MECHANISMS AND CONSEQUENCES OF SURVIVING FREEZING IN THE BAY MUSSEL, *MYTILUS TROSSULUS*

by

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Mechanisms and Consequences of Surviving Freezing in the Bay Mussel, *Mytilus trossulus*

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Abstract

Many intertidal invertebrates are freeze tolerant, meaning that they can survive ice formation within their bodies when exposed to freezing air temperatures during low tides. In my thesis I addressed two key questions regarding intertidal invertebrate freeze tolerance using the intertidal mussel *Mytilus trossulus*. First: What biochemical mechanisms enable freeze tolerance in intertidal invertebrates? Second: How do sublethal single and repeated freeze exposures negatively impact intertidal invertebrates? To address the first question, I investigated the role of osmolytes in mussel freeze tolerance, which may be cryoprotective by mitigating osmotic stress caused by freezing. I sought to determine if different osmolytes are interchangeable cryoprotectants (acting as colligative cryoprotectants), or if each osmolyte has unique a cryoprotective role, beyond just contributing to increased intracellular osmolarity (and thus act as non-colligative cryoprotectants). I did this by manipulating the composition of mussels' intracellular osmolyte pools, and then testing how mussel freeze tolerance changed. I found that mussel freeze tolerance did not change after taurine and betaine increased in concentration, significantly decreased after alanine and glycine increased in concentration, and increased with increasing TMAO concentrations, indicating that TMAO may be cryoprotective. Overall, my findings indicate that osmolytes are non-colligative cryoprotectants. Next, I explored how mussels are impacted by sublethal freezing. I found that mussels do not filter feed for the first four hours post-freeze, but resume filter feeding 24 hours after freezing, which corresponds to my microscopic examinations of mussel gill tissues after freezing which reveal freeze-related damage. I also found that freezing decreased mussel posterior adductor strength, although this effect did not lead to an increase in mussel susceptibility to sea star predation. Finally, I found that mussels survived shorter, repeated freezes (where mussels received 1 day for recovery

between freezes) better than prolonged freezes, when total time frozen is held constant. Thus, mussels are well-adapted to survive the short freezing events which they regularly encounter in their habitat, and one mechanism behind this survival could be TMAO accumulation. Further, the effects of sublethal freezing on mussel performance are limited, although how these effects scale up to entire mussel beds remains unknown.

Lay Summary

Intertidal mussels can survive being frozen, which is remarkable since freezing causes extreme stress to most organisms. To better understand how mussels survive freezing, in my thesis I tested whether a group of molecules called osmolytes increase mussel freeze tolerance. I found that artificially manipulating the concentration of osmolytes in mussels generally had either no effect or a negative effect on mussel freeze tolerance. I was, however, able to identify one osmolyte, TMAO, whose concentration correlated with increased mussel freeze tolerance. Additionally, I tested whether freezing negatively impacts mussels' ability to function normally post-freeze. I discovered that mussels do not filter feed until 24 hours post-freezing, but that mussels are not significantly more vulnerable to being preyed upon by sea stars post-freeze. Lastly, I found that mussels survive shorter, repeated freeze exposures better than prolonged freezes when total time frozen is held constant.

Preface

The sub-sections of my general introduction titled “Osmolytes as Potential Cryoprotectants” and “Anaerobic By-Products as Potential Cryoprotectants” were taken from my writing for an upcoming review article on intertidal invertebrate freeze tolerance which is currently in preparation. This review paper is being prepared in collaboration with Lauren Gill, Isaiah Box and Dr. Katie Marshall, although the sections that I’ve included in my thesis are sections that I wrote independently.

The mussel osmolyte incubation experiments detailed in chapter 1 were completed by me, under the guidance of Drs Katie Marshall and Christopher Harley. I completed these experiments primarily in the Marshall lab on the fourth floor of the Biological Sciences building at UBC Vancouver, and stored mussels in the Harley lab seawater tables on the first floor of the UBC’s Biodiversity Research Center. The ^{1}H NMR spectra for these experiments were all collected by Oleg Sannikov at UBC Chemistry’s NMR facility. I prepared the NMR samples, processed the NMR spectra, and quantified metabolite concentrations.

The experiments detailed in chapter 2 investigating the effects of repeated vs. prolonged freezing on mussel survival were completed in collaboration with Lauren Gill, another student in the Marshall lab. We split the work of designing the experiment and completing the lab work evenly between the two of us, but all the writing and analysis in this thesis were completed by me. The data from these survival assays are included in Lauren’s BSc Honours thesis, and in the publicly available pre-print: **Gill, L. T., Kennedy, J. R. and Marshall, K. E.** (2022). Proteostasis in ice: The role of heat shock proteins and ubiquitin in the freeze tolerance of the intertidal mussel, *Mytilus trossulus*. *bioRxiv* https://doi.org/10.1101/2022.02.03.478032. Additionally, the sea star feeding rate experiment in chapter 2 was completed with the help of the

Biology 326 undergraduate class; students in the class helped me set up and monitor this experiment, but again all of the writing and analysis regarding this experiment is my own. The rest of the experiments detailed in my second chapter were completed by me, under the guidance of my supervisors Drs Katie Marshall and Christopher Harley. Again, I completed these experiments primarily in the Marshall lab on the fourth floor of the Biological Sciences building, and stored mussels in the Harley lab seawater tables on the first floor of the UBC's Biodiversity Research Center. Additional suggestions and feedback from Dr. Patricia Schulte were extremely helpful in designing my experiments and during the editing process for both chapters of my thesis.

Appendix figure A.2 which shows intertidal temperature traces will also appear in an upcoming article which is currently in preparation for journal submission, led by Danja Currie-Olsen and various co-authors (the data displayed in figure A.2 was collected by myself). Appendix figures A.3 and A.4 displaying mussel freezing apparatus and temperature traces were taken from my BSc Honours thesis.

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List of Abbreviations

ANOVA	Analysis of variance
DAPI	4′,6-diamidino-2-phenylindole
DSS	Sodium trimethylsilylpropanesulfonate
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
HSP	Heat shock protein
LLT_{50}	Lower lethal temperature
PB	Phosphate buffer
PBT	Phosphate buffer with 0.5% Triton X-100
PCA	Principal component analysis
SCP	Supercooling point
TMA	Trimethylamine
TMAO	Trimethylamine *N*-oxide

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Thanks to the Natural Sciences and Engineering Research Council of Canada (NSERC) for awarding me a Canada Graduate Scholarship (CGS-M) which contributed to my funding during the second year of my Masters. Thanks also to UBC for awarding me the McLean Fraser Memorial Scholarship.

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Thanks to my friends, both within the Zoology department and outside of UBC, for the great memories and excellent company that really got me through my degree by giving me things to look forward to and laugh about to myself later- you know who you are!

Thanks to my family for being so supportive, and occasionally even reading my papers! In particular, thanks to my mom Michelle, my dad Todd, my sisters Katie and Ava, my grandmas Marie and Marlene, and my great uncle Gilbert. I'm so grateful to have such a great family.

Chapter 1: General Introduction

Ectotherms are animals which almost entirely unable to physiologically regulate their body temperature, meaning that their body temperatures vary with the temperature of their surroundings (Schmidt-Nielsen, 1975). When subjected to sub-zero environmental temperatures, ectotherms are susceptible to experiencing freezing body temperatures and, subsequently, internal ice formation. There are a select few ectotherm species that can survive internal ice formation and recover after thawing, despite the incredible amount of physiological stress that comes along with freezing live tissues. These organisms are referred to as freeze tolerant (Salt, 1961).

Freeze tolerant organisms have evolved strategies to survive the multiple types of damages that internal ice formation inflicts on normal physiological processes and structures. These freeze-inflicted damages are referred to as cryoinjuries (Lee, 2010). Typically, freeze tolerant organisms restrict freezing to their extracellular spaces to avoid intracellular freezing. Because of this, cell dehydration is one key consequence of freezing; as ice forms in extracellular spaces this concentrates the extracellular fluid, since locking up extracellular water into ice excludes solutes. This creates an osmotic gradient, causing water to leave cells, and leading to cell dehydration (Storey and Storey, 1988). Mechanical damage to cell membranes from ice crystals can also occur during freezing (Storey and Storey, 2013). Protein denaturation is another consequence of being exposed to extreme cold and/or freezing temperatures, which can render proteins dysfunctional (Ramløv, 2000). Similarly, cell membrane denaturation (i.e., changes to membrane permeability) is also a problem caused by exposure to extreme low temperatures and/or freezing itself (Ramløv, 2000).

1.1 Freeze Tolerance in Intertidal Invertebrates

The intertidal zone is the area along the ocean shore that is submerged under seawater during high tides yet exposed to air during low tides. This means that organisms inhabiting the intertidal zone need to survive periods of submersion and emersion (i.e., exposure to air). Each of these two phases of the tidal cycle come along with their own unique abiotic and biotic stressors that intertidal organisms must be able to tolerate (Kinuthia, 2018). Despite these stressors, a diverse array of algae, invertebrates and fish inhabit the intertidal zone and form complex, productive ecosystems.

One abiotic stressor faced by intertidal organisms is the threat of freezing during low tide air exposures. Intertidal organisms are susceptible to freezing when the tide recedes, and air temperatures are below the freezing point of that organism's tissues. Furthermore, since intertidal organisms are suddenly exposed to freezing air temperatures when the tide recedes, this subjects organisms to quite a rapid cooling rate (intertidal rocks cool at approximately -0.1 ºC/minute during freezing low tides; see Figure A.2), which imposes additional physiological stress (Murphy and Johnson, 1980). Additionally, because most intertidal organisms maintain high levels of moisture in their bodies during low tides to prevent desiccation, freezing is extremely difficult to avoid in the intertidal zone, since water readily nucleates into ice (Loomis, 1995). Mobile intertidal invertebrate species such as intertidal snails and crabs can move to warmer microhabitats to avoid or lessen the severity of cold temperatures they experience (Reid and Harley, 2021). However, intertidal invertebrates which are sessile or have limited mobility, such as intertidal mussels and barnacles, are unable to behaviourally avoid freezing during low tides. Thus, many sessile intertidal invertebrate species found in temperate and/or Arctic climates are freeze tolerant.

Intertidal organisms are exposed to freezing for relatively short, repeated freezing exposures in accordance with daily low tide exposures. When the tide returns and submerges organisms under seawater again, the organisms thaw, which may provide important reprieve for organisms to repair freeze-related damage and/or up-regulate protective mechanisms for subsequent freezes. However, these repeated sets of daily cool-freeze-thaw cycles may pose unique physiological stresses, as compared to more prolonged freezes, since the processes of cooling down to reach freezing body temperatures when the tide goes out and then thawing when the tide returns both come with their own set of unique damages (Lee, 2010; Storey and Storey, 1988). First, cooling down to low temperatures, even before freezing occurs, can be very physiologically damaging to ectotherms since extreme low temperatures can denature proteins, disrupt cell membrane fluidity, interfere with metabolic processes, and perturb cytoskeletal structure (Al-Fageeh and Smales, 2006; Ramløv, 2000). As frozen organisms begin to thaw, further damage can occur due to an accumulation of reactive oxygen species, in addition to the mechanical damage that ice recrystallization during thawing may cause (Doelling et al., 2014; Mazur, 2010). However, we currently lack a good understanding of the effects of repeated freezing in intertidal invertebrates, as compared to single freezing exposures, which represents a key lacking in our understanding of how ecologically relevant freezing regimes impact intertidal invertebrates.

While death is the most extreme result of experiencing the extreme abiotic stressor of freezing, there are may be other negative impacts of freezing on organism performance, which are still important to consider because they may have important ecosystem-level impacts. Since freezing results in so many different negative consequences on tissue, cellular, and macromolecular levels, it may cause a host of negative consequences to an organisms'

performance, apart from just increasing mortality, which may represent a largely under-appreciated impact of freezing to populations of freeze tolerant organisms and the ecosystems which they are a part of. Coping with damage inflicted by freezing and/or upregulating cryoprotective mechanisms can be energetically costly (Toxopeus and Sinclair, 2018) and may lead to an energetic limitation for freeze tolerant organisms, resulting in decreased performance. For instance, in the goldenrod gall fly, *Eurosta solidaginis*, freezing stress results in an energetic trade-off wherein up-regulation of cryoprotectants corresponds with lowered egg production (Marshall and Sinclair, 2018). Similarly, in the California mussel, *Mytilus californianus,* exposure to high temperature stress leads to reduced shell growth rates, highlighting another energetic trade-off between coping with abiotic stress and lowered organism performance (Fitzgerald-Dehoog et al., 2012). Alternatively, the direct damage that freezing inflicts on to tissues, cells, and macromolecules could directly inhibit an organisms' ability to performance normally. Thus, an important aspect of intertidal invertebrate freeze tolerance, which remains severely understudied, are the impacts of sublethal freezing stress on intertidal invertebrate performance.

1.2 Mechanisms of Freeze Tolerance

To survive the extreme stress of freezing, freeze tolerant animals employ a range of protective and reparative physiological mechanisms to avoid and cope with the negative consequences brought about by freezing. One key mechanism is the use of cryoprotectants, which are molecules used by freeze tolerant species to prevent some of the cryoinjuries inflicted by freezing. While the use of cryoprotectants in insects has been well established in the freeze tolerance literature (Lee, 2010), much less is known about intertidal invertebrate freeze tolerance, including which cryoprotectants are important in this group of animals. In the freeze

tolerance literature, cryoprotectants are often categorized into two broad types based on molecular weight: high molecular weight and low molecular weight cryoprotectants.

High molecular weight cryoprotectants include ice binding and heat shock proteins (Lee, 2010). Ice binding proteins come in many forms: some are cryoprotective by inoculating ice at relatively high temperatures which allows for more controlled ice formation, and they also enable organisms to restrict freezing to certain parts of their bodies only, such as extracellular spaces (Zachariassen, 1992). Alternately, other ice binding proteins prevent ice crystallization and/or recrystallization (Devries, 1982). Heat shock proteins (HSPs) are also thought to be cryoprotective since they are up-regulated in response to extreme cold and freezing in many freeze tolerant organisms (in *M. trossulus* Gill et al., 2022; in *Drosophila melanogaster* Štětina et al., 2015; in the goldenrod gall moth, *Epiblema scudderiana* Zhang et al., 2018). HSPs protect against protein denaturation caused by heat stress (Fabbri et al., 2008), and so it follows that they may also protect proteins during freezing and/or extreme cold stress.

Important low molecular weight cryoprotectants identified in insects include polyhydric alcohols such as glycerol and sorbitol, sugars such as trehalose and glucose, and amino acids such as proline and arginine (Lee, 2010; Toxopeus and Sinclair, 2018). Intertidal invertebrates fail to accumulate the same types of low-molecular weight cryoprotectants that are common in insects, at least not to the concentrations which would be required for substantial cryoprotection (Storey and Storey, 1988). This may be due to the marked differences between terrestrial and intertidal stressors, which necessitate different cryoprotective mechanisms. Some key differences include a) the length of freeze exposures (continuous for terrestrial organisms, separated by high tide submersion for intertidal organisms) and b) the essentially unavoidable presence of water in the bodies and surroundings of intertidal organisms, as opposed to terrestrial organisms which

can lower their body water content and thereby prevent, or at least delay, ice nucleation (Storey and Storey, 1988). While much less is known about the low molecular weight cryoprotectants used by freeze intertidal invertebrates, as compared to insects, previous studies have proposed two classes of potentially important low molecular weight cryoprotectants in intertidal invertebrates: osmolytes (Yancey, 2005) and anaerobic by-products (Anchordoguy et al., 1988; Storey et al., 2013).

1.2.1 Osmolytes as Potential Cryoprotectants

To cope with osmotic stress, most intertidal invertebrates osmoconform to their environment (Deaton, 2009; Foster, 1970) meaning that they modify the osmolarity of their cells to match the osmolarity of their surroundings (Yancey, 2005). Osmoconforming organisms primarily use organic osmolytes to adjust their intracellular osmolarity, however inorganic ions such as Na^+, Cl^-, K^+ can also be used for short-term osmoconformation. Osmoconformers preferentially use organic osmolytes for long-term osmoconformation since organic osmolytes are not perturbing to macromolecules, while inorganic ions typically are (Yancey, 2001). This means that as salinity conditions vary, intertidal invertebrates either increase or decrease the size of their intracellular pools of organic osmolytes to raise or lower the osmolarity of their cells to match their surroundings. Common organic osmolytes used by intertidal invertebrates include amino acids such as glycine, taurine, and alanine, as well as the methylamines betaine and trimethylamine *N*-oxide (TMAO) (Kube et al., 2006; Stickle et al., 1985; Sundell et al., 2019; reviewed in Yancey, 2005).

Since a consequence of freezing is osmotic stress, it follows that osmolytes may be cryoprotective insofar as they may protect against the osmotic stress associated with freezing. Support for this hypothesis comes from various previous studies which have shown that after

high salinity acclimation (~30-35 ppt seawater), intertidal invertebrates exhibit increased freeze tolerance relative to animals acclimated to lower salinity, brackish seawater (*Mytilus trossulus* in Kennedy et al., 2020; *Littorina littorea* and *Nassarius obsoletus* in Murphy, 1979; *Modiolus demissus* in Murphy and Pierce, 1975; *Balanus balanoides* in Cook and Lewis, 1971; *Mytilus edulis* in Williams, 1970; *Macoma baltica, Mytilus edulis, Mya arenaria,* and *Cardium edule* in Theede and Lassig, 1967). Additionally, Stickle et al. (2015) found that the osmolytes taurine and glycine were elevated in winter-collected *Littorina sitkana* and *Nucella lima*, corresponding to a seasonal increase in freeze tolerance. In the bivalve *Mytilus trossulus*, the osmolytes taurine, TMAO, betaine, alanine, and glycine all increased in concentration in the winter and after high salinity acclimation, corresponding to an increase in freeze tolerance in both cases (Kennedy et al., 2020). In the bivalve *Macoma balthica* alanine and glycine concentrations increased in the autumn and winter (although this was only true for 2/5 of the tested populations) (Kube et al., 2007). Taken together, these findings provide strong evidence that osmolytes play a cryoprotective role in freeze tolerant, osmoconforming intertidal invertebrates.

1.2.2 Anaerobic By-Products as Potential Cryoprotectants

To survive hypoxic conditions during daily low tide exposures, intertidal invertebrates, particularly sessile ones, must be able to anaerobically respire. There are multiple pathways utilized by marine invertebrates to generate ATP in hypoxic conditions, meaning that a variety of different anaerobic by-products accumulate in animals' tissues during hypoxia. One such pathway is the succinate pathway which results in succinate, propionate, and acetate accumulation, which is often coupled with the reduction of glycogen to alanine (Gäde, 1983; Kluytmans et al., 1975; Livingstone, 1991; Muller et al., 2012). Many intertidal invertebrates also utilize the lactate fermentation pathway during anaerobiosis, resulting in an accumulation of

lactate in tissues (Livingstone, 1991). Another common anaerobic pathway is the formation of opines such as strombine, octopine, and alanopine (Muller et al., 2012). Since intertidal invertebrates are exposed to hypoxia on a regular, often daily, basis, it is no surprise that their anaerobic metabolism pathways are so well-developed.

One reason why one might expect that anaerobic by-products are cryoprotective is because animals undergoing freezing for any significant amount of time must be able to anaerobically respire to maintain ATP supply, because freezing results in reduced oxygen supply since ice impairs oxygen transport (Storey and Churchill, 1995). Also, freezing and hypoxia exposure co-occur during low tides, so intertidal invertebrates would be accumulating anaerobic by-products in their tissues during the same time that they would be experiencing freezing, which lends further support to the hypothesis that anaerobic by-products are potential cryoprotectants.

