deviance from background respiration). The data were normalized to the individual by subtracting the pre-treatment oxygen consumption rates from the post-treatment consumption rates to obtain the absolute change in aerobic metabolic rate. Then a two-way ANOVA (difference ~ outcome of treatment * time) was conducted. The R package “ggplot2” was used to generate figure 2 and “sciplot” was used for generating figure 3. (Ginestet, 2011), “respR” was used to analyze the respirometry data.

Results:

A two-way ANOVA indicated that there were no significant differences between outcome of treatment (p=0.393). There also was not a significant difference with time after treatment (p=0.101) despite an increasing trend most evident with the supercooled mussels with a positive oxygen consumption rate 40 mins post-chill. The interaction between outcome of treatment and time after treatment was not significant (p=0.435). Means of net O_2 change (mg O_2/L/h) for mussels which froze starting from the first 10 minutes post-freeze are -0.051, -0.034, -0.29, and -0.024 with standard deviations of 0.17, 0.063, 0.041, and 0.068, respectively. Means O_2 change for supercooled mussels starting from the first 10 minutes post-chill are -0.040, -0.036, -0.0092, 0.050 with standard deviations of 0.10, 0.15, 0.10, and 0.079, respectively.

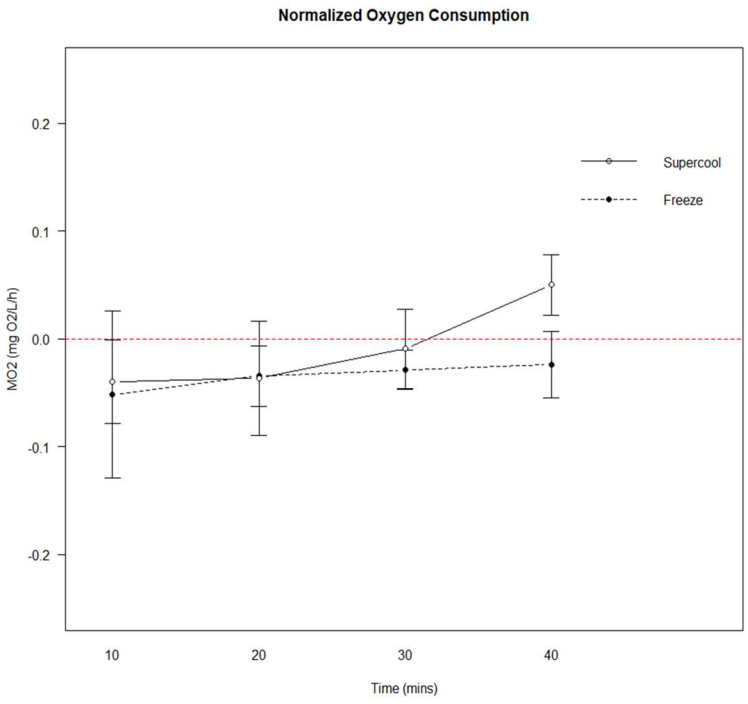

Figure 3. Normalized Oxygen Consumption Means of net O_2 change (mg O_2/L/h) relative to O_2 consumption rates prior to cold treatment for frozen mussels N=5 (dotted line, filled) and supercooled mussels N=8 (solid line, hollow). Time (minutes) immediately after cold treatment on the x-axis. Error bars are 95% confidence intervals. Red dotted line represents the point where there is no increase nor decrease of O_2 consumption rates. No significant interactions were found between any factors (p>0.05).

Discussion:

In this study, I investigated the effects of crossing the freezing threshold on the post-freeze metabolic rates of *M. trossulus*. I hypothesized that crossing the freezing threshold would drive metabolic changes, as freezing is expected to cause more damage than cooling and this would manifest as a metabolic increase in mussels which froze (Storey & Storey, 1996). However, the results do not support this hypothesis as there is no specific response that coincides with whether the animal is frozen or supercooled. I found that subjecting mussels to a cold exposure of -5.5°C does not significantly change their metabolic rate relative to their baseline regardless of whether they experienced freezing or were supercooled (Fig. 3). This was surprising as it is contradictory to the results found in Irwin and Lee (2002) where metabolic rates differed between frozen or supercooled goldenrod gall fly larvae. As well, other studies measuring post-freeze metabolic rates observed either an increase or decrease (MacMillan et al., 2012; Sinclair et al., 2004). Although there appears to be a slight increasing trend of metabolic rate through time most evident in the supercooled mussels (Fig. 3), it is not supported by the statistical analyses. It is possible a longer monitoring period after freezing could yield a different conclusion.