Previous studies have provided some evidence for the cryoprotective role of anaerobic by-products. First, one study found that *M. edulis* increases freeze tolerance after being exposed to air or to oxygen-deficient water (Theede, 1972). Another study showed that in the intertidal bivalve, *Modiolus demissus*, acclimation to low temperatures caused a decrease in oxygen consumption, an accumulation of anaerobic by-products, and increased freeze tolerance (Murphy, 1977a). This observed increase in freeze tolerance might be due to the accumulation of anaerobic by-products. Alternatively, increased concentrations of calcium ions in the hemolymph (which accumulate due to anaerobiosis) may preserve membrane integrity during freezing (Murphy, 1977b). Finally, the observed increase in tolerance may be due to the absence of oxygen free radicals, which may reduce oxidative damage during freezing (Storey & Churchill, 1995).

To investigate this hypothesis further, Storey and Churchill (1995) determined how the metabolite profile of the intertidal mussel *Geukensia demissus* changed after exposure to either anoxia or freezing at -6 ºC for 2 and 12 hours by assaying anaerobic by-product concentrations immediately after mussels were exposed to each stressor. They found that most anaerobic by-products accumulated in all mussel tissues, except for gill tissues, after both stressors. However the relative change in the concentration of each by-product differed significantly between the freezing and anoxia groups. Churchill and Storey (1996) performed a similar experiment on the intertidal periwinkle *Littorina littorea* and found that lactate and succinate concentrations significantly increased in *L. littorea* foot muscle tissues after 72 hrs of freezing at -8 ºC, while almost no change in metabolite concentrations, apart from a very slight increase in lactate, occurred after 72 hrs of anoxia. By contrast, after 3 hours of freezing at -6 ºC followed by 24 hours of recovery time, very little change in anaerobic by-products was observed in *Mytilus trossulus*, with aspartate (an osmolyte) being the only metabolite to significantly increase in concentration following freezing (Kennedy et al., 2020). Overall, it remains unclear how or if metabolic processes and anaerobic by-product concentrations change in response to freezing, thus more careful study is necessary.

Lastly, Loomis et al. (1989) demonstrated that various anaerobic by-products stabilized small unilamellar vesicles and maintained the activity of the freeze-labile enzymes lactate dehydrogenase and phosphofructokinase during freeze/thaw cycles *in vitro,* which lends support to the hypothesis that anaerobic by-products may be cryoprotective *in vivo*. The anaerobic by-products that were best at stabilizing unilamellar vesicles were alanopine, alanine, acetate, lactate, and propionate, while octopine and strombine also had a protective effect, but to a lesser

extent than the aforementioned group. All by-products, however, worked to maintain enzyme activity to a similar degree (Loomis et al., 1989).

1.3 Study Organism: *Mytilus trossulus*

To better understand the mechanisms and consequences of surviving freezing in intertidal invertebrates, I used the intertidal mussel *Mytilus trossulus,* the bay mussel, as a model organism throughout my thesis. *M. trossulus* is common intertidal mussel and has a range which extends along the west coast of North America as far north as the Gulf of Alaska (Suchanek et al., 1997). *Mytilus trossulus* and its closely-related congener *Mytilus edulis* are freeze tolerant (Bourget, 1982; Williams, 1970). Furthermore, Mytilid mussels are ecosystem engineers in the intertidal since mussel beds (which are dense aggregations of mussels that commonly form in the intertidal) increase habitat structural diversity and therefore increase intertidal species richness and biodiversity (Borthagaray and Carranza, 2007; Buschbaum et al., 2009; Gutiérrez et al., 2003). I chose *M. trossulus* as my model organism not only because Mytilid mussels are a commonly used model organism in other freeze tolerance studies, but also because their ecological importance suggests that their ability to survive freezing has important downstream impacts on intertidal ecosystems.

One of the main biotic factors shaping intertidal mussel density on rocky shorelines in the Northwest Pacific ocean is predation by sea stars (Paine, 1974). Sea stars consume mussels by using their tube feet to pry open mussels' shells to access the mussel's flesh (Fig C.1; Feder, 1955). Thus, mussels' primary defence against predation by sea stars is to use their posterior adductor muscle to hold their shells shut, working against the pulling force exerted by sea stars. Thus, mussels' posterior adductor muscle strength directly relates to how susceptible mussels are

to sea star predation, particularly since mussels are sessile and therefore cannot move to avoid sea star predation.

Mussels are filter feeders, meaning that they consume phytoplankton from the water column by pumping water into their bodies using their siphons, then trapping phytoplankton in their gills for subsequent ingestion (Jørgensen, 1996). Mussel gills are made up of two lamellae which line each half of the mussels' mantle cavity. Each lamella is made up of many rows of filaments running parallel to one another that are each lined with cilia for capturing food particles. More specifically, the latero-frontal cilia of neighbouring gill filaments form a lattice with one another which enables the mussel to "catch" plankton cells from the water column (Riisgård et al., 2015). Since the gills are freely suspended in the mussel's mantle cavity *in vivo*, and are not supported by another organ, mussel gills have small pieces of connective tissue termed "ciliary junctions" or "cross struts" which run perpendicular to adjacent mussel gill filaments to ensure that gill filaments remain parallel to one another to enable filter feeding (Riisgård et al., 2015; Silverman et al., 1996).

A mussel's ability to filter feed is important to maintain adequate energy supply, but moreover, a mussel bed's collective filtration rate can also affect the whole ecosystem since it can significantly increase local water quality by taking up suspended clay and silt along with food particles while filter feeding (Al-Mamun and Khan, 2011). A single mussel can filter up to 40 L of water per day, and so the daily filter feeding capacity of an entire mussel bed is considerable, particularly in areas with high phytoplankton densities (Jansen et al., 2018; Tankersley and Dimock, 1993). In eutrophic areas, high mussel densities can significantly reduce phytoplankton densities and therefore mussel farms have been identified as a potential mitigation tool to counteract eutrophication (Petersen et al., 2014). Another reason why filtration by benthic

invertebrates such as mussels is ecologically important is because it serves as a key link between benthic and pelagic marine ecosystems (Menge et al., 1997), allowing carbon fixed by phytoplankton to enter other portions of the food web (e.g., marine ducks which rely on mussels as a food source; Nilsson et al., 1972).

Mussel gills are important organs not only due to the fact that they are critical for feeding, but mussel gills are also respiratory organs and key sites of osmoconformation (Neufeld and Wright, 1996). Mussel gills are optimal tissues for osmoconforming since they are directly exposed to the external environment, and because mussel gill tissues are also respiratory surfaces, gills have a relatively large surface-area-to-volume ratio and thin epithelium which allows for optimal exchange of ions and osmolytes between mussels and their external environment (Bradley, 2010). There are multiple transporters on the surface of mussel gills which can transport amino acids such as taurine, alanine, and glycine (Rice and Stephens, 1988) as well as betaine (Wright et al., 1992) between the surrounding seawater and mussel cells, which is the primary method of osmoconformation in mussels.

1.4 Main Objectives

While an increasingly robust body of literature on intertidal invertebrate freeze tolerance is currently being established, there are still many notable gaps in the literature that limit our understanding of intertidal invertebrate freeze tolerance. To fill these knowledge gaps, in my thesis I investigated the mechanisms and consequences of surviving freezing in the intertidal mussel *Mytilus trossulus*.

1.4.1 Objective 1: Cryoprotective Role of Osmolytes in Intertidal Invertebrates

To better understand how mussels survive freezing, the first main objective of my thesis was to determine what the cryoprotective role of osmolytes are in intertidal mussels. While

previous literature lends solid support to the hypothesis that osmolytes may increase freeze tolerance capacity intertidal invertebrates (Kennedy et al., 2020; Loomis et al., 1988), it is still not clear what the exact cryoprotective mechanism of osmolytes is. Osmolytes may function colligative basis wherein each osmolyte is interchangeable with one another and the collective pool of osmolytes is what aids in cryoprotection, or osmolytes may function cryoprotectively in a non-colligative way, wherein each different osmolyte offers its own, unique cryoprotective function, beyond just protecting against osmotic stress. To better understand the role of osmolytes in intertidal invertebrate freeze tolerance, I artificially manipulated the osmolyte pool of *M. trossulus* and then tested the mussels' freeze tolerance. This experiment was designed to inform us as to whether one or some osmolytes are uniquely cryoprotective (i.e., non-colligative), as evidenced by a change in freeze tolerance after the relative proportions of osmolytes changes. However, if mussel freeze tolerance does not change after their osmolyte pool composition is manipulated, then this would indicate that osmolytes are colligative cryoprotectants, given that mussel's total intracellular osmolarity does not change.

1.4.2 Objective 2A: Effects of Sublethal Freezing on Performance

Next, to test the hypothesis that responding to the stress of freezing may either elicit energetic trade-offs in mussels and/or cause direct damage that results in lowered performance, I sought to quantify two types of potentially negative and biologically relevant impacts of freezing on mussel performance. The first performance metric I examined was filtration rate, since if freezing lowers filtration rate, this would reduce mussel energy supply, and also potentially decrease local water quality. Relatedly, to characterize the damage that freeze inflicts on to mussel gills, I visualized changes to mussel gill tissue morphology after freezing using microscopy. The second response I measured was how mussel susceptibility to sea star predation

changed post-freezing, which I quantified by measuring the strength of mussels' posterior adductor muscles and by determining if sea star feeding rate increases when sea stars are fed recently frozen mussels.

1.4.3 Objective 2B: Effects of Repeated Freezing

Another main objective of my thesis was to investigate how *M. trossulus* are impacted by multiple repeated freezing exposures, and how this compares to the effects of a single, prolonged freezing exposure. This is an ecologically important question to investigate since a cold snap (i.e., a series of particularly cold winter days) may align with a low tide series, which would expose intertidal invertebrates to several successive freezing exposures over the course of a set of consecutive daily low tides (see Fig A.2 for an example intertidal temperature trace which shows that intertidal rocks reach freezing temperatures multiple times over the course of subsequent daily low tides during a winter cold snap).

Chapter 2: Are Osmolytes Colligative or Non-Colligative Cryoprotectants in Mussels?

2.1 Introduction

Freeze tolerance refers to the ability of an organism to survive internal ice formation. Survival of ice formation involves coping with a wide array of physiological stressors, including, but not limited to, mechanical damage from ice, osmotic stress, protein and membrane damage, and oxidative damage (Lee, 2010; Storey and Storey, 2002). Thus, to survive freezing, organisms need to employ sophisticated physiological strategies to cope with these damages. Much remains unknown about what strategies enable freeze tolerance in animals, and how exactly these strategies work. One general mechanism that freeze tolerant organisms use to survive freezing is to up-regulate molecules termed cryoprotectants, which are molecules that help organisms cope with freeze-related damages. Cryoprotectants are generally split into two groups, based on molecular weight. High molecular weight cryoprotectants include large molecules such as ice binding proteins and heat shock proteins (Storey and Storey, 2013). Low molecular weight cryoprotectants include smaller molecules such as sugars, polyols, and amino acids (Lee, 2010). Much remains unknown about the exact identity and mechanism of these cryoprotectants across different types of freeze tolerant animals. This is especially true in the case of the severely understudied group of freeze tolerant organisms, intertidal invertebrates.

Intertidal invertebrates need to be freeze tolerant if air temperatures in their habitat often drop below the freezing point of their tissues, since winter low tide air exposures make them susceptible to freezing. Further, the constant presence of ice nucleators (e.g., water) in their environment means that they will experience freezing in their tissues and must be able to tolerate

this stressor. Moreover, while mobile organisms such as intertidal snails or crabs can behaviourally avoid freezing either by moving into subtidal regions or moving to relatively warmer microhabitats such as underneath rocks or seaweed (Reid and Harley, 2021), sessile species such as mussels and barnacles are unable to behaviourally avoid sub-zero temperatures. Despite the prevalence of freeze tolerance in intertidal invertebrates, little is known about which cryoprotectants are used by this group of freeze tolerant animals.

Previous work has identified osmolytes as a potentially important group of cryoprotectants in intertidal invertebrates. Many studies have found that acclimation to high salinity significantly increases freeze tolerance in intertidal invertebrates (Kennedy et al., 2020; Murphy and Pierce, 1975; Williams, 1970). To understand why this might happen, it is important to note that freezing live tissues leads to cell dehydration as extracellular water is locked away into ice which concentrates the extracellular fluid and causes osmotic stress (Storey and Storey, 1988). Many intertidal invertebrates are osmoconformers, which means that they will match the osmolarity of their cells to their external environment (Deaton, 2009). Osmoconforming marine invertebrates modify the osmolarity of their cells using organic osmolytes (which are inert molecules such as amino acids and methylamines), as well as inorganic ions such as sodium, chloride, and potassium ions (Yancey, 2001). Most organisms utilize inorganic ions only for short-term osmoconforming, since high concentrations of inorganic ions can disrupt normal protein functioning, which is why organic osmolytes, which are much less protein-perturbing, are preferred for long-term osmoconformation (Yancey, 2001). Intertidal invertebrates such as bivalves can take up organic osmolytes from the surrounding seawater using gill transporters which can actively transport various amino acids into mussel gills, even against large concentration gradients (Wright and Pajor, 1989). Gills are the primary site of osmoconformation

in marine bivalves for this reason, and because the large surface area and thin epithelium of the gills is suited to osmoconform by exchanging molecules with the surrounding seawater (Bradley, 2010).

After acclimation to high salinity, osmoconforming intertidal invertebrates increase their intracellular osmolarity using organic osmolytes, and these organic osmolytes may protect cells from dehydrating during freezing, explaining the link between high salinity acclimation and increased survival after freezing. My previous study demonstrated that there is a correlative link between high salinity acclimation, increased intracellular osmolyte concentrations, and increased freeze tolerance in intertidal *Mytilus trossulus* mussels (Kennedy et al., 2020). Additionally, I showed that osmolyte concentrations increase in winter-collected mussels, corresponding to a seasonal increase in freeze tolerance, which provides further support for the cryoprotective role of osmolytes. This finding is supported by *in vitro* studies which identified that osmolytes such as taurine, betaine, glycine, and alanine protect unilamellar vesicles during freeze-thaw cycles, suggesting that they are cryoprotective since they may protect cell membranes from damage during freezing *in vivo* (Anchordoguy et al., 1987; Anchordoguy et al., 1988; Loomis et al., 1988).

Another proposed group of potentially important cryoprotectants in intertidal invertebrates are anaerobic by-products (Loomis et al., 1988; Storey and Churchill, 1995), which accumulate in invertebrate tissues as by-products of anaerobic metabolism during hypoxia caused by low tides. Anaerobic by-products commonly found in intertidal invertebrates include alanine, succinate, octopine, propionate, acetate, and strombine (Muller et al., 2012). However, in my previous study I did not find evidence that freeze tolerance in *M. trossulus* was correlated with anaerobic by-product accumulation, apart from alanine, which did correlate with freeze

tolerance and serves as both an osmolyte and anaerobic by-product (Kennedy et al., 2020). However, mussels produce a wide array of anaerobic by-products, not all of which I was able to measure, and so whether changes in these anaerobic by-products are correlated with freeze tolerance remains unknown.

Osmolytes correlate with increased freeze tolerance in intertidal invertebrates, but their exact cryoprotective role is still unknown. There are, however, two possibilities regarding the cryoprotective role of osmolytes. First, osmolytes may function as colligative cryoprotectants, meaning that osmolytes only offer cryoprotection through increasing intracellular osmolarity, and the identity of the osmolytes which make up an organism's intracellular osmolyte pool is irrelevant in terms of freeze tolerance, only the size of the osmolyte pool matters. An alternate possibility is that osmolytes are non-colligative cryoprotectants, meaning that different osmolytes have their own unique cryoprotective functions, beyond just protecting cells against osmotic stress from freezing. In this case, the composition of an organism's osmolyte pool would impact that organism's freeze tolerance. The non-colligative hypothesis is supported by the fact that many osmolytes have unique cytoprotective functions that may protect cells from freezing damage. For instance, taurine from hemolymph of the intertidal mussel *Mytilus edulis* preserves the integrity of unilamellar vesicles during freeze thaw cycles *in vitro*, meaning that taurine may also preserve cell membranes during freezing *in vivo* (Loomis et al., 1988). Betaine, glycine, and alanine all reduce unilamellar vesicle fusion and leakage *in vitro* (Anchordoguy et al., 1987; Anchordoguy et al., 1988). Taurine also has antioxidative properties which might protect cells against the oxidative damage that may result from freezing (Schaffer et al., 2003). Additionally, the osmolytes TMAO and betaine are protein stabilizing, and thus may preserve protein structure during and after freezing stress (Bennion et al., 2004; Street et al., 2006).

To better understand the specific role of osmolytes in intertidal invertebrate freeze tolerance, I set out to test whether osmolytes function as cryoprotectants on a colligative or non-colligative basis in *Mytilus trossulus*, a freeze tolerant intertidal mussel. My approach was to artificially manipulate the intracellular concentrations of osmolytes in mussels, and then determine if mussel freeze tolerance changed. If any of the tested osmolytes are uniquely cryoprotective (i.e., non-colligative cryoprotectants), this would be indicated by an increase in freeze tolerance caused by an increased concentration of that osmolyte in mussel tissues. If osmolytes are colligative cryoprotectants, then one would expect to see no change in freeze tolerance after composition of the osmolyte pool changes, given that the cumulative intracellular osmolyte pool concentration remains constant. I predicted that osmolytes are non-colligative cryoprotectants, given the fact that many osmolytes have unique cytoprotective attributes that may protect cells from freezing damage.

2.2 Methods

2.2.1 *Mytilus trossulus* Collection

All intertidal *Mytilus trossulus* specimens were collected from Tower Beach, which is in the Point Grey area of Vancouver, British Columbia, Canada (49°16′26.1″N, 123°15′23.7″W; Fig A.1). All collections were completed during low tides, when tidal height was <1 m above the chart datum. Vancouver has relatively mild winters, but winter temperatures can reach lows of −10 °C to −15 °C (Environment and Climate Change Canada; see Figure A.2). The tides in this area are mixed semi- diurnal, with two low and two high tides per day. In the winter, the lower daily low tide usually happens during the night, meaning that intertidal invertebrates are exposed to some of Vancouver's coldest air temperatures during winter low tides. Due to this site's proximity to the Fraser River, which inputs fresh water into the surrounding seawaters, Tower

Beach is an estuarine site with maximal salinities around 27 ppt in the winter and minimal salinities as low as 6 ppt during the summer, due to the increased river outflow resulting from the spring freshet (Covernton and Harley, 2020; Kennedy et al., 2020).

Mussels were collected from the same outcropping of rocks at Tower Beach on each sampling date. All mussels used in these experiments were collected at approximately 1 m above Canadian chart datum (mean lower low water) on the rock outcropping which is directly in front of the abandoned searchlight tower at Tower Beach. Only mussels with shell lengths between 2 and 3.5 cm were collected. This size range was intentionally chosen so that mussels could fit into plastic vials for experimental cold exposures. All mussel collections were completed under Scientific Licences, Management of Contaminated Fisheries Regulations from the Department of Fisheries and Oceans Canada (licence number XMCFR 33 2020 covered mussel collections up until August 9, 2021, and licence number XMCFR 34 2021 covered collections from September 21, 2021, onwards).

2.2.2 Osmolyte Uptake by Gill Tissues

To modify mussel intracellular osmolyte concentrations, I incubated mussels in seawater that had been enriched with osmolytes. I used alanine, betaine, glycine, taurine, and TMAO, which are the most dominant osmolytes found in mussel gill tissues (Kennedy et al., 2020). First, to ensure that incubating mussels in osmolyte-enriched seawater resulted in increased intracellular osmolyte concentrations, I measured the low-molecular weight metabolite profile of mussel gills that had been incubated in seawater enriched with each of the five osmolytes daily across a three-day incubation period to determine how long I would need to incubate mussels in osmolyte-enriched seawater before I tested mussels' freeze tolerance.

Mytilus trossulus used in this experiment were collected from Tower Beach, Vancouver, BC on October 15, 2021. They were kept in the recirculating seawater tables in the Biodiversity building at UBC (7 ºC, 32 ppt) for four days before experimentation. In five separate 1 L glass beakers (one beaker per osmolyte), 750 mL of natural seawater sourced from UBC's Biosciences building was combined with one of the five osmolytes (alanine, betaine, glycine, taurine, or trimethylamine *N*-oxide (TMAO)) to achieve a 40 mM concentration of each osmolyte. 40 mM was the chosen concentration since previous work has shown that mussel tissues took up osmolytes when incubated in seawater enriched with osmolytes at this concentration (Dr. Inna Sokolova, pers. comm.). Then, dechlorinated water was added to the seawater so that a salinity of 29 ppt was maintained across treatments (± 1 ppt). Salinity was measured using a refractometer (Tangxi, Sanming, China). One control 1 L beaker with 750 mL of 29 ppt seawater was also prepared. L-alanine, betaine, glycine, and taurine were all sourced from Sigma-Aldrich and were ≥99.0 % purity. TMAO was sourced from Fisher Scientific and was ≥95.0 % pure.

Twelve randomly selected mussels per treatment were placed in each of the osmolyte enriched seawater beakers. Four mussels were placed in the control beaker. The beakers were kept in Panasonic incubators (MIR-254) set to 10 ºC, and the beakers were all strongly aerated using air stones and pumps. To prevent possible water evaporation and excessive splashing from the air stones, beakers were covered with plastic saran wrap and secured using a rubber band.

To determine the time course for osmolyte uptake into gill tissues, I incubated mussels in each of the osmolytes for 24, 48, and 72 hours. At each time point, four mussels were removed from their treatment, their gills were immediately excised, the gill tissues were rinsed for 2 × 5 min in plain seawater, and then dabbed dry with a KimWipe. Gills were weighed out to 100 mg

and frozen at -80 ºC for later NMR analysis. The four control mussels were removed from their beaker after 72 hours, after which their gills were excised, dabbed dry, weighed, and frozen.

I then incubated mussels in seawater that had been enriched with a combination of all five osmolytes, proportional to the natural concentrations of these osmolytes in mussel gills, which I refer to as the "osmolyte cocktail" treatment. To create the "osmolyte cocktail" seawater solution, I used similar protocol as above. Mussels used here were collected on December 1, 2021, from Tower Beach. Using the results from Kennedy et al. (2020) I calculated the average gill concentrations of each of the five most-dominant osmolytes across a sample of 10 *Mytilus trossulus* individuals that had been acclimated to 30 ppt salinity seawater (see Table 2.1). I then calculated the relative proportion of each of these osmolytes, compared to the cumulative concentration of all the five dominant osmolytes. I used these proportions to then calculate how much of each osmolyte to add to 750 mL seawater to reach a cumulative 40 mM concentration of osmolytes. I incubated four mussels in the osmolyte cocktail for 72 hours, before excising, washing, weighing, and freezing the mussels' gill tissues at -80 ºC for subsequent NMR analysis.

Table 2.1 Mean concentrations (± SE) of the five most prominent osmolytes in *Mytilus trossulus* gill tissues. These concentrations were then used to calculate the concentrations of osmolytes which were combined to create the "osmolyte cocktail" seawater solution. Data are from mussels collected November 29, 2019 from Tower Beach, Vancouver, BC (*N*=10; Kennedy et al., 2020).

Osmolyte	Mean Gill Concentration (mM/100 mg)	Percentage of Gill Osmolyte Pool (%)	Concentration in "Osmolyte Cocktail" Seawater Solution (mM)
Taurine	27.3 ± 0.844	31.5	12.8
TMAO	14.8 ± 0.483	17.1	6.8
Betaine	13.7 ± 1.02	15.8	6.4
Alanine	9.9 ± 0.643	11.4	4.4
Glycine	21.1 ± 2.00	24.3	9.6
Total	86.8	100	40

2.2.3 ^{1}H NMR Metabolomics

To ensure that mussels were taking up osmolytes in their gill tissues after incubation in osmolyte-enriched seawater, I measured mussel gill metabolite profiles using one-dimensional, 600 MHz proton nuclear magnetic resonance spectroscopy (^{1}H NMR). ^{1}H NMR is ideal for measuring low molecular weight, polar metabolites such as osmolytes. I chose gills as the representative tissue since one of the main functions of bivalve gills is to enable mussels to osmoconform to the surrounding seawater (Cappello et al., 2013; Rice and Stephens, 1988).

Sample preparation was based on Cappello et al. (2013). 100 mg of mussel gill tissue was excised, dried with a Kimwipe to remove excess water and frozen at −80°C. Frozen tissue was homogenized in 400 µL cold methanol and 85 µL cold water-xylitol solution (5 mM xylitol, used as a standard to reduce inter-sample variation caused by potential sample loss during sample preparation) using a bead homogenizer (Bullet Blender 50 Gold Model: BBX24, Next Advance) with approximately 200 µL of 3.2 mm round stainless steel beads for 10 min at setting 8 in 1.5 mL microcentrifuge vials. After adding 400 µL chloroform and 200 µL water to the samples, they were vortexed for 60 s, left on ice for 10 min for phase separation, and centrifuged for 5 min at 2000 rpm. The upper methanol layer (600 µL) containing the polar metabolites was transferred into new vials, dried in a centrifugal vacuum concentrator (Eppendorf 5301), and then stored at −80°C. Immediately prior to NMR analysis, the dried polar extracts were resuspended in 600 µL of 0.1 mol/l sodium phosphate buffer (pH 7.0, 50% deuterium oxide, Sigma-Aldrich) containing 1 mmol/l 2,2-dimethyl-2-sila- pentane-5-sulfonate (DSS; Sigma-Aldrich) as internal reference. The mixture was vortexed for 60 s and transferred to a 5 mm NMR tube.

^{1}H NMR spectra were acquired using Bruker Avance 600 with cryoprobe and Bruker Avance III 600 spectrometers (Bruker, Billerica, Massachusetts, USA) at the UBC Chemistry

NMR facility. TopSpin software version 2.1 (Bruker, Billerica, Massachusetts, USA) was used to process spectra collected with the Bruker Avance 600 spectrometer with cryoprobe, and TopSpin version 3.5 (Bruker) was used with the Bruker Avance III 600 spectrometer. Experiments required 15 minutes of acquisition time and were performed at room temperature.

Peak identification of the NMR spectra was performed with Chenomx NMR Suite 9.0 (Chenomx, Edmonton, Alberta, Canada) that uses the Human Metabolome Database compound spectral reference library (Wishart et al., 2018). First, line broadening of 2.5 Hz, automatic phase correction, and manual baseline correction were performed with Chenomx Processor (within the Chenomx NMR Suite software). Then, determination of metabolite concentrations was performed using Chenomx Profiler, which determines the concentrations of individual metabolites using the concentration of a known DSS signal. Metabolite concentrations are reported as mmol/100 mg gill wet mass. More specifically, for each sample, a standardization value was calculated by dividing that sample's measured xylitol concentration by the average xylitol concentration across all samples. Then, I multiplied all metabolite concentrations in each sample by that sample's xylitol standardization value.

2.2.4 How Osmolyte Incubation Impacts Freeze Tolerance

Next, I tested mussel freeze tolerance after incubation in osmolyte-enriched seawater. For all treatments except the osmolyte cocktail treatment, I used mussels that were collected on November 3, 2021. Mussels used in the osmolyte cocktail treatment were collected on December 1, 2021. The set-up for this experiment was the same as the osmolyte uptake experiment that proceeded it, apart from a few differences. 24 mussels were placed in each of the beakers (one beaker per osmolyte treatment, plus one control treatment beaker). All mussels were removed

from the osmolyte-enriched seawater after 72 hours of incubation. Also, mussel shell length was recorded for these experiments and mussels were labelled with ID numbers using nail polish.

After 72 hours, mussels were removed from their respective treatments, their shells were dried with paper towels and then split evenly into two groups, one group of 12 mussels was frozen at -10 ºC for 3 hours, and the other 12 were frozen at -12 ºC. These temperatures were chosen since the LLT_{50} (which is the temperature predicted to cause 50% mortality in a population of organisms) of mussels from Tower Beach on November 29, 2019 was -11.6 ± 0.3 ºC (Kennedy et al., 2020).

For the freezing exposures, mussels were placed in 35 mL vials (diameter=2.2 cm), and the vials were placed into wells in an aluminium head (insulated by foam) that was cooled using a methanol and water mixture (60:40, v/v) that was circulated by a refrigerated bath (ECO Silver: RE 415 S Model, Lauda, Wurzburg, Germany; Fig A.3). All mussels were emersed during my laboratory cold exposures to mimic a low tide. The bath cooling rate was −1.5°C per minute. The length of cold exposure was measured as the time after the cooled bath reached the set temperature, as measured by thermocouples that were attached with putty to the aluminium head.

Mussel freezing was indicated by the presence of a supercooling point (SCP). The SCP is defined as the lowest temperature immediately prior to the exothermic release of energy due to ice formation, visualized as a sharp spike in temperature on the temperature trace graph (Fig A.4). Thus, the presence of a SCP indicates that internal ice formation has occurred (Lee, 2010). Mussel body temperatures were monitored using a 16 gauge Type T thermocouple that had been taped to each mussel's shell and connected to Picolog TC-08 thermocouple interfaces, so that mussel body temperatures could be recorded continuously during freeze exposures using PicoLog 6 beta software for Windows (Pico Technology, Cambridge, UK). Thermocouple

sampling rate was once per second. Since previous experiments have verified that all mussels exposed to temperatures lower than -8 ºC for three hours freeze, as evidenced by a SCP, I only continuously monitored eight representative mussel body temperatures per cold exposure, to confirm ice formation.

Immediately after the low temperature exposure, mussels were returned to the sea water table and separated by treatment using 150 mL plastic containers with mesh windows to allow water flow. Survival was assessed daily after the cold exposure for 1 week. Mussels were considered dead when they were unable to hold their shells shut after removing them from the seawater.

For the follow-up osmolyte cocktail freeze tolerance experiment, mussels were collected on December 1, 2021, and the osmolyte cocktail seawater solution was prepared as described above. To ensure that collection date effects did not interfere with results, I tested the freeze tolerance of another group of control mussels that had also been collected on December 1, 2021. I incubated these control mussels in a beaker of natural seawater under the same conditions (750 mL seawater at 29 ppt in a 10 ºC incubator) for 72 hours. Mussels tested here were also frozen at -10 ºC and -12 ºC for 3 hours (n=12), with the same experimental freezing methods as stated above.

2.2.5 Statistical Analyses

Data analysis was performed using R (c. 4.0.3; R Development Core Team, 2021). Packages from the "tidyverse" suite were used to analyse and visualize data (Wickham et al., 2019).

To determine how incubation time impacted mussel gill metabolite concentrations, I performed separate ANOVAs for each group of osmolyte-incubated mussels, followed by Tukey

post-hoc tests where appropriate, for all gill metabolites found in >20% of samples. For each osmolyte-incubation treatment, I tested whether different incubation times (24 h, 48 h, 72 h or control) had significantly different concentrations of each gill metabolite, with a separate model used for each metabolite. Lastly, an ANOVA with a Tukey post-hoc test was used to determine whether the total organic metabolite pool concentration in mussel gills changed after 72 hours of incubation in any of the osmolyte treatments, relative to control mussels incubated in plain seawater. Gill osmolyte concentrations were all cube-root transformed to better adjust for normality before ANOVA analyses were conducted.

Logistic regressions were used to test how different osmolyte incubation treatments and test temperatures impacted mussel survival after freezing, with mussel shell length used as a covariate. Then, a generalized linear model was used to test if mean total gill organic metabolite pool concentration was a significant predictor of the proportion of mussels which survived after freezing at either test temperature. Next, a principal component analysis was generated using all mussel gill metabolite profiles across all osmolyte-enriched seawater treatments and timepoints. I extracted the mean PC scores for mussels from the 72-hour timepoint, and then performed a logistic regression to determine if any of the first 4 mean principal component scores significantly predicted the number of mussels which survived after freezing at either -10 ºC or -12 ºC.

Finally, the mean concentration of each metabolite across the various osmolyte-incubation treatments was used as a predictor variable in a logistic regression model to determine if any metabolite concentration significantly predicted mussel survival after freezing. Test temperature was also included as a covariate in these models, unless the interaction term between

metabolite concentration and test temperature was found to be significant, in which case the interaction term was included in the model.

2.3 Results

2.3.1 Osmolyte Uptake by Gill Tissues

Only metabolites found in > 20% of mussel gill tissue samples were analysed. These metabolites were: alanine, betaine, glycine, malonate, succinate, taurine, trimethylamine, trimethylamine *N*-oxide, and β-alanine. As in Kennedy et al. (2020) the dominant metabolites in gill tissues were taurine and glycine, followed by betaine, alanine, and TMAO. A representative ^{1}H NMR spectrum is shown in Figure B.1.

For each osmolyte incubation treatment, I first tested how well mussel gills took up the osmolyte that they were incubated in (just considering the single osmolyte incubation experiments) by comparing how the concentration of the osmolyte they were incubated in changed as incubation time increased (Table 2.2). For alanine incubated mussels, incubation time was a significant predictor of mussel gill alanine concentration, with alanine concentrations increasing as incubation time increased ($F_{3,12}$=54.15, $p<0.001$; Fig 2.1). Similarly, for betaine, glycine, and taurine incubated mussels, longer incubation times also resulted in significantly increased betaine, glycine, and taurine concentrations, respectively (betaine $F_{3,11}$=36.12, $p<0.001$; glycine $F_{3,12}$= 7.72, $p=0.00389$; taurine $F_{3,11}$=13.01, $p<0.001$). However, for TMAO incubated mussels, incubation time was not a significant predictor of mussel gill TMAO concentration ($F_{3,12}$=2.402, $p=0.119$).

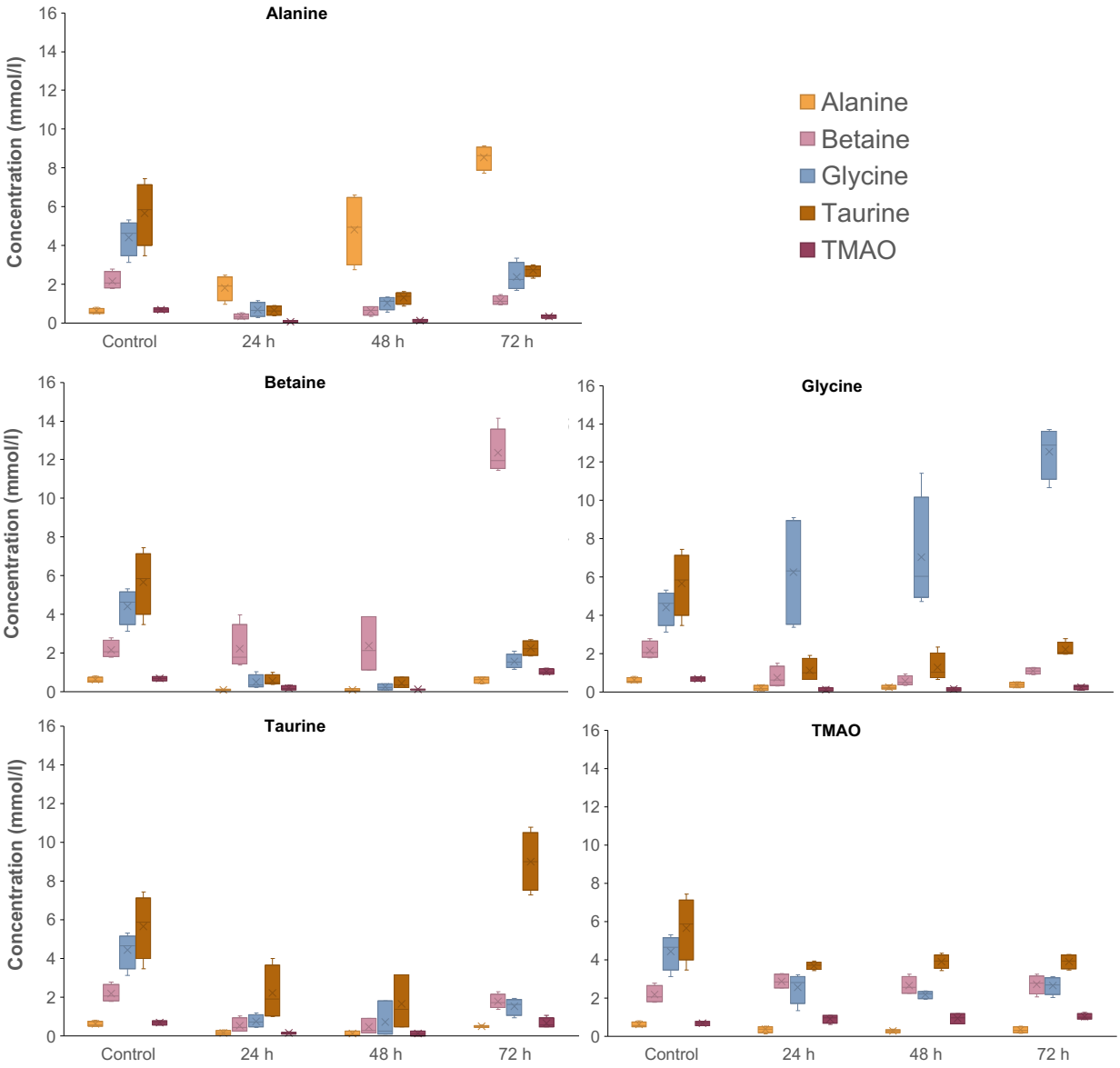

Figure 2.1 Osmolyte uptake occurs in *Mytilus trossulus* gill tissues after incubation in seawater enriched with 40 mM of that osmolyte, however compensatory decreases in the concentrations of other osmolytes also occurs (apart from in TMAO-incubated mussels). The horizontal axes indicate how long mussels were incubated in the osmolyte-enriched seawater; maximal uptake occurs after 72 hours of incubation. apart from TMAO. The title of each plot indicates which osmolyte that group of mussels was incubated in. Osmolyte concentrations are per 100 mg of gill tissue. Control mussels were incubated in plain seawater (n=4).

Table 2.2 Tukey post-hoc test results indicate how *Mytilus trossulus* osmolyte concentrations change over time when mussels are incubated in seawater enriched with 40 mM of that osmolyte. Mussels incubated in TMAO enriched seawater did not significantly increase their TMAO gill concentrations and are excluded from this table. Mussels were collected from Tower Beach, Vancouver, BC (n=4). Bolded text indicates significant p values.

Osmolyte Incubation Treatment	Comparison	p value
Alanine	**24 h - Control**	**0.0180**
	48 h - Control	**<0.001**
	72 h - Control	**<0.001**
	24 h - 48 h	**0.00298**
	24 h – 72 h	**<0.001**
	48 h – 72 h	**0.0129**
Betaine	24 h - Control	1.00
	48 h - Control	1.00
	72 h - Control	**<0.001**
	24 h - 48 h	1.00
	24 h – 72 h	**<0.001**
	48 h – 72 h	**<0.001**
Glycine	24 h - Control	0.662
	48 h - Control	0.35
	72 h - Control	**0.00286**
	24 h - 48 h	0.939
	24 h – 72 h	**0.0211**
	48 h – 72 h	**0.0573**
Taurine	**24 h - Control**	**0.0464**
	48 h - Control	**0.0185**
	72 h - Control	0.307
	24 h - 48 h	0.864
	24 h – 72 h	**0.00229**
	48 h – 72 h	**<0.001**

In general, after mussels were incubated in seawater enriched with one osmolyte, the other osmolytes found in gill tissues significantly decreased in concentration over time (Fig 2.1), although there were many variations in specific cases. A summary of how different osmolytes changed in concentration in mussel gills over time across the different osmolyte incubation treatments can be found in Table 2.3.