Rather than considering freezing as discrete checkpoints, it may be more accurate to consider freezing on a continuous scale using the percentage of internal ice formation. This idea was also suggested by Irwin and Lee (2002) as the relationships between internal ice formation and metabolic shifts were investigated and found that a higher percentage of body water converted to ice may be driving the metabolic

changes. Supercooling and frozen mussels would likely have similar percentages of internal ice formation, given that -5.5°C is a relatively mild freeze with only 5/14 (36%) mussels frozen and 0% mortality. Specifically, mussels frozen at this temperature would likely yield a low percentage of internal ice formation and supercooled mussels would have no internal ice formation. Using this metric, we can also quantify the severity of the freeze (higher percentage frozen equates to higher severity) which may be the key driver of post-freeze metabolism. Therefore, further experiments testing lower temperatures on the metabolic shifts of mussels will be required to provide support for the consideration of this metric.

There are some limitations of the experimental design. Due to logistical constraints, mussels sampled must be a certain size to fit inside the freezing apparatus. This can limit confidence in the results as the effects of body size are not captured in this experiment. Body size has been shown to be a potential factor in determining subsequent responses to freezing due to differences in their thermal inertia (Murphy & Johnson, 1980). To better extrapolate these results, knowledge of the body size of these variables on the experimental design will be required. Additionally, a logistical constraint in this study is that after freezing, there was a brief delay before oxygen consumption was measured. Although transported in a pre-chilled container, it is possible that some thawing may have occurred. The relative importance of this delay to the results needs to be evaluated, although it is important to note that the majority of thawing occurs when the mussel is placed in the water and that respiration does not begin until the mussel is returned to the water. Confirmation of this caveat by measuring

the temperature of the mussel during transport and setup could aid in estimating whether significant thawing has occurred.

With a predicted increased frequency of extreme weather events due to climate change (Francis & Vavrus, 2012), temperate bivalves are likely to experience a cold snap in their lifetime. *M. trossulus* are known ecosystem engineers who increase intertidal biodiversity through their role in the food chain or by providing shelter for other smaller animals (Borthagaray & Carranza, 2007; Harley, 2011). Therefore, their ability to survive freezing will greatly impact the viability of mussel populations and the ecosystem along our shores in the future. Expanding beyond these mussels in the intertidal zone, the ecological importance of surviving sub-zero temperatures is often overlooked. Climate change is predicted to cause range shifts in organisms and the ability to survive freezing may be an important determinant of an organism's poleward range limit (Parmesan & Yohe, 2003; Sunday et al., 2012).

Conclusions:

This study furthers our understanding of freezing physiology by determining how the aerobic metabolism of the bay mussel shifts after a cold exposure. A mild cold treatment resulting in either freezing or supercooling is not sufficient in causing an observable metabolic shift, which may suggest a continuous scale of freezing using percentage internal ice formation may be more accurate when studying freezing physiology. Given the similarity in outcomes and absence of mortality, these results may imply that mild freezes are in fact well tolerated by mussels. However, further work

would be needed to better extrapolate these findings into other species of the intertidal and to solidify the ecological relevance of this study.

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Supplemental Material:

Table 1. Summary of cold exposures and environmental measurements on sampling days.

Collection Date	September 8, 2022	September 22, 2022
Site Salinity (ppt)	19.5	19.6
Site air temperature (°C)	18.3	15.5
Site water temperature (°C)	19.3	14.1
Acclimation salinity (ppt)	20	20
Acclimation temperature (°C)	15	15
Average Weight (g)	4.27	3.79
Average Shell Length (cm)	3.06	3.1
Experiment Date	September 13, 2022	September 30, 2022