Table 2.3 How various metabolites change in concentration over time when *Mytilus trossulus* are incubated in seawater enriched with 40 mM of one of the five osmolytes listed in the far left column, either for 24, 48 or 72 hours (*n*=4). The first row of data for each osmolyte incubation treatment contains the ANOVA results, the following rows contain p-values obtained from Tukey post-hoc tests. Arrows indicate the directionality of change for significantly different comparisons. The "Comparison" column indicates which incubation timepoints are being compared in that row ("C" stands for control mussels). TMA stands for trimethylamine, TMAO stands for trimethylamine *N*-oxide.

Osmolyte Incubation Treatment	***Comparison***	***Gill Osmolyte*** **Alanine**	**Betaine**	**Glycine**	**Malonate**	**Succinate**	**Taurine**	**TMA**	**TMAO**	**β-Alanine**
Alanine		$F_{3,12}$= 54.15, p<0.001	$F_{3,12}$= 34.30, p<0.001	$F_{3,12}$= 25.43, p<0.001	$F_{3,12}$= 7.076, p=0.0054	$F_{3,12}$= 28.81, p<0.001	$F_{3,12}$= 43.96, p<0.001	$F_{3,12}$= 0.365, p= 0.78	$F_{3,12}$= 37.77, p<0.001	$F_{3,12}$= 7.73, p= 0.0039
	C - 24 h	↑ **0.018**	↓ **<0.001**	↓ **<0.001**	↓ **0.0039**	↓ **0.0030**	↓ **<0.001**		↓ **<0.001**	↓ **0.0058**
	C- 48 h	↑ **<0.001**	↓ **<0.001**	↓ **<0.001**	↓ **0.076**	0.89	↓ **<0.001**		↓ **<0.001**	↓ **0.067**
	C - 72 h	↑ **<0.001**	↓ **0.011**	↓ **0.034**	0.461	↑ **0.0026**	↓ **0.0038**		↓ **0.017**	0.95
	24 h - 48 h	↑ **0.0030**	0.101	0.47	0.36	↑ **<0.001**	↑ **0.073**		0.21	0.52
	24 h – 72 h	↑ **<0.001**	↑ **<0.001**	↑ **0.0021**	↑ **0.053**	↑ **<0.001**	↑ **<0.001**		↑ **<0.001**	↑ **0.015**
	48 h – 72 h	↑ **0.013**	↑ **0.033**	↑ **0.028**	0.63	↑ **0.0089**	↑ **0.016**		↑ **0.0072**	0.16
Betaine		$F_{3,11}$= 36.53, p<0.001	$F_{3,11}$= 36.12, p<0.001	$F_{3,11}$= 40.84, p<0.001	$F_{3,11}$= 16.21, p<0.001	$F_{3,11}$= 18.37, p<0.001	$F_{3,11}$= 44.04, p<0.001	$F_{3,11}$= 2.566, p= 0.11	$F_{3,11}$= 49.85, p<0.001	$F_{3,11}$= 6.094, p=0.011
	C - 24 h	↓ **<0.001**	1.00	↓ **<0.001**	↓ **<0.001**	↓ **0.0061**	↑ **<0.001**		↓ **<0.001**	↓ **0.011**
	C- 48 h	↓ **<0.001**	1.00	↓ **<0.001**	↓ **<0.001**	↓ **0.032**	↑ **<0.001**		↓ **<0.001**	↓ **0.032**
	C - 72 h	1.00	↑ **<0.001**	↓ **0.0023**	0.15	0.15	↓ **0.0031**		↑ **0.068**	↓ **0.098**
	24 h - 48 h	1.00	1.00	0.50	0.74	0.90	0.89		0.45	0.98
	24 h – 72 h	↑ **<0.001**	↑ **<0.001**	↑ **0.0086**	↑ **0.043**	↑ **<0.001**	↑ **0.0020**		↑ **<0.001**	0.56

Osmolyte Incubation Treatment	*Comparison*	*Gill Osmolyte*								
		Alanine	**Betaine**	**Glycine**	**Malonate**	**Succinate**	**Taurine**	**TMA**	**TMAO**	**β-Alanine**
Betaine	48 h – 72 h	↑ **<0.001**	↑ **<0.001**	↑ **0.0014**	↑ **0.012**	↑ **<0.001**	↑ **<0.001**		↑ **<0.001**	0.82
Glycine		$F_{3,12}$= 6.151, p=0.0089	$F_{3,12}$= 10.50, p=0.0011	$F_{3,12}$= 7.724, p=0.0039	$F_{3,12}$= 11.6, p<0.001	$F_{3,12}$= 5.72, p= 0.011	$F_{3,12}$= 17.4, p<0.001	$F_{3,12}$= 2.88, p= 0.080	$F_{3,12}$= 13.91, p<0.001	$F_{3,12}$= 7.35, p= 0.0047
	C - 24 h	↓ **0.0091**	↓ **0.0035**	0.66	↓ **0.0012**	0.86	↓ **<0.001**	↓ **0.067**	↓ **<0.001**	0.366
	C- 48 h	↓ **0.027**	↓ **0.0012**	0.35	↓ **0.0036**	0.40	↓ **<0.001**	0.82	↓ **<0.001**	↓ **0.0037**
	C - 72 h	0.28	↓ **0.062**	↑ **0.0029**	0.344	0.15	↓ **0.0085**	0.34	0.13	↓ **0.037**
	24 h - 48 h	0.93	0.92	0.939	0.905	0.84	0.99	0.27	0.96	↓ **0.071**
	24 h – 72 h	0.23	0.39	↑ **0.021**	↑ **0.023**	↑ **0.040**	0.13	0.78	0.22	0.50
	48 h – 72 h	0.51	0.16	↑ **0.057**	↑ **0.073**	↑ **0.0093**	0.20	0.81	0.42	0.57
Taurine		$F_{3,11}$= 12.82, p<0.001	$F_{3,11}$= 13.95, p<0.001	$F_{3,11}$= 13.42, p<0.001	$F_{3,11}$= 7.708, p= 0.0048	$F_{3,11}$= 13.49, p<0.001	$F_{3,11}$= 13.01, p<0.001	$F_{3,11}$= 3.419, p= 0.056	$F_{3,11}$= 16.03, p<0.001	$F_{3,11}$= 8.525, p= 0.0033
	C - 24 h	↓ **0.0062**	↓ **0.0024**	↓ **0.0014**	↓ **0.020**	0.18	↓ **0.046**	↓ **0.045**	↓ **0.0032**	↓ **0.010**
	C- 48 h	↓ **0.0016**	↓ **0.0020**	↓ **<0.001**	↓ **0.036**	↓ **0.0017**	↓ **0.0185**	0.32	↓ **0.0012**	↓ **0.0079**
	C - 72 h	0.86	0.86	↓ **0.022**	1.00	0.83	0.307	0.76	1.00	0.65
	24 h - 48 h	0.65	0.96	0.87	1.00	↓ **0.047**	0.864	0.74	0.77	0.97
	24 h – 72 h	↑ **0.023**	↑ **0.0085**	0.36	↑ **0.020**	↑ **0.046**	↑ **0.0023**	0.22	↑ **0.0045**	↑ **0.072**
	48 h – 72 h	↑ **0.0050**	↑ **0.0063**	0.14	↑ **0.036**	↑ **<0.001**	↑ **0.0012**	0.81	↑ **0.0017**	↑ **0.049**
TMAO		$F_{3,12}$= 3.36, p=0.055	$F_{3,12}$= 1.92, p=0.18	$F_{3,12}$= 7.10, p= 0.0053	$F_{3,12}$= 0.16, p=0.92	$F_{3,12}$= 25.7, p<0.001	$F_{3,12}$= 4.22, p=0.030	$F_{3,12}$= 1.00, p= 0.43	$F_{3,12}$= 2.40, p=0.12	$F_{3,12}$= 3.19, p=0.063
	C - 24 h	0.17		↓ **0.018**		↓ **0.029**	↓ **0.037**			↓ **0.075**

Osmolyte Incubation Treatment	*Comparison*	*Gill Osmolyte*								
		Alanine	**Betaine**	**Glycine**	**Malonate**	**Succinate**	**Taurine**	**TMA**	**TMAO**	**β-Alanine**
TMAO	C- 48 h	**↓ 0.056**		**↓ 0.0054**		**↓ <0.001**	**↓ 0.073**			**↓ 0.097**
	C - 72 h	0.12		**↓ 0.033**		**↓ <0.001**	**↓ 0.074**			0.49
	24 h - 48 h	0.90		0.89		**↑ 0.0016**	0.98			1.00
	24 h – 72 h	1.00		0.99		**↑ 0.059**	0.98			0.60
	48 h – 72 h	0.97		0.73		0.21	1.00			0.69

For mussels incubated in the "osmolyte cocktail" treatment, samples were only taken at the 72-hour timepoint. Only alanine ($F_{1,6}$=29.04, p = 0.00168) and betaine ($F_{1,6}$=5.72, p=0.0539) significantly increased in concentration following 72 h incubation in the osmolyte cocktail, while malonate ($F_{1,6}$=11.97, p=0.0135), trimethylamine ($F_{1,6}$=6.64, p=0.0419), and β-alanine ($F_{1,6}$=7.93, p=0.0305) all significantly decreased in concentration (Fig 2.2). Finally, glycine ($F_{1,6}$=0.837, p=0.396), taurine ($F_{1,6}$=2.91, p=0.139), TMAO ($F_{1,6}$=1.208, p=0.314), and succinate ($F_{1,6}$=1.30, p=0.298) did not significantly change following 72 h incubation in the osmolyte cocktail.

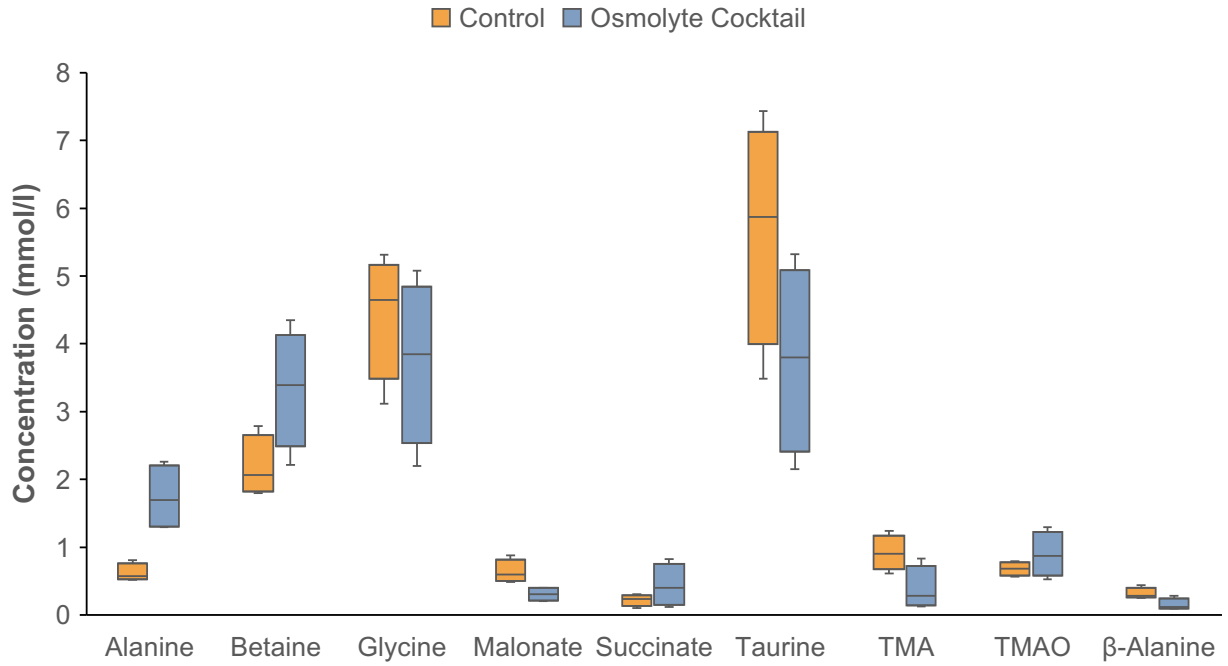

Figure 2.2 When incubated an "osmolyte cocktail", which refers to seawater enriched with a combination of the following five osmolytes: alanine, betaine, glycine, taurine and TMAO (totalling to 40 mM), *Mytilus trossulus* increase the concentration of some osmolytes in their gill tissues, but they also lower the concentration of others, after 72 hours of incubation. Asterisks denote significant differences between control and treatment (n=4). Metabolite concentrations are per 100 mg of gill tissue. Mussels were collected from Tower Beach, Vancouver, BC.

After 72 h of incubation in each osmolyte treatment, an ANOVA showed that there was a trend towards a significant difference between the total gill organic metabolite pool concentration across different osmolyte-incubation treatments ($F_{6,21}$=2.54, p=0.0522; Fig 2.3). However, a Tukey post-hoc test revealed that only two treatment groups significantly differed in terms of total organic metabolite pool concentration; betaine-incubated mussels had a larger total organic metabolite pool as compared to the TMAO incubated mussels (p=0.0471). All other osmolyte-incubation treatments did not result in a significant change in total gill organic metabolite pool concentrations.

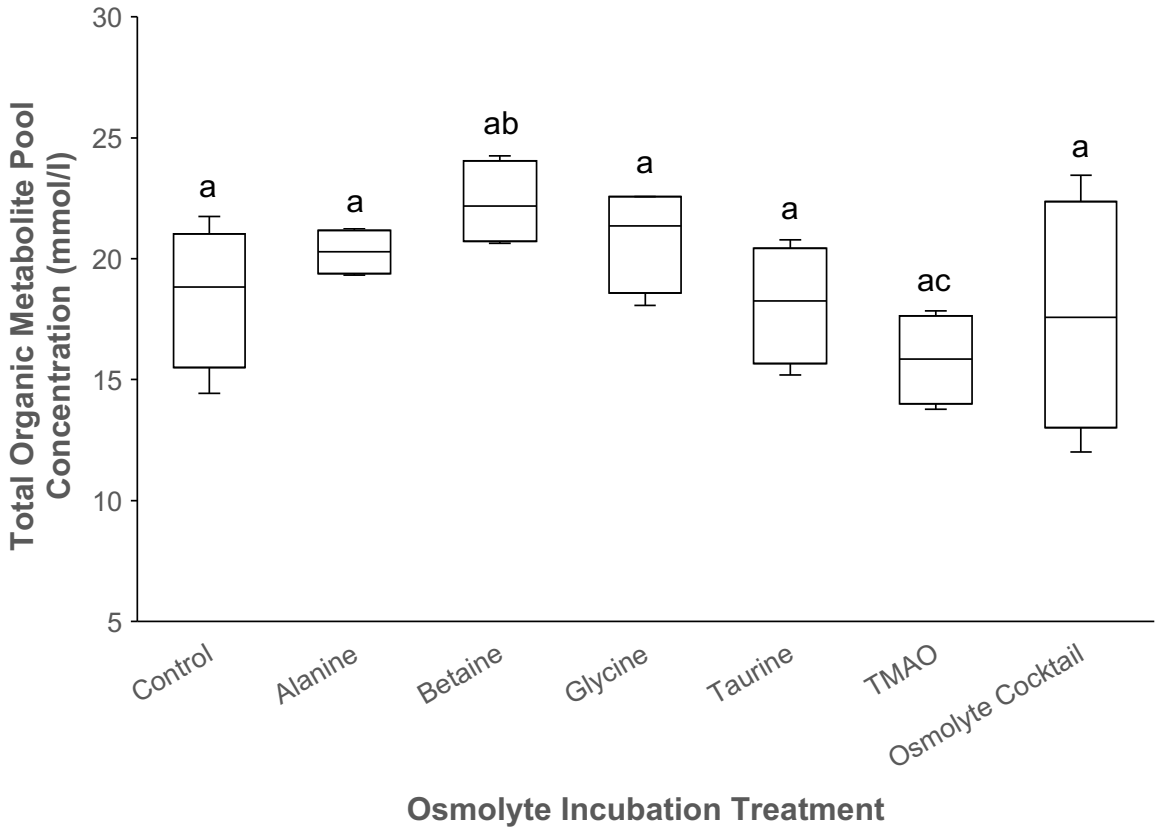

Figure 2.3 None of the osmolyte-incubation treatments significantly altered the total organic metabolite pool concentration in *Mytilus trossulus* gill tissues. Mussels were incubated in seawater enriched with 40 mM of each osmolyte for 72 hours, in addition to an "osmolyte cocktail" treatment which was a combination of all the five osmolytes, and control mussels which kept in plain seawater (n=4). Letters denote statistically significant differences between treatment groups. Concentrations are per 100 mg of gill tissue. Mussels were collected from Tower Beach, Vancouver, BC.

2.3.2 How Osmolyte Incubation Impacts Freeze Tolerance

The body temperatures of eight representative mussels per treatment were monitored in these experiments, and all monitored mussels froze, as indicated by the presence of a supercooling point, SCP, in the body temperature trace graphs (see Fig A.4 for a representative mussel body temperature trace graph). One mussel from the taurine-enriched seawater treatment and one mussel from the betaine-enriched seawater treatment died after 72 hours of incubation, before they could be frozen.

A logistic regression revealed that osmolyte incubation treatment (excluding the osmolyte cocktail treatment data which were analysed against a set of time-matched control mussels) was a significant predictor of mussel survival after freezing ($df=5$, deviance=19.79, $p=0.0014$; Fig 2.4). Lower test temperature resulted in lower survival ($df=1$, deviance=36.61, $p<0.001$) and larger mussels survived better ($df=1$, deviance=6.16, $p=0.013$). The interaction between test temperature and osmolyte-incubation treatment was insignificant ($df=5$, deviance=6.51, $p= 0.30$).

A Tukey post-hoc test revealed that, after incubation in glycine for 72 h, mussel survival was significantly lower than in the control mussels ($p=0.0053$). Mussels incubated in alanine also had lower survival, although this effect was marginally insignificant ($p= 0.067$). There was no significant difference in survival after freezing between control mussels and mussels incubated in the other 3 osmolytes: betaine ($p=0.99$), taurine ($p=0.26$), and TMAO ($p=0.99$).

Survival after freezing for mussels incubated in the osmolyte-cocktail seawater treatment was compared against a different set of control mussels that had been collected on the same date. Osmolyte-cocktail incubated mussels had significantly lower survival after freezing ($df=1$, deviance=8.59, $p=0.0034$; Fig 2.5), and mussels had lower survival at the lower test temperature

(df=1, deviance=6.76, p=0.0093). Shell length was not a significant predictor of survival (df=1, deviance=0.14, p=0.71), and the interaction between test temperature and osmolyte treatment was also insignificant (df=1, deviance=0.61, p=0.43).

Additionally, mean total gill organic metabolite pool concentration was not a significant predictor of the proportion of mussels which survived after freezing at either test temperature, across all osmolyte incubation treatments (-10 ºC $F_{1,6}$=0.366, p=0.567; -12 ºC $F_{1,6}$=0.28, p=0.616; Fig 2.6).

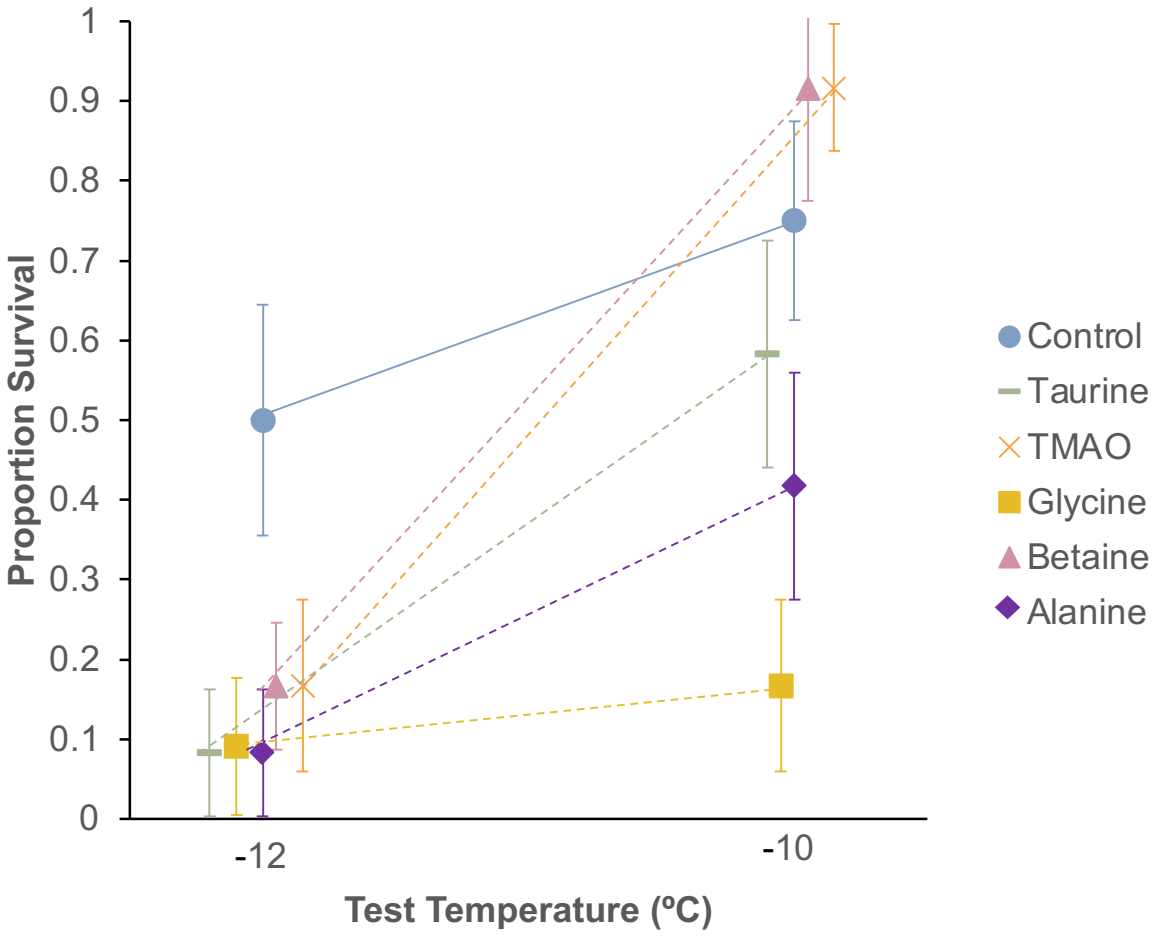

Figure 2.4 After being incubated in osmolyte-enriched seawater (40 mM) for 72 h, *Mytilus trossulus* freeze tolerance decreases (glycine and alanine) or else does not significantly differ from control mussels (taurine, TMAO, and betaine; statistics described in text). Error bars are standard error of the proportion. Mussels were collected from Tower Beach, Vancouver, BC on October 15, 2021, and were frozen for 3 hours (n = 12).

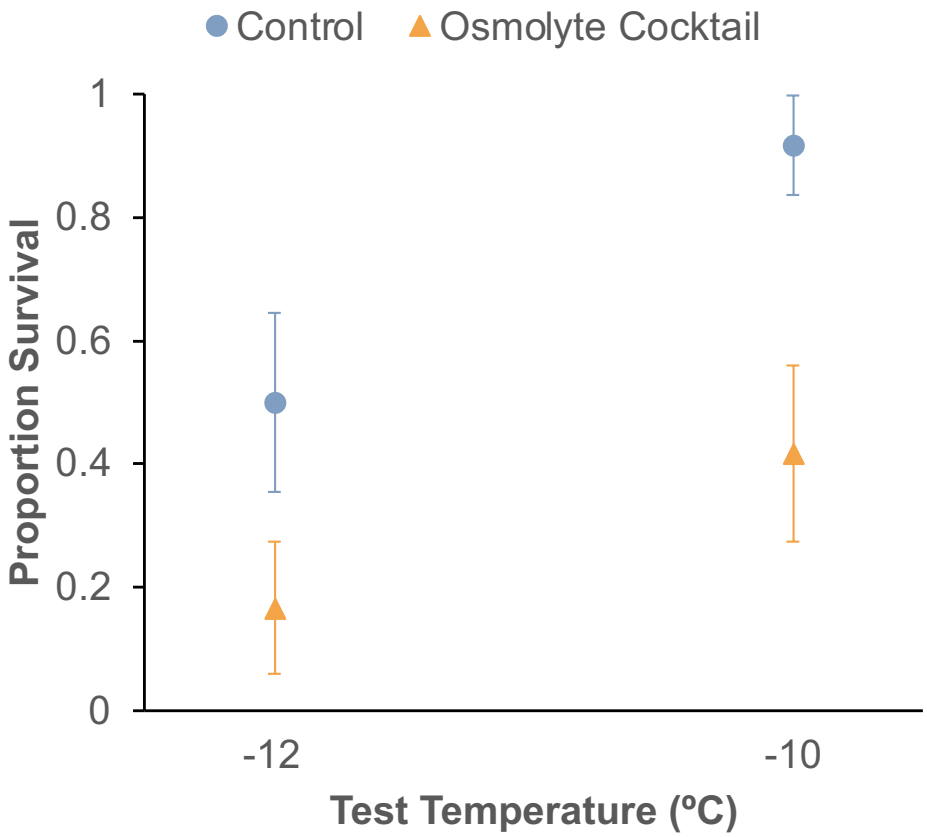

Figure 2.5 Mussel survival after freezing decreases after being incubated for 72 h in seawater enriched with a combination of the following five osmolytes: taurine (12.8 mM), TMAO (6.8 mM), betaine (6.4 mM), glycine (9.6 mM), and alanine (4.4 mM). Error bars are standard error of the proportion. Mussels were collected from Tower Beach, Vancouver, BC, on December 1, 2021 and were frozen for 3 h (n = 12).

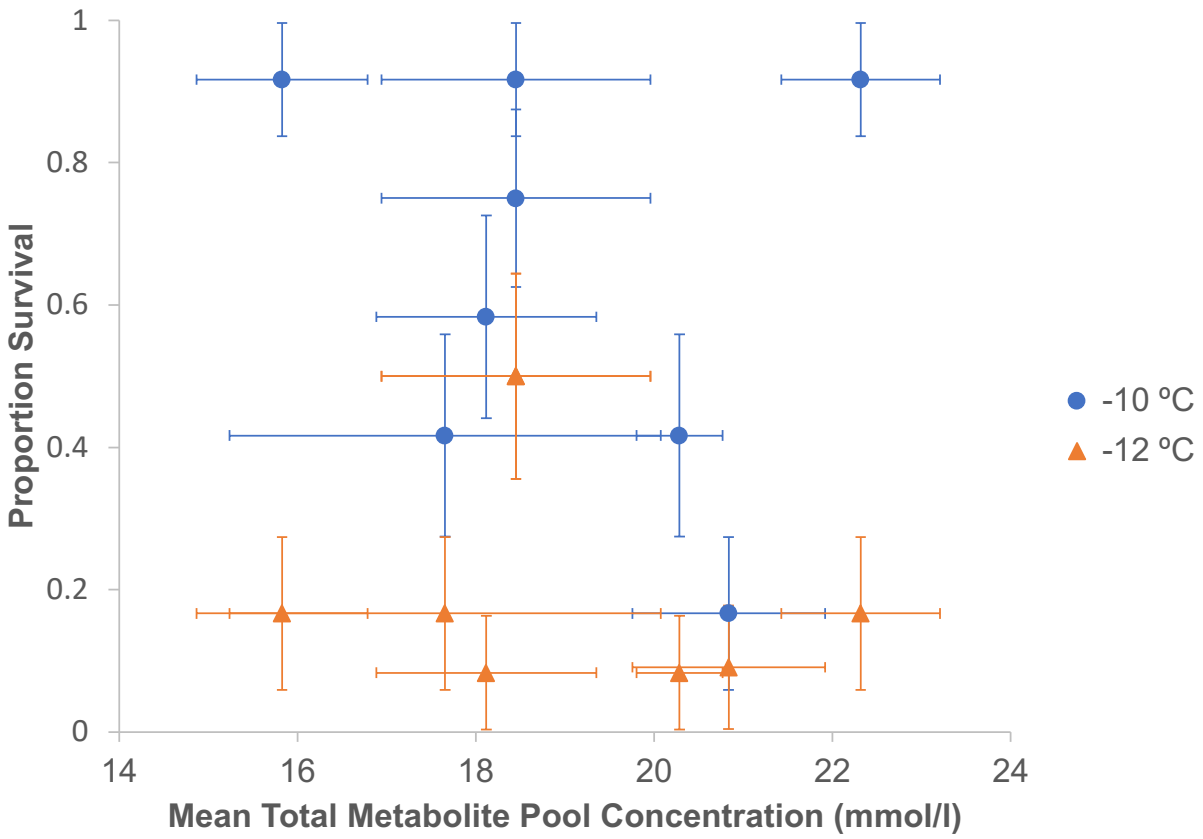

Figure 2.6 Mean *Mytilus trossulus* gill total organic metabolite pool concentration does not significantly relate to mussel survival proportion after mussels are frozen for 3 hours at either -10 or -12 ºC. Each point represents the mean metabolite concentration (n = 4 per treatment) and mean survival proportion (n = 12 per treatment) for each osmolyte incubation group. Vertical error bars are standard error of the proportion and horizontal error bars are standard error of the mean. Concentrations are per 100 mg of gill tissue. Mussels were collected from Tower Beach, Vancouver, BC.

I used a principle components analysis of the metabolite profiles of mussels across all osmolyte-incubation treatment groups to characterize changes in metabolite levels across osmolyte-incubation treatments. How different gill metabolites load on the nine different principal components is described in Table 2.4. The first PC axis largely explains total metabolite content, accounting for 35.7% of the variation in the data, with malonate, TMAO, β-alanine, and taurine all loading negatively. Next, the second axis accounts for 18.2% of the variation in the data, and alanine and succinate concentrations both load positively on to the second axis. The third axis explains succinate and betaine concentrations, and both metabolites load positively on this third PC axis which explains 14.1 % of variation in the data. Finally, the fourth axis explains 12.6% of the variation in the data, with glycine loading strongly negatively on to this axis.

Analysis using a generalized linear model revealed that none of the first 4 principal component scores significantly predicted the number of mussels which survived after freezing at either -10 ºC or -12 ºC (Table 2.5). Furthermore, visual examination of the heat map shown in Table 2.6 reveals that there is no clear relationship between mean PC scores across the various osmolyte-incubation treatments and mussel survival after freezing at -10 ºC.

Table 2.4 Loadings of all nine principal components from a principle components analysis of changes to *Mytilus trossulus* gill metabolite concentrations after mussels are incubated in osmolyte-enriched seawater. Bolded values > |0.4|.

Metabolite	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Alanine	-0.05270	**0.6720**	-0.0302	0.3686	-0.2213	0.1734	-0.2749	0.1716	**-0.4740**
Betaine	-0.2554	-0.1025	**0.7230**	0.0423	0.3099	0.0294	0.0543	-0.3490	**-0.4220**
Glycine	-0.0747	0.1981	-0.1734	**-0.8558**	0.1763	0.2044	-0.0085	0.1048	-0.3299
Malonate	**-0.4468**	-0.2050	-0.2274	0.0017	0.1300	-0.0563	**-0.7996**	-0.2000	0.0849
Succinate	-0.2209	**0.5378**	**0.4021**	-0.2648	-0.1889	-0.2804	-0.0647	-0.0668	**0.5543**
Taurine	**-0.4388**	-0.1100	-0.2039	-0.0367	-0.3420	**-0.6592**	0.2760	0.03520	-0.3514
TMA	-0.2291	0.3143	-0.2748	0.2259	**0.7744**	-0.1835	0.2539	0.0961	0.1232
TMAO	**-0.4745**	-0.2267	0.2302	0.0495	-0.0407	0.2993	0.0562	**0.7475**	0.1217
β-Alanine	**-0.4558**	0.0715	-0.2505	0.0710	-0.2304	**0.5363**	0.3661	**-0.4733**	0.1432
Proportion Variance Explained	35.7%	18.2%	14.1%	12.6%	8.88%	4.62%	3.53%	1.63%	0.772%

Table 2.5 How *Mytilus trossulus* survival after freezing at either -10 ºC or -12 ºC is predicted by the mean principal component score for that group. The PCA is based on the metabolite profiles of mussels from different osmolyte incubation treatments. Reported values are from general linear models (df=5). Mussels were collected from Tower Beach, Vancouver, BC (for osmolyte incubation experiments n = 4 per treatment, for survival experiments n = 12 per treatment).

Test Temperature (ºC)	Predictor	t value	p value
-10	PC 1	-4.80	0.131
	PC 2	2.16	0.276
	PC 3	0.588	0.662
	PC 4	3.99	0.156
-12	PC 1	-1.66	0.345
	PC 2	3.78	0.165
	PC 3	-1.47	0.381
	PC 4	3.67	0.169

Table 2.6 A heat map displaying how survival proportion after *Mytilus trossulus* are frozen at -10 ºC relates to the mean principal component scores for the top 5 PCs, only considering mussels incubated for 72 hours. "Cocktail" refers to the osmolyte-cocktail treatment (i.e., combination of all five osmolytes added to seawater). Blue denotes higher PC scores and red denotes lower PC scores. Green denotes higher survival and yellow denotes lower survival. Mussels were collected from Tower Beach, Vancouver, BC (for osmolyte incubation experiments $n = 4$ per treatment, for survival experiments $n = 12$ per treatment).

Osmolyte Incubation Treatment	**Mean PC1**	**Mean PC2**	**Mean PC3**	**Mean PC4**	**Mean PC5**	**Survival Proportion after -10 ºC Freeze**
Alanine	1.97	-0.774	0.404	0.522	0.262	0.417 ± 0.14
Betaine	-0.871	0.213	1.04	-0.510	-1.09	0.917 ± 0.080
Control	1.98	0.261	-0.357	0.851	0.397	0.75 ± 0.13
Glycine	1.52	0.209	-0.547	-1.13	0.889	0.167 ± 0.11
Taurine	1.69	-0.640	-0.343	0.456	0.0724	0.583 ± 0.14
TMAO	-1.60	-1.28	-0.647	0.505	0.330	0.917 ± 0.080
Cocktail	-1.74	-1.25	-0.534	0.359	0.0127	0.417 ± 0.14

I used logistic regressions to test whether gill metabolite concentrations at 72 hours post-incubation and test temperature significantly predicted mussel survival after freezing, using a separate model for each metabolite. Higher mussel survival after freezing was significantly predicted by higher TMAO concentrations (df=10, t=3.40, p=0.0068; Fig 2.8), test temperature did not significantly predict survival after freezing (t=0.168, p=0.87), but the interaction term between TMAO concentration and test temperature was marginally-insignificant (t=-2.08, p=0.0641). However, none of the other metabolite concentrations significantly predicted mussel survival after freezing (Table 2.7). For all other comparisons apart from TMAO, only test temperature was a significant predictor of survival, with lower survival at -12 ºC (Table 2.7). Also, for all other comparisons, the interaction term between metabolite concentration and test temperature was insignificant and so test temperature was used as a covariate for all other metabolite comparisons, apart from TMAO.

Table 2.7 Logistic regression results testing the effect of *Mytilus trossulus* gill metabolite concentration and test temperature on mussel survival across all osmolyte-incubation treatments. No significant effect of metabolite concentration on survival was found except for TMAO. Mussels were collected from Tower Beach, Vancouver, BC. Bold values indicate $p<0.05$ ($df = 11$; for osmolyte incubation experiments $n = 4$ per treatment, for survival experiments $n = 12$ per treatment).

Gill Metabolite	Effect of Mean Metabolite Concentration on Survival		Effect of Test Temperature on Survival	
	t value	p value	t value	p value
Alanine	-1.00	0.338	-3.46	**0.00538**
Betaine	1.31	0.216	-3.56	**0.00449**
Glycine	-1.64	0.130	-3.69	**0.00357**
Malonate	1.60	0.137	-3.67	**0.00366**
Succinate	-1.57	0.146	-3.66	**0.00377**
Taurine	0.510	0.620	-3.35	**0.00652**
TMA	0.861	0.407	-3.42	**0.00575**
TMAO	2.39	**0.0361**	-4.08	**0.00183**
β-Alanine	1.61	0.137	-3.68	**0.00366**

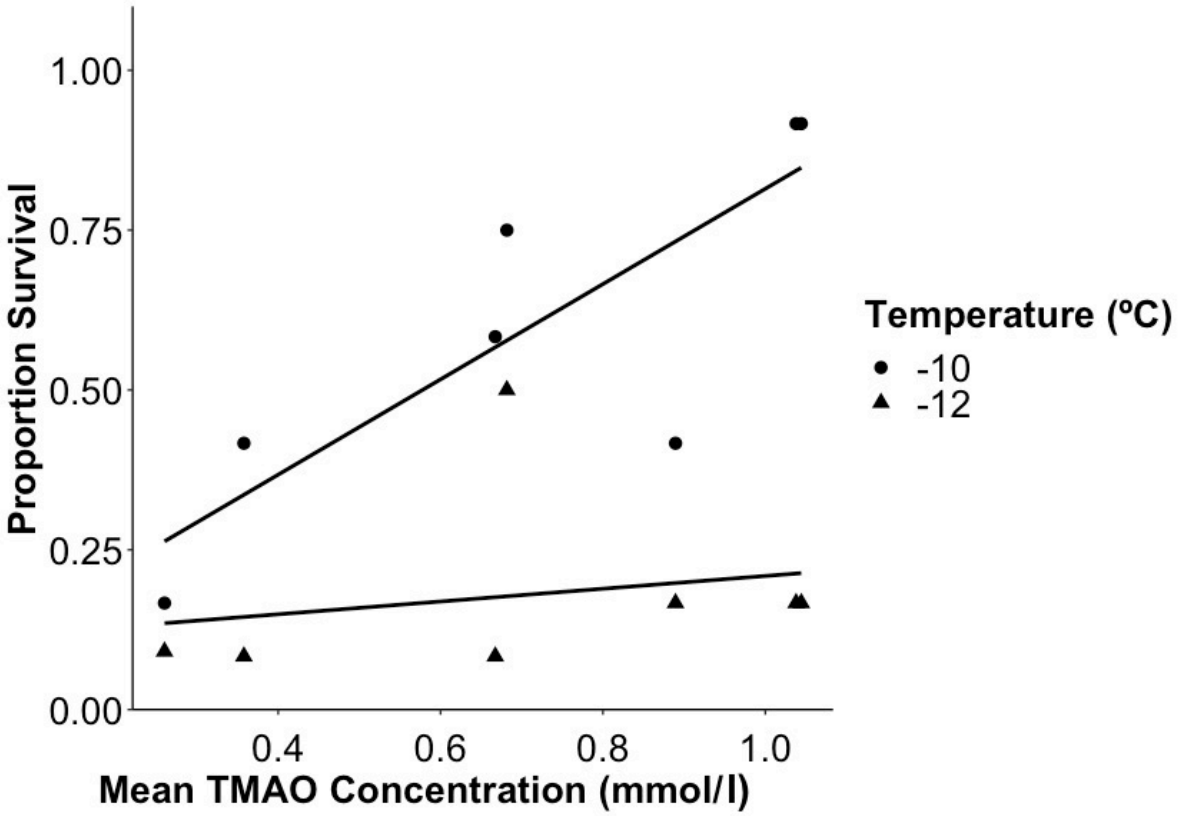

Figure 2.7 After *Mytilus trossulus* metabolite pools are artificially manipulated by incubating mussels in osmolyte-enriched seawater, higher TMAO concentrations significantly improve mussel freezing survival. Concentrations are per 100 mg of gill tissue. Mussels were collected from Tower Beach, Vancouver, BC (for osmolyte incubation experiments $n = 4$ per treatment, for survival experiments $n = 12$ per treatment).

2.4 Discussion

In this chapter I investigated the potential cryoprotective role of osmolytes in the intertidal mussel *Mytilus trossulus*. Specifically, I wanted to determine if osmolytes are colligative cryoprotectants (i.e., different osmolytes are interchangeable with one another with respect to their cryoprotective capacity) or if osmolytes are non-colligative cryoprotectants (i.e., different osmolytes have different cryoprotective roles). To do this, I artificially manipulated mussels' intracellular osmolyte concentrations by incubating mussels in seawater enriched with osmolytes, which enabled me to test how the composition of intracellular osmolyte pools relates to freeze tolerance. I found that mussel gill tissues take up alanine, betaine, glycine, and taurine after mussels are incubated in seawater enriched with the osmolytes for 72 hours, but they do not take up external TMAO. Although mussel gills increased the intracellular concentrations of the osmolyte that they were incubated in, mussels showed compensatory decreases in the concentrations of other gill metabolites after incubation in osmolyte-enriched seawater, presumably to maintain osmotic balance.

Further, I found either no effect of osmolyte-incubation on mussel freeze tolerance (for taurine, TMAO, and betaine incubated mussels), or decreases in freeze tolerance for glycine, alanine, and the osmolyte-cocktail treatment (the effect of alanine was marginally insignificant). Despite the significant changes to metabolite levels that I induced across various osmolyte-incubation treatments, the only metabolite that was predictive of increased freeze tolerance was TMAO. Overall, I found more support for the non-colligative cryoprotectant hypothesis since a) only TMAO is predictive of survival after freezing, and b) some osmolyte incubation treatments lowered mussel freeze tolerance (which may indicate that the specific composition of osmolytes normally found in mussel gills is important to freeze tolerance, since disrupting the relative

proportions of the various osmolytes can lower freeze tolerance). However, since no change in freeze tolerance was observed in some osmolyte-incubation treatments, despite significant changes to osmolyte pool composition, we cannot rule out the colligative cryoprotectant hypothesis because in some cases changes to composition of the osmolyte pool did not impact mussel freeze tolerance. Overall, however, I found comparatively more evidence to support the hypothesis that osmolytes are non-colligative cryoprotectants in *Mytilus trossulus* than the alternative explanation, but the mechanisms enabling freeze tolerance in intertidal mussels most likely extend beyond just osmolyte accumulation.

2.4.1 Osmolyte Uptake by Gill Tissues

I saw very pronounced uptake of each osmolyte in mussel gill tissues when mussels were incubated in seawater that had been enriched with 40 mM of that osmolyte, in every case except for TMAO. It has been well established that mussels of the genus *Mytilus* take up organic osmolytes, including various amino acids as well as betaine, from the surrounding seawater using gill transporters which actively transport amino acids (plus betaine) into mussel gills, even against large concentration gradients (Rice and Stephens, 1988; Wright and Pajor, 1989; Wright et al., 1992). It remains unclear if mussels would continue to take up more osmolytes with longer incubation periods, since other work has shown that it may take up to 6 days for the mussel *Modiolus demissus* to fully osmoconform to high salinity seawater (Baginski and Pierce, 1977). However, since I incubated mussels in relatively high concentrations of osmolytes (40 mM) perhaps uptake might be faster than in natural seawater, which has much lower amino acid concentrations, approximately <1 μM (Wright and Pajor, 1989). One caveat here is that I only measured osmolyte uptake in mussel gill tissues, and so I cannot be certain that the osmolyte uptake patterns observed in gill tissues are representative of the uptake patterns in the whole

body. However, unpublished data shows that *Mytilus edulis* gills, hepatopancreas, and adductor muscle tissues all take up taurine and glycine when mussels are incubated in 40 mM osmolyte-enriched seawater (Dr. Inna Sokolova, pers. comm.). Further studies should measure the osmolyte uptake patterns of various mussel tissues, not just gills since this would give further insight into how mussels osmoconform.

The summed concentration of all the organic gill metabolites which were detected using ^{1}H NMR did not change after 72 hours of osmolyte incubation. While betaine-incubated mussels did have significantly higher total organic metabolite pool concentrations relative to TMAO-incubated mussels, this increase was relatively small at only +6.5 mmol/100 mg and was not significantly different than the total concentration of detected organic metabolites in control mussels (Fig 2.3). Since mussels maintain constant intracellular osmolarity, if extracellular osmolarity remains constant, as mussels took up the osmolyte that they were incubated in, other metabolites in mussel gills had compensatory decreases in concentration (Fig 2.1). In other words, mussels prevent their cells from becoming hyperosmotic to their surroundings by reducing the intracellular concentrations of other metabolites in their gills as they increase the intracellular concentration of the osmolyte that they're incubated in, to keep their intracellular metabolite pool size constant. Previous studies which tested osmolyte uptake patterns in bivalves only measured the concentration of the one or two osmolytes of interest and did not take a full metabolomic profile of gill tissues as I have done here, and so it is not clear if the mussels used in prior experiments testing mussel osmolyte uptake patterns also had compensatory decreases in other, non-target metabolites (Rice and Stephens, 1988; Wright et al., 1992). In the future, wherever possible, measuring the full metabolite profile of tissues when manipulating potentially-cryoprotective metabolite concentrations in organisms will provide a more nuanced

understanding of how artificially increasing the concentration of one metabolite may lead to changes in the rest of the organism's metabolite profile.

Since manipulating just one osmolyte resulted in disrupting the balance of other osmolytes in mussel gills, I decided to incubate mussels in an "osmolyte cocktail" treatment, which was seawater that had been enriched with a combination of all five osmolytes corresponding to the relative proportions of osmolytes found naturally in mussel gills. Seventy-two hours of incubation in the osmolyte cocktail resulted in some significant changes to mussel metabolite profiles, although these changes were not as drastic as in the single osmolyte-incubation treatments. Alanine and betaine both significantly increased in concentration following incubation (although each osmolyte only increased by ~2 mmol/100 mg in concentration), and the metabolites malonate, trimethylamine, and beta-alanine all decreased in concentration after incubation. The fact that these three metabolites decreased makes sense considering that none of these three metabolites were added to the osmolyte cocktail. As in the other osmolyte-incubation treatments, after incubation in the osmolyte-cocktail, gill total organic metabolite pool concentration did not change after 72 hours of incubation. Therefore, perhaps mussels lowered the concentrations of those three metabolites to avoid disrupting osmotic balance as they increased their alanine and betaine intracellular concentrations.

2.4.2 How Osmolyte Incubation Impacts Freeze Tolerance

To test whether osmolytes act as colligative or non-colligative cryoprotectants, I measured how mussel freeze tolerance changed after mussel osmolyte concentrations were artificially manipulated by incubating mussels in seawater enriched with osmolytes for 72 hours. If osmolytes are non-colligative cryoprotectants, then different osmolytes contribute to freeze tolerance through unique cryoprotective mechanisms, and freeze tolerance depends on the

composition of an organism's osmolyte pool. However, if osmolytes are colligative cryoprotectants, then all osmolytes are interchangeable with one another in terms of their cryoprotective function and manipulating the composition of the osmolyte pool would not affect freeze tolerance. In my study, I found that osmolyte-incubation treatments either a) had no effect on mussel freeze tolerance, which was the case in the taurine, TMAO, and betaine treatments, or b) osmolyte incubation led to a decrease in mussel freeze tolerance, which was the case in the alanine (this effect was marginally insignificant), glycine, and osmolyte cocktail treatments. Additionally, I found that across all osmolyte incubation treatments, TMAO was the only metabolite which significantly predicted mussel survival after freezing, with higher mussel survival predicted by higher TMAO gill concentrations.

2.4.2.1 Evidence for the Colligative Cryoprotectant Hypothesis

Some evidence for the colligative-cryoprotectant hypothesis comes from my finding that the betaine- and taurine- incubation treatments had no effect on mussel freeze tolerance. This finding suggests that osmolytes act as interchangeable cryoprotectants and the composition of the osmolyte pool is not important to freeze tolerance since after incubation in either betaine-enriched or taurine- enriched seawater, the relative proportions of various osmolytes in mussel gill tissues was modified significantly, although no change in freeze tolerance occurred. In other words, since mussel freeze tolerance was un-affected by changes in the composition of their osmolyte pool, this seems to indicate that osmolytes are colligative cryoprotectants. Under the colligative cryoprotectant hypothesis, only increasing intracellular osmolarity by increasing the concentration of the cumulative metabolite pool would result in increased freeze tolerance, and since neither of these osmolyte-incubation treatments resulted in a change in intracellular osmolarity, one would expect to see no change in freeze tolerance under this hypothesis. One

important caveat is that there was an insignificant decrease in survival, particularly for the -12 ºC treatment, after mussels were exposed the betaine- and taurine- incubation treatments.

2.4.2.2 Evidence for the Non-Colligative Cryoprotectant Hypothesis

The finding that mussel freeze tolerance decreases after mussels are incubated in alanine-enriched seawater, glycine-enriched seawater, and the osmolyte-cocktail lends support to the non-colligative cryoprotectant hypothesis. Incubation in single-osmolyte-enriched seawater led to an increase in the intracellular concentration of the osmolyte that the mussels were incubated in, but most other metabolites decreased in concentration, which disrupted the normal ratio of intracellular metabolites in mussel gills. The osmolyte cocktail treatment also resulted in significant changes to the relative proportions of various metabolites, however these changes were not as pronounced as in the single-osmolyte incubation treatments. Since disrupting the relative proportions of osmolytes without changing the total amount of organic osmolytes in mussel gill tissues led to a decrease in freeze tolerance, this suggests that the specific proportion of osmolytes found in mussel gills normally may be optimal for cryoprotection. It may not be that any one osmolyte is individually cryoprotective on its own, but that the normal, specific relative proportions of osmolytes in mussel cells provides optimal protein protection, and/or protection against other freezing-related damages. However, as previously mentioned, survival data from the taurine- and betaine- incubated mussels contradicts this hypothesis, since significant changes to the osmolyte pool composition in those treatments did not result in significant changes to freeze tolerance.

The glycine incubation treatment significantly lowered mussel freeze tolerance. Interestingly, glycine shows the largest proportional increases in concentration after mussels are incubated in higher salinity (30 ppt *vs.* 15 ppt), which corresponds to increased freeze tolerance

in mussels (Kennedy et al., 2020), which makes the fact that glycine-enrichment had such negative effects on mussel survival after freezing a counterintuitive result. Various *in vitro* studies on cell cultures and *in vivo* studies on animals have shown that glycine has a multitude of cytoprotective roles including protecting against damage from hypoxia, mediating ion influx, and acting as an antioxidant (Pérez-Torres et al., 2016). However, at high concentrations, glycine can have toxic effects on study organisms, such as leading to increased bradycardia and death in mice and rabbits (Pérez-Torres et al., 2016). More studies on glycine's cytoprotective effects in invertebrates, and how these effects are mediated by the concentration of glycine, are needed.

Mussel freeze tolerance also decreased after incubation in alanine- enriched seawater (albeit only marginally-significantly so). Additionally, in the osmolyte cocktail-incubated mussels, which was another treatment which caused a significant decrease in freeze tolerance, alanine was one of the only osmolytes to significantly increase in concentration, along with betaine. This means that there might be something about alanine, or higher-than-normal concentrations of alanine specifically, that hinders mussels' ability to survive freezing. One study showed that alanine protects against damage from hypoxia in rats (Nichols et al., 1994), but research on the cytoprotective role of alanine in invertebrates are lacking, and so more research is necessary to contextualize these results.

Mussels incubated in the "osmolyte cocktail" treatment experienced significant changes to their gill metabolite profile, but these changes were less pronounced than the osmolyte composition disruptions caused by the other osmolyte-incubation treatments. However, mussel survival did significantly decrease after mussels were exposed to the osmolyte cocktail treatment. Why this happened is not clear, but it could indicate that incubating mussels in osmolyte-enriched seawater has some unknown, negative effects on mussel survival, even

without the added stressor of freezing. This is a real possibility, since one mussel died (out of 24 total) after 72 hours of incubation in both betaine- and taurine- enriched seawater treatments. Perhaps adjusting osmolyte concentrations is energetically costly for mussels, which could explain the low levels of non-freeze-related mortality I observed, as well as could potentially account for the lowered survival post-freeze caused by some of the osmolyte incubation treatments. Further study investigating changes in mussels' energy stores after incubation in osmolyte-enriched seawater may be help determine if this the case.

2.4.2.3 The Effect of TMAO on Freeze Tolerance

Another piece of evidence which lends additional support for the non-colligative cryoprotectant hypothesis was that across all osmolyte-incubation treatments, higher TMAO gill concentrations correlated with higher mussel survival after freezing, suggesting that TMAO is a uniquely important cryoprotectant for mussels. TMAO is a widely-known to be a protein stabilizing osmolyte (Bennion et al., 2004; Street et al., 2006), and so its cryoprotective role likely involves reducing freezing-induced protein denaturation. In plants, TMAO directly increases tolerance to freezing, drought, and high salt stress, providing further support for TMAO's role in cryoprotection *via* reducing protein denaturation (Catalá et al., 2021). Further evidence that TMAO has a cryoprotective role in marine organisms comes from the fact that Antarctic teleost fishes have significantly higher TMAO concentrations, as compared to similar species from temperate habitats (Raymond and DeVries, 1998). Interestingly, the fact that the change in TMAO concentrations that I measured across treatments was relatively small (<1 mM) and yet TMAO still significantly increased freeze tolerance, suggests that TMAO is a very potent cryoprotectant, particularly considering that insects accumulate cryoprotectants on a molar scale (up to 5 M in some wasp species; Salt, 1961). Thus, my study adds to the growing body of

evidence that TMAO is an important low-molecular weight cryoprotectant in a variety of freeze tolerant organisms. Although, since I did not measure the concentrations of all potential high- and low- molecular weight cryoprotectants, I cannot be sure that changes in freeze tolerance across different treatments are caused by some other un-measured cryoprotectant and the relationship TMAO concentrations and freeze tolerance is a type 1 error.

Unfortunately, mussel gills did not significantly take up TMAO in the TMAO-enriched seawater treatment. Thus, the fact that mussels from the TMAO-incubation treatment showed no significant change in freeze tolerance was expected, since TMAO-incubation resulted in only a few changes in the metabolite profiles of mussels. This finding may indicate that mussels do not actually have TMAO transporters and do not typically take up TMAO from the external environment, which aligns with the findings of a previous study which found that the cockles *Cerastoderma edule* and *Cerastoderma lamarcki* synthesize TMAO using trimethylamine (TMA) which they derive from the phytoplankton that they ingest (De Vooys, 2002). Fishes are also thought to accumulate TMAO primarily through dietary sources (Groninger, 1959; King, 1988). Thus, further studies should try to use other methods to artificially increase mussel TMAO concentrations (perhaps through dietary supplementation) to further explore the cryoprotective role of TMAO. Another future avenue of study would be to investigate the cryoprotective role of TMAO *in vitro* to better understand how TMAO functions as a cryoprotectant.

However, the cryoprotective role of osmolytes such as TMAO may be complicated by the fact that, in some cases, osmolytes' cytoprotective roles are dependent upon their concentration, and at higher-than-normal concentrations, osmolytes can have a negative effect on cells. Higher-than-normal intracellular TMAO concentrations have been found to cause protein aggregates,

rather than stabilize proteins, as is TMAO's function at normal concentrations (Devlin et al., 2001; Yancey, 2005). Furthermore, TMAO counteracts urea's de-stabilizing effects on macromolecular structures, but only when the two molecules are found in specific ratios relative to each other (Yancey et al., 2002). Thus, *in vivo*, the cytoprotective role of osmolytes, namely TMAO, might be more complex than is currently understood and might be dependent not only on the concentration of that osmolyte, but also the relative concentration of other osmolytes.

2.4.2.4 The Effect of Body Size

Interestingly, I found that body size influenced freeze tolerance, with larger mussels surviving freezing better, when considering the pooled data across all single osmolyte-incubation treatments. I did not find a significant effect of mussel body size in the osmolyte cocktail vs. control freeze tolerance comparison, though. This finding partially aligns with the findings of my previous study where, again only in some cases, larger *Mytilus trossulus* mussels survived freezing better than smaller ones (Kennedy et al., 2020). There are a few potential explanations behind this trend: first, body size often correlates to age in mussels (Richardson et al., 2007), and so larger mussels are likely older and perhaps more freeze tolerant since they would have previously survived at least one winter (Bourget, 1982). Or, bigger mussels may experience slightly slower body cooling rates because they have more thermal inertia, compared to smaller mussels (Murphy and Johnson, 1980). Larger mussels may also take longer to reach equilibrium ice content (i.e., maximum ice content) as compared to small mussels (Claussen and Zani, 1991). However, I am limited in my ability to make conclusions about how body size influences freeze tolerance in mussels since I only froze mussels within a narrow range of body sizes, due to equipment limitations. Therefore, the effect of body size on freeze tolerance in mussels is still not clear.

2.4.3 Conclusion

Here I have shown that mussel gill tissues take up osmolytes if they are incubated in seawater that has been artificially enriched with the osmolytes alanine, betaine, glycine, or taurine, but not TMAO, within 72 hours of incubation. Metabolomics data revealed that as mussel gills take up the osmolyte that they are incubated in, they reduce the concentration of other metabolites in their gills, presumably to maintain equal osmolarity between their cells and the surrounding seawater.

Overall, my data lends support to the hypothesis that osmolytes are non-colligative cryoprotectants, however there are clearly other aspects of mussel freeze tolerance that are still not well characterized. My results partially align with the findings of a recent study which found that after injecting crickets with various cryoprotectants which accumulate in the tissues of winter-acclimated, freeze tolerant crickets, no single cryoprotectant or combination of cryoprotectants was sufficient to confer freeze tolerance in freeze intolerant crickets (Toxopeus et al., 2019). Similarly, here, I was unable to increase mussel freeze tolerance after enriching mussel tissues with osmolytes, which have been proposed to be an important group of cryoprotectants in mussels. As speculated by Toxopeus et al. (2019), organisms likely need to employ a whole host of cryoprotective strategies to tolerate freezing, since it comes along with such a varied set of damages, which explains why just up-regulating one cryoprotectant or even one type of cryoprotectant is not sufficient to increase freeze tolerance. Since many cellular modifications and cryoprotectants likely all work in tandem to increase mussel freeze tolerance, this might explain why manipulating osmolytes did not increase in mussel freeze tolerance.

In summary, mussel freeze tolerance decreased after incubation in glycine-enriched seawater, alanine-enriched seawater, and the osmolyte-cocktail seawater solution, which seems

to lend support to the hypothesis that osmolytes are non-colligative cryoprotectants since disrupting the normal ratios of various osmolytes in mussels' tissues leads to lower freeze tolerance. This finding could indicate that the role of osmolytes in mussel freeze tolerance is much more complex than previously understood and the specific, normal composition of osmolytes in mussel cells is an important driver of freeze tolerance. Another piece of evidence supporting the non-colligative cryoprotectant hypothesis is the finding that higher TMAO concentrations correspond to higher mussel survival rates after freezing. This indicates that TMAO has a uniquely cryoprotective role, since the concentrations of none of the other metabolites correlated to changes in mussel freeze tolerance. Alternately, support for the colligative cryoprotectant hypothesis comes from the fact that the betaine- and taurine- incubated mussels did experience significant changes to their metabolite profile following osmolyte incubation, and yet they showed no significant change in freeze tolerance. This finding supports the hypothesis that osmolytes are colligative cryoprotectants since even after significant changes to the composition of mussels' osmolyte pools, mussels experienced no significant change in freeze tolerance. Taken together, the findings of my study lend more support for the hypothesis that osmolytes are non-colligative cryoprotectants, but the exact role of osmolytes as cryoprotectants in intertidal mussels is likely much more complicated than we currently understand. Mussel freeze tolerance likely is a resultant of many cellular modifications, not just osmolyte accumulation, and so more study investigating a broader range of cryoprotective mechanisms is needed to better understand how mussels survive freezing.

Chapter 3: Impacts of Single and Repeated Freezing Exposures on *Mytilus trossulus* Survival, Posterior Adductor Strength, and Filtration Rate

3.1 Introduction

Intertidal invertebrates need to be able to survive freezing to survive in areas where air temperatures regularly drop below the freezing point of their bodies. More specifically, intertidal invertebrates are vulnerable to freezing during low tide air exposures, particularly so if they are sessile and cannot move to warmer microhabitats during winter low tides. To allow them to survive in these habitats, many intertidal invertebrates are freeze tolerant, meaning that they can survive ice formation within their bodies. However, even if an organism does not die after being frozen, there may be other sublethal, negative effects of experiencing freezing. This is because freezing is an incredibly physiologically stressful event for an organism to endure as it can come along with a host of cell- and tissue- level damages (Storey and Storey, 2002). Thus, experiencing freezing may have subsequent negative effects on organism performance because an organisms' body may not be functioning normally after freezing due to the direct damage that freezing inflicts on to tissues, cells, and macromolecules. Additionally, organisms may be energy limited post-freeze since they need to dedicate energy to repair freeze related damages and/or up-regulate protective mechanisms (Toxopeus and Sinclair, 2018) and may not have adequate energy supplies to perform other functions normally.

Understanding if and how an organism's performance is impacted by experiencing freezing can have also have important implications for our understanding of how the wider ecosystem might be impacted after a freezing event. One direct consequence of freezing could be a reduction in that organism's feeding rate, which would not only have impacts on the

organisms' energy supply but might have broader ecosystem impacts. For example, in the case of filter feeders such as intertidal barnacles and mussels, a significant reduction in filtration rate can negatively impact local water quality (Al-Mamun and Khan, 2011). Another indirect consequence of freezing might be a decreased ability to avoid predation, which could have significant impacts on population dynamics and community structure (Flynn, 2016; Paine, 1966). This is particularly true if the organism of interest is a keystone predator or an ecosystem engineer, since changes to the performance of that organism are more likely to have disproportionately large impacts on the rest of the ecosystem. Therefore, understanding the impacts of sublethal freezing on intertidal invertebrate performance will help to shed light on how intertidal ecosystems may be impacted after a freeze exposure.

3.1.1 Impacts of Freezing on Filtration Rate

One direct negative effect of experiencing freezing could be a subsequent reduction in feeding rate. Intertidal mussels, for instance, utilize their gills to filter feed by capturing suspended plankton particles from the water column in their gills and then moving those food particles to their digestive gland (Riisgård et al., 2015). Mussel filter feeding is important to maintain proper energy supply to the organism, and entire mussel beds can significantly improve local water quality by filter feeding and depositing pollutants into the sediment (Al-Mamun and Khan, 2011). Mussel filter feeding is also an important link between benthic and pelagic marine communities since energy flows from the open ocean into the benthic intertidal ecosystem when mussels consume plankton (Menge et al., 1997). Limited work has been conducted on the effects of freezing on organisms' feeding rates, particularly since many freeze tolerant insects cease feeding during the winter, but one study showed that in the intertidal sea stars *Pisaster ochraceus* and *Evasterias troschelii* and the intertidal dog whelk *Nucella lamellosa,* feeding rate

significantly decreased for two weeks following exposure to a cold shock of -0.5 ºC for three hours, which is sublethal yet stressful cold exposure (Currie-Olsen, 2021).

Mussels may be unable to filter feed properly post-freezing if their gill structures are damaged by cryoinjuries. In insects, freezing and cold stress have been shown to disrupt cytoskeletal structure in many instances (Cottam et al., 2006; Des Marteaux et al., 2018; Kayukawa and Ishikawa, 2009). If a mussel gill's cytoskeleton is damaged by cryoinjuries, this may disrupt the normal positioning of parallel gill filaments after freezing. This may then disrupt the lattice arrangement of neighbouring gill filaments' cilia, thereby reducing mussel's ability to "catch" plankton cells from the water column and impeding the mussel's ability to filter feed properly (Riisgård et al., 2015).

Thus, to further understand what some consequences of freezing may be in the intertidal zone, I sought to understand if, and for how long, mussel filter feeding was impaired following sublethal freezing stress. I also qualitatively investigated how gills are damaged post-freezing. This will lead to a better understanding of how mussel energy supply and/or local water quality may be significantly impacted after a mussel bed experiences freezing.

3.1.2 Impacts of Freezing on Mussel Susceptibility to Predation

An additional way that freezing may reduce mussel performance would be a reduction in mussels' capacity to avoid predation. Mussels rely on their posterior adductor muscle to hold their shells shut and resist sea star predation since sea stars eat mussels by prying open mussels' shells using their tube feet to access the flesh of the mussel (Feder, 1955; Reimer and Tedengren, 1996; Fig C.1). Thus, due to mussels' largely sessile nature, a mussel's ability to hold itself shut and resist this pulling force is its primary defence against predation by sea stars. This is an ecologically-relevant topic to investigate since intertidal sea star predation is the main biotic

factor influencing mussel abundance and distribution in the rocky intertidal zone in the Northeast Pacific ocean (Menge et al., 1994; Paine, 1974). If a significant increase in predation by sea stars leads to reductions in mussel density and/or distribution in the intertidal, this could impact the biodiversity of the entire intertidal ecosystem, since mussels are both competitive dominants that can limit the diversity of primary space-holders and ecosystem engineers which can enhance the diversity of interstitial fauna (Buschbaum et al., 2009; Harley, 2011; Paine, 1966). Sea stars likely do not experience a similar decrease in their capacity to prey upon mussels after a freezing low tide since they typically retreat down shore during low tides and therefore do not experience as much extreme cold and freezing stress as mussels do (Paine, 1974).

Posterior adductor muscle strength can be quantified by measuring the time it takes for mussels to open under a constant "pulling" force. Previous work has established that mussels which take a longer time to open under a constant pulling force also take longer to be opened by sea stars of the species *Pisaster ochraceus* (Flynn, 2016). In my thesis I investigated whether freezing impacted mussel susceptibility to sea star predation first by measuring mussel posterior adductor strength after freezing and then by testing whether sea stars eat recently-frozen mussels at a higher rate, compared to control mussels.

3.1.3 Impacts of Single & Repeated Freezing Stresses on Mussel Survival

Another under-studied, yet ecologically relevant, aspect of intertidal invertebrate freeze tolerance is the impact of subsequent, repeated freezes on intertidal invertebrates, rather than just the effects of a single freeze exposure. During multi-day periods of cold weather, intertidal organisms are exposed to sets of relatively short, repeated freezes during low tides, separated by recovery periods when the tide returns. However, in all currently published studies on intertidal invertebrate freeze tolerance, the authors only exposed their study organisms to a single freeze

(Bourget, 1982; Stickle et al., 2010; Waller et al., 2006), rather than a series of repeated freezing events separated by recovery periods, to mimic what intertidal invertebrates would actually experience in the field during a winter low tide series.

Furthermore, the intertidal shore height that an organism inhabits determines the thermal regime that organism experiences, which is because intertidal organisms living higher up on the shore are exposed to air for longer durations during low tides, as compared to organisms located lower down on the shore, which are submerged underwater for longer. This means that high intertidal organisms would be subject to longer and more severe sub-zero temperatures during low tides, as compared to organisms found lower down on the shore (Reid and Harley, 2021; Fig A.2). Accordingly, organisms from the high intertidal are expected to be better able to cope with extreme temperature stressors such as freezing, as compared to low intertidal organisms. Previous studies have found this to be the case in a variety of intertidal invertebrates such as intertidal mussels, gastropods, and barnacles (Davenport and Davenport, 2005; Kennedy et al., 2020).

3.1.4 Main Objectives & Predictions

To shed light on the sublethal impacts of freezing on intertidal invertebrate performance, I used the freeze tolerant intertidal mussel species, *Mytilus trossulus,* as my model organism. *M. trossulus* is an ecosystem engineer since the species forms dense aggregations in the intertidal termed mussel beds which have been shown to increase intertidal biodiversity (Buschbaum et al., 2009). My over-arching hypothesis was that after sublethal freezing, mussel performance would decrease, since freezing results in a myriad of cell and tissue level damages and these damages may either directly affect an organism's ability to perform normally, or indirectly reduce performance due to energetic constraints that might be imposed by the need to repair the damage

and/or up-regulate cryoprotective mechanisms in preparation for subsequent freezes. Therefore, I predicted to see lowered performance post-sublethal-freeze in *M. trossulus*, as evidenced by a) lower filtration rates, in accordance with gill damage and b) increased susceptibility to predation by sea stars due to reduced mussel posterior adductor muscle strength.

Next, I predicted that shorter, repeated freezes would result in lower survival, as compared to longer continuous freeze exposures of the same length, since mussels would need to survive being repeatedly cooled, frozen, and thawed, in the repeated freezing experiments as compared to the single, prolonged freezing treatment which would only subject mussels to a single round of cool/freeze/thaw. I predicted that high intertidal mussels would survive freezing better than low intertidal mussels, since higher intertidal mussels would be more acclimatized to experiencing more severe cold and freezing exposures in the field.

3.2 Methods

3.2.1 *Mytilus trossulus* Collection

Intertidal *Mytilus trossulus* specimens were collected from Tower Beach (49°16′26.1″N, 123°15′23.7″W) as well as Jericho Beach, near the Royal Vancouver Yacht Club (49°16’24.1”N 123°11’15.7”W). Both sites are in the Burrard Inlet in Vancouver, British Columbia, Canada (Fig A.1). All collections were completed during the lowest low tide of the day, when tidal height was <1 m above the chart datum. Vancouver has relatively mild winters, however winter temperatures can reach lows of −10 °C to −15 °C (Environment and Climate Change Canada; see Figure A.2). The tides in this area are mixed semi-diurnal, with two low and two high tides per day. In the winter, the lower daily low tide usually happens during the night, meaning that intertidal invertebrates are exposed to some of Vancouver’s coldest air temperatures during winter low tides. Both sites are near the mouth of the Fraser River which inputs fresh water into

the surrounding seawaters resulting in an estuarine habitat. Thus, both sites can reach salinities lower than 15 ppt during the spring summer when river outflow is high due to melting mountain snow and ice which drains into the Fraser River (Covernton and Harley, 2020; Kennedy et al., 2020).

Mussels were collected from the same outcropping of rocks at Tower Beach on each Tower Beach sampling date. High intertidal mussels were collected along the uppermost edge of the mussel bed at Tower Beach (approximately 3.5 m above chart datum) and low intertidal mussels were collected at approximately 1 m above chart datum. Both collection areas are directly in front of the abandoned searchlight tower at Tower Beach. Mussels were collected from low intertidal dock pilings at Jericho Beach. Only mussels with shell lengths between approximately 2 and 3.5 cm were collected. This size range was intentionally chosen so that mussels could fit into plastic vials for experimental cold exposures. All mussel collections were completed under Scientific Licences, Management of Contaminated Fisheries Regulations from the Department of Fisheries and Oceans Canada (licence number XMCFR 33 2020 covered mussel collections up until August 9, 2021, and licence number XMCFR 34 2021 covered collections from September 21, 2021 onwards).

To better understand the temperature regimes that mussels at Tower Beach would experience during a Vancouver winter, HOBO Pendant MX Temp temperature loggers (MX2201; Bourne, Massachusetts, USA) were drilled into intertidal rocks directly adjacent to mussel beds at Tower Beach. Temperature was logged over the period of December 1, 2021 to January 28, 2022, with a logging interval of 45 seconds. 4 “high intertidal” loggers were drilled into rocks at approximately 3 m above chart datum, and 4 “low intertidal” loggers were placed at

approximately 1 m above chart datum. The resulting temperature traces from these temperature loggers are displayed in Figure A.2.

As a part of my thesis, I tried to construct "frobo-mussels" which were HOBO pendant temperature loggers surrounded by a container filled with a volume of seawater equal to the average volume of body water contained within a mussel. This was done in hopes of accurately detecting and recording potential mussel freezing events in the field. To roughly calculate the wet weight of mussels, I weighed 8 mussels, dissected them, removed their wet mass, and then re-weighed just the shell. I calculated that the wet weight of each mussel by subtracting the mussel's total weight by the weight of just the shell. I found the average wet weight to be 3.07 ± 0.515 g. Unfortunately, due to the awkward size of the HOBO logger, finding a hard plastic container which fit the logger was difficult. In addition, the relatively small volume of water meant that the logger was not fully submerged in seawater. I did set out three HOBO loggers in December 2020 which I had placed in double layered plastic baggies filled with 3 mL of natural seawater that I secured to intertidal rocks underneath two layers of plastic mesh which were bolted to rocks, but on returning to collect the loggers the baggies had leaked.

3.2.2 Laboratory Acclimation and Freezing Procedure

All mussels, apart from those used in the repeated freezing experiments, were kept in the circulating sea water system at the University of British Columbia's Biodiversity Research Centre. Seawater was sourced from the Vancouver Aquarium; salinity was 30 ± 3 ppt and seawater temperature was 8-7 ºC. Salinity was measured with a refractometer (Tangxi, Sanming, China). Mussels were not fed while kept in the laboratory. Neither sex nor age were controlled for in any treatments, although shell size may be a rough proxy for age (Richardson et al., 2007).

To conduct experimental low temperature exposures, mussels were removed from seawater and their shells were dried with a paper towel. Mussel shells were then measured with calipers to the nearest 0.5 mm. The longest part of the shell was measured and recorded, and then mussels were labelled with an ID number using nail polish. Next, a 16-gauge Type T thermocouple were secured with Sticky Tack to each mussel's shell and connected to Picolog TC-08 thermocouple interfaces, so that mussel body temperatures could be recorded continuously during freeze exposures using PicoLog 6 beta software for Windows (Pico Technology, Cambridge, UK). Thermocouple sampling rate was once per second. Mussels were frozen using refrigerated circulating baths, which cooled at a rate of −1.5°C per minute. The mussels were placed in 35 mL vials (diameter=2.2 cm), and the vials were placed into wells in an aluminium head (insulated by foam) that was cooled using a methanol and water mixture (60:40, v/v) that was circulated by a refrigerated bath (ECO Silver: RE 415 S Model, Lauda, Wurzburg, Germany; Fig A.3). All mussels were emersed during the laboratory cold exposures to mimic a low tide, which is when freezing and/or cold stress would occur in the field. The length of cold exposure was measured as the time after the cooled bath reached the set temperature, as measured by thermocouples that were attached with putty to the aluminium head.

I continuously monitored all mussel body temperatures throughout all cold exposures so that I could determine which mussels froze, as indicated by the presence of a supercooling point (SCP). The SCP is defined as the lowest temperature immediately prior to the exothermic release of energy due to ice formation, visualized as a sharp spike in temperature on the temperature trace graph (see Figure A.4 for a representative mussel body temperature trace during a freeze exposure). Thus, the presence of a SCP indicates that internal ice formation has occurred (Lee, 2010). Immediately after the low temperature exposure, mussels were returned to their aerated

aquaria or to the sea water table and separated by treatment using 150 mL plastic containers with mesh windows to allow water flow. Survival was assessed daily after the cold exposure for 1 week. Mussels were considered dead when they were unable to hold their shells shut after removing them from the seawater.

3.2.3 Impacts of Freezing on Filtration Rate

Filtration rate data collection methods were loosely based on a recent study using the same mussel species (Marshall et al., 2021). I scraped off any epibiotic barnacles growing on the shells of all mussels used in this experiment before testing took place to ensure that I was only measuring mussel feeding rate, and not barnacle and mussel filter feeding rates. Mussels were randomly assigned to treatments and body size was controlled for to ensure that mussel body sizes remained consistent between treatments. Mussels used here were collected on February 13, 2022 from dock pilings near the Royal Vancouver Yacht Club at Jericho Beach, Vancouver, BC (Fig A.1).

To measure how filtration rate changes after freezing I compared the filtration rate of mussels which had been frozen at -10 ºC for three hours (as described in section 3.2.2), and a control, unfrozen group of mussels. Eight beakers filled with seawater and phytoplankton were prepared by combining 6 mL of PHYTOGOLD phytoplankton concentrate (Brightwell Aquatics, Alabama, US) and 500 mL of natural seawater in a 1 L beaker to achieve an approximate phytoplankton concentration of 22,000 cells/mL. This algal concentration is within the range of concentrations which results in optimal mussel filter feeding rates (Clausen and Riisgård, 1996). Four beakers were allocated to each treatment and five mussels were placed in each beaker. After a 15-minute rest period in a tank filled with plain sea water at 10 ºC (to allow mussels to clear their gills of any residual phytoplankton/particles before testing began) mussels were placed in

their appropriate beaker filled with phytoplankton-seawater solution. Actual starting algal cell concentrations were measured for all beakers before mussels were placed in the beakers; the average starting phytoplankton concentrations was 21,441.6 ± 1030 cells/mL. Both beakers were strongly aerated and placed on stir plates set to medium-high in Panasonic MIR-154 incubators set to 10 ºC. The seawater salinity was 31 ppt. Mussels were also visually monitored throughout the experiment and the visibility of mussel siphons was recorded periodically throughout the experiment. Also, to better understand how long it took mussels to thaw after freezing, three extra frozen mussels were dissected and visually examined for ice 45 minutes post-freezing, while the filtration rate experiment was underway.

Water samples were taken at the following timepoints to measure algal cell drawdown over time: 0 min (before mussels were added), 15 min, 1 hour, and 4 hours. 1.5 mL of seawater was removed from each beaker at each timepoint and stored in a labelled Eppendorf 1.5 mL tube. The water samples were later dried down using dried in a centrifugal vacuum concentrator (Eppendorf 5301) until the sample volumes reached 0.1 mL (which was approximately 6-8 hours of drying time).

Algal cell concentration was measured using a Countess II automated cell counter (ThermoFisher Scientific). The samples were dried down (as described above) to increase accuracy of the Countess cell counts, since the minimum cell concentration countable by the Countess is 10,000 cells/mL. The dried-down water samples were vortexed and then 10 µL of each sample was pipetted into a chamber of a Countess cell counting chamber disposable slide. The Countess cell counter cut-off for cell size was set to 5-20 µm since this is the size range of the phytoplankton in the phytoplankton concentrate that was used. Although Trypan Blue staining is recommended to measure cell counts with the Countess, I did not stain my samples

with Trypan Blue, since the addition of Trypan Blue resulted in large precipitates forming in the seawater solution, and the algal cells had enough contrast with the background for the Countess to accurately count the cells. This means that the cell count values given by the Countess were divided by two, to account for the lack of added Trypan Blue solution, since the Countess calculates cell counts assuming that the samples are diluted with 50% Trypan Blue. Cell counts given by the Countess were further divided by 15 to account for the fact that the samples had been dried down from 1.5 mL to 0.1 mL. One technical replicate was measured for each control beaker seawater sample, and two technical replicates were measured for each of the seawater samples from the frozen treatment beakers, due to equipment limitations.

After 4 hours in the phytoplankton-seawater beakers, all mussels were removed from the phytoplankton-seawater beakers and placed in an aerated tank filled with plain seawater at 10 ºC and left overnight so that the frozen mussels had more time to recover from freezing. This was done since a pilot study indicated that mussels did not filter feed for the first three hours following freezing. The next morning, new beakers were set-up with fresh seawater and phytoplankton (such that the concentration was again at 22,000 cells/mL). Mussels were again separated into four groups of five for each treatment and algal cell drawdown was measured at the following timepoints: 24 hours post freeze (before mussels were placed in the beakers), 24 hours plus 30 min post-freeze (30 minutes into the experiment), 25 hours post freeze (one hour into the experiment), 26 hours post-freeze (two hours into the experiment), and 27 hours post freeze (three hours into the experiment). All frozen mussels were monitored for survival 1 week after freezing to ensure that this was a sublethal freeze and mussels' inability to filter feed was not due to lethal freezing injury.

3.2.4 Gill Morphology Changes Post-Freeze

To compare how gill tissue structure changed after freezing, 20 mussels were frozen at -10 ºC for three hours using the refrigerated circulating baths (freezing protocol described in section 3.2.2). Mussels were allowed to recover for either 2 hours, 24 hours, or 7 days to determine if gill structure repair occurs after a certain amount of time post-freeze (with five mussels per treatment group). Five control, un-frozen mussel gill tissue slides were also prepared, for a total of four treatment groups. Mussels used here were collected from dock pilings at Jericho Beach on February 13, 2022.

Gill tissues were stained with phalloidin for actin and DAPI for nuclei and then examined under the microscope using the following protocol, which was adapted from (Des Marteaux et al., 2018). Whole mussels were opened by cutting their posterior adductor muscle, and the whole mussel was fixed overnight (17–18 h) at room temperature in jars with 4% paraformaldehyde (Sigma Aldrich) in PB (0.8 mol/l $Na_2HPO_4 \cdot 7H_2O$, 0.2 mol/l $NaH_2PO_4 \cdot 2H_2O$, pH 7.4). Then, mussel gills were carefully dissected out of the mussel body and, using a paintbrush to move the tissues, gill tissues were rinsed for 3×15 min in PB, and then permeabilized for 20 min in PBT (PB with 0.5% Triton X-100). Next, tissues were stained with a 1:100 dilution of Alexa Fluor® 488 phalloidin (Life Technologies, Carlsbad, CA, USA) in PBT for 20 min and rinsed again for 3×15 min in PBT. The gill tissue was then arranged so that it laid flat in one single layer on glass slides, in a drop (50 µL) of ProLong™ Diamond antifade mountant with DAPI (Life Technologies) under a cover slip. Slides were kept in the dark in the fridge (4°C) until imaging. Five biological and technical replicates were used for each treatment. Slides were imaged using an Olympus BX53 fluorescence microscope (Olympus Life Science Solutions, Tokyo, Japan).

For visualizing DAPI staining 375/435 nm excitation/emission wavelengths were used, and for phalloidin 470/525 nm excitation/emission wavelengths were used.

3.2.5 Impacts of Freezing on Posterior Adductor Strength

To measure how mussel posterior adductor muscle strength changes after freezing, I used similar methods to those described in Flynn (2016). To measure posterior adductor muscle strength, I used an Instron (model 5500R, Instron Corp., Canton, MA, USA; Fig C.1). Mussels used in these experiments were collected from Tower Beach, Vancouver, BC across two collection dates in the winter of 2020-2021 (see Table 3.1).

Mussels were exposed to one of the freeze exposures listed in Table 3.1, given a specified amount of time for post-freeze recovery in the seawater table, and then their posterior adductor muscle strength was tested. Before Instron testing, plastic beads were attached to the mid-point of each side of the mussels' shells (i.e., halfway length-wise and width-wise) using super glue (All-Purpose Krazy Glue, Elmers, Westerville, Ohio, USA). Then, fishing line was threaded through the bead and tied into a loop for the Instron clamps to attach to. I waited 1 hour before testing to allow the glue to dry. For all treatments, mussel shell length was measured and recorded before testing.

To perform a trial, one mussel was attached to the Instron clamps using the loops of fishing line previously attached to their shell. Then the mussel was submerged in seawater (30 ppt and 10 ºC, cooled using ice packs) for the duration of the trial. The mussel was then put under a constant pulling force of 4 N with a creep rate of 0.1 N/second by the Instron. 4 N was chosen based on the force that *Pisaster ochraceous*, a common sea star species known for preying on mussels, is capable of exerting and the force that mussels of this size class can withstand (Feder, 1955).

The time it took the mussel to open was recorded in seconds and used as a proxy for posterior adductor muscle strength. Mussels were given ten minutes to open, during which time the mussel was closely monitored for even the smallest gap between the two shells, since sea stars can consume bivalves through gaps as small as 0.1-0.6 mm (Feder, 1955). Mussels that exceeded ten minutes were assigned a value of 600 seconds. Mussels that opened before the Instron reached a force of 4 N were recorded as "compromised" and assigned a value of 0 seconds to open. Additionally, in some trials the bead was pulled from the mussel's shell before the mussel opened, in which case that trial was excluded from the analysis, resulting in variable sample sizes across some treatments. Mussels that "died" due to the freezing treatment were also excluded from the analysis (i.e., mussels that gaped immediately when removed from the seawater). Sample sizes for each treatment and collection date are listed in Table 3.1. Lastly, only in the March trials, each mussel was dissected, and the diameter of its posterior adductor was measured with callipers and recorded to the nearest mm (Fig C.2), after being measured with the Instron.

Table 3.1 List of treatments used in the *Mytilus trossulus* posterior adductor strength Instron experiments. All freezing durations were 2 hours. Recovery time refers to the amount of time mussels were given to recover in the seawater system after being frozen and before being tested with the Instron. For the repeated freezing treatments, mussels were given a 24-hour recovery period between freezes. "Compromised" mussels were mussels that opened almost immediately when the Instron trial began, before the Instron reached the pulling force of 4 N. *M. trossulus* specimens were collected from Tower Beach, Vancouver BC.

Collection Date	**Freezing Treatment (ºC)**	**Recovery Time Post-Freeze (hours)**	**Sample Size**
December 12, 2020	Control	Control	22
	-8	24	22
	-8	15	23
	-8	7	18
	-8 × 2	7	23
March 8, 2021	Control	Control	18
	-8	3	18
	-8 × 2	3	17
	-10	3	15

3.2.6 Sea Star Feeding Rate Experiment

For the sea star feeding rate experiment, mussels were collected from dock pilings at the Royal Vancouver Yacht Club at Jericho Beach in Vancouver on February 13, 2022. *Evasterias troschelii* were collected from the low intertidal zone at Stanley Park, Vancouver, BC, near the "Girl in a Wetsuit" statue (49°18'08.7"N 123°07'33.8"W) on January 30, 20222. *E. troschelii* was chosen as the predator species because it is one of the most common intertidal sea star species in the Burrard Inlet, and is a key consumer of intertidal mussels (Kay et al., 2019).

I set up eight 20 L aquaria filled with natural seawater at 32 ppt salinity and 10 ºC in the Biosciences building at UBC Vancouver campus, with four aquaria allocated to each treatment. All tanks were strongly aerated using air stones. Two *E. troschelii* specimens were placed in each tank, after their longest leg length was measured to the nearest mm using callipers and recorded. Four of the tanks contained two sea stars that were fed ten control, un-frozen mussels, and in the other four tanks the two sea stars were given ten mussels that had been frozen at -10 ºC for three hours (freezing procedure described in section 3.2.2). The frozen mussels were given an hour to recover in the seawater table before being fed to the sea stars. The number of dead mussels was assessed daily for four days, and all fully consumed mussels were removed from the tanks daily.

3.2.7 Time Course of Post-Freeze Recovery

During the Instron trials, some mussels were unable to hold their shells shut when removed from seawater three hours post-freeze, even in the absence of any external pulling force. To further understand how long after freezing mussels exhibit this behaviour and at what frequency, a test group of mussels were monitored for their ability to hold their shells shut upon removal from seawater (without being subjected to any external pulling force) at several time

points after being frozen. 20 low intertidal mussels were frozen at -12 ºC for three hours, were then immediately returned to the sea water system and were monitored for recovery at 5 time points post-freeze; 30 min, 1 h 15 min, 2 h 15 min, 2 h 45 min and 25 h. Mussels used here were collected on December 1, 2021, from Tower Beach.

3.2.8 Repeated Freezing Experiments

For repeated freezing treatments, mussels were collected from Tower Beach and kept in strongly aerated 20 L aquaria filled with sea water (28 ± 1 ppt salinity) in Panasonic MIR-154 incubators set to 7 ºC in UBC's Biological Sciences building. Full seawater changes were performed every 2 days. Mussels used in this set of repeated freezing survival experiments were collected on November 14, 2020, and December 12, 2020. Mussels were frozen either at -8 ºC or -10 ºC for either 2, 4, or 8 hours using refrigerated circulating baths (as described in section 3.2.2). See Table 3.2 for a complete list of repeated freezing treatments. Mussels were returned to their aquaria and given 22 hours of recovery time for the 2 hour long freezes and 20 hours of recovery time for 4 hour freezes (recovery time = 24 hours – time spent frozen) before being exposed to the next freeze. Sample sizes were 12 mussels per treatment per intertidal zone. These freezing temperatures were chosen since they are slightly milder than the LLT_{50} (which is the temperature that causes 50% mortality) values for *Mytilus trossulus* from Tower Beach; the LLT_{50} for three hour freeze-exposures is -12 ºC for high intertidal mussels and -11.5 ºC for low intertidal mussels from November 29, 2019 (Kennedy et al., 2020).

Table 3.2 List of repeated freezing treatments performed for this study. *Mytilus trossulus* were given a 24-hour recovery period between repeated freezes. Sample size is 24 mussels per treatment (12 high intertidal and 12 low intertidal mussels). Mussels were collected from Tower Beach Vancouver, BC.

Test Temperature	Repeated Freezing Treatments	Collection Date
-8 °C	8 hrs × 1	Nov 14, 2020
	4 hrs × 1	
	4 hrs × 2	
	2 hrs × 1	
	2 hrs × 2	
	2 hrs × 3	
	2 hrs × 4	
-10 °C	8 hrs × 1	Nov 14, 2020
	4 hrs × 1	Dec 12, 2020
	4 hrs × 2	Dec 12, 2020
	2 hrs × 1	Nov 14, 2020
	2 hrs × 2	Nov 14, 2020
	2 hrs × 3	Nov 14, 2020
	2 hrs × 4	Nov 14, 2020

3.2.9 Time Course of Ice Formation During Freezing

A qualitative assessment of the amount of body water frozen in mussels during a 2-hour exposure to -8 ºC was performed by dissecting mussels immediately after removing them from a cooling bath set at -8 ºC. This to ensure that mussels were completely freezing during the relatively short, 2-hour freeze exposures used in my study. Two mussels were taken out of the cooling bath every 15 minutes, starting 45 minutes after the cooling bath reached -8 ºC. These two mussels were then quickly dissected and photographed to qualitatively assess the relative proportion of their body that was visibly frozen. Mussels used here were collected on March 8, 2021, from Tower Beach. Mussels ranged from 26-39.5 mm in shell length (mean= 34.5 ± 1.35 mm).

3.2.10 Statistical Analysis

Data analysis was performed using R (c. 4.0.3; R Development Core Team, 2021). Packages from the "tidyverse" suite were used to analyse and visualize data (Wickham et al., 2019). The package "sciplot" was used to generate Figures 3.7, 3.8, and 3.9 (Morales, 2020). I used the command "zeroinfl" from the R package "pscl" to run zero-inflated negative binomial regression models.

To test if significant algal cell drawdown over time occurred, a nonlinear regression implemented using the "aomisc" package in R was used (Onofri, 2020). Change in phytoplankton cell concentration over time was modelled as a power law function (cell concentration = $a \times \text{time}^b$). To test if treatment significantly impacted algal cell drawdown rate, I compared two models using ANOVA: one model which estimated the coefficients *a* and *b* for data pooled across both treatments, and one model that estimated a separate coefficient value for each treatment level (frozen and control mussels). A separate model was produced for day 1

(immediately after freezing) and day 2 (after 24 hours of recovery). A paired t-test was used to confirm that starting phytoplankton concentrations (before mussels were added) did not significantly differ between treatments. Sample size was four beakers per treatment with five mussels per beaker.

A zero-inflated negative binomial generalized linear model was used to test if freezing treatment and shell length impacted the time it took mussels to open under the 4 N pulling force exerted by the Instron for the trials done using December-collected mussels. Another zero-inflated negative binomial generalized linear model was used to test if freezing treatment, posterior adductor diameter, and shell length impacted time to open for the trials done using March-collected mussels. Model selection was performed using likelihood ratio tests. How the proportion of mussels that recovered post-freezing changed over time after freezing was analysed using an ANOVA (results from this test are in the Appendix C.2).

An ANOVA was used to assess if the number of mussels consumed per day per sea star (the number of mussels consumed daily per tank was divided by two to account for there being two sea stars in each tank) was significantly impacted by whether the mussels had been recently frozen. Experiment day, sea star body size, the interaction term between treatment and experiment day, and tank number (which was included as a random effect) were also included as predictor variables in this ANOVA. Feeding rate was log transformed before ANOVA analysis to better adjust for normality. Sample sizes were four tanks per treatment, with two sea stars and ten mussels per tank.

Logistic regressions were used to test for significant differences in survival after exposure to the different repeated freezing treatments. Then, a chi-squared test was used to test for significant differences in survival using mussel shell length, freezing treatment, and shore height

as potential predictors of survival after freezing. Tukey post-hoc tests were then used to identify statistically significant differences between freezing treatment groups. Sample size was 12 mussels/treatment/intertidal zone.

3.3 Results

3.3.1 Impacts of Freezing on Filtration Rate

Nonlinear regression showed that for both day 1 (immediately after freezing) and day 2 (24 hours after freezing) there was significant algal cell drawdown over the course of the experiment (Fig 3.1). An ANOVA analysis showed for both experimental days, a model with separate coefficient estimates for each treatment was a better fit to the data than a model where the treatments had the same estimated coefficients (Day 1: $F_{24,26}$=13.86, p>0.001; Day 2: $F_{36,38}$=3.56, p=0.0390). Results from Table 3.3 indicate that the rate of algal cell drawdown for frozen mussels was slower immediately after freezing (experimental Day 1), as compared to control mussel filtration rates (as evidenced by a less negative *b* estimate for frozen mussels). Although, after 24 hours of recovery post-freezing, the *b* estimates for frozen and control mussels become much more similar, indicating the effect of treatment on filtration rate becomes smaller after recovery (Table 3.3).

Table 3.3 Estimated coefficient values for the power law functions describing mussel filtration rates, i.e., changes in algal cell concentration over time (cell concentration = $a \times \text{time}^b$). "Frozen" mussels were frozen at -10 ºC for three hours. Coefficient estimates are reported as ± standard error, p values indicate whether or not the coefficient is significantly different from zero. The last two rows report models which estimated the coefficients *a* and *b* for data pooled across both control and frozen treatments. Mussels were collected from Jericho Beach, Vancouver, BC (n=4 beakers per treatment, 5 mussels per beaker).

Time Tested	Treatment	Estimate for a	p value	Estimate for b	p value
Immediately after freezing	Control	2.132×10^4 ± 2512	<0.001	-0.3241 ± 0.07876	<0.001
	Frozen	2.342×10^4 ± 2410	<0.001	-0.06526 ± 0.03150	0.04920
24h after freezing	Control	1.899×10^4 ± 1957	<0.001	-0.1648 + 0.03429	<0.001
	Frozen	2.145×10^4 ± 1947	<0.001	-0.1180 ± 0.02729	<0.001
Immediately after freezing	Both	2.219×10^4 ± 2456	<0.001	-0.1497 ± 0.04207	0.001460
24h after freezing	Both	2.016×10^4 ± 1470	<0.001	-0.1375 ± 0.02281	<0.001

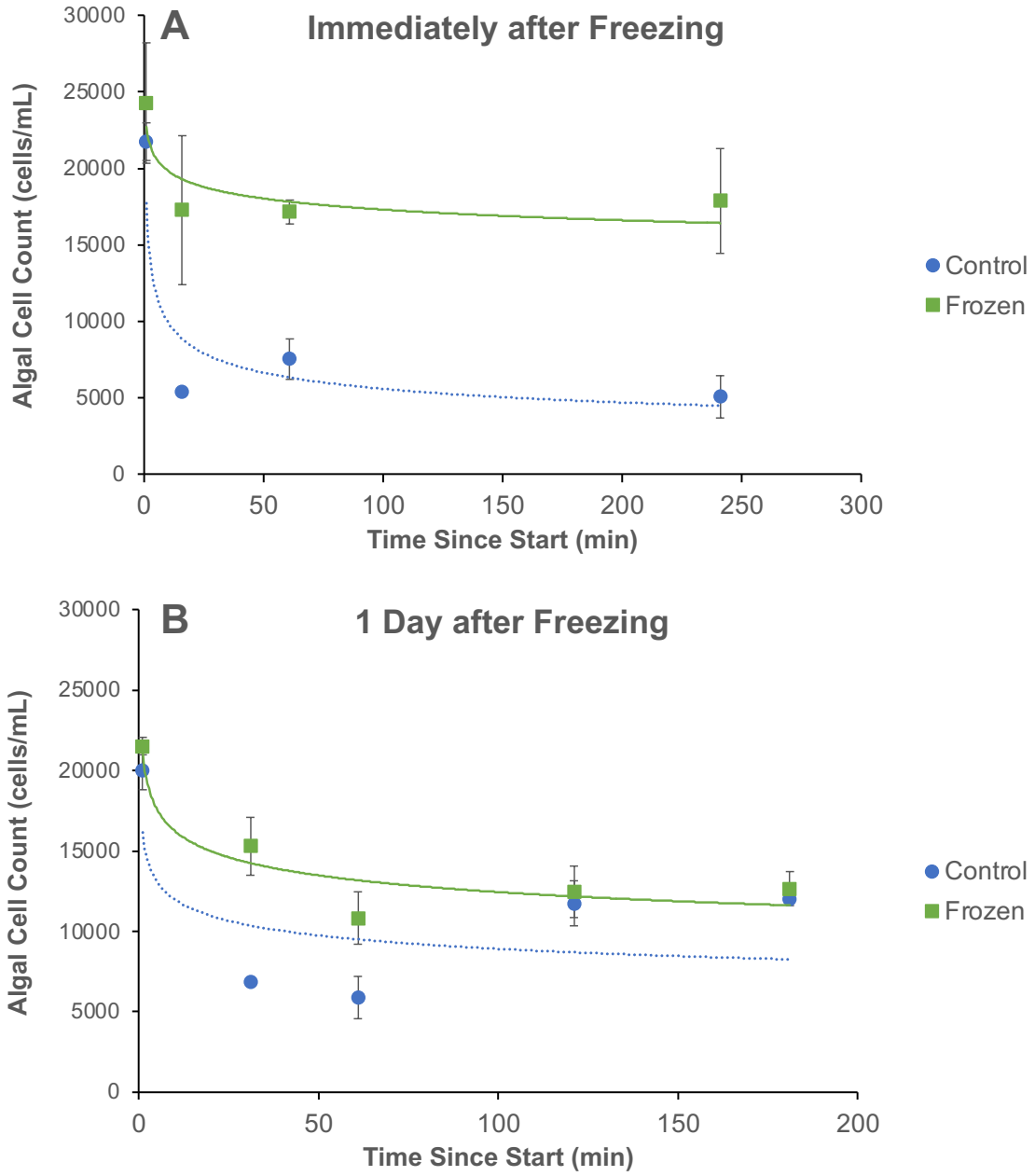

Figure 3.1 *Mytilus trossulus* have negligible filtration rates for the first 4 hours post freezing at -10 ºC for three hours, but after 24 hours of recovery post-freeze, mussels do begin to resume filter feeding, as evidenced by the algal cell drawdown of the frozen treatment over time in Panel B but not Panel A. Time from start indicates how mussels have spent in the phytoplankton seawater solution; mussels had 15 minutes to recover post-freezing before the experiment began. The relationship between algal cell count and time from start is modelled using power curves. Mussels were collected from Jericho Beach, Vancouver, BC (n=4 beakers per treatment, 5 mussels per beaker). Error bars are standard error.

3.3.2 Impacts of Freezing on Gill Morphology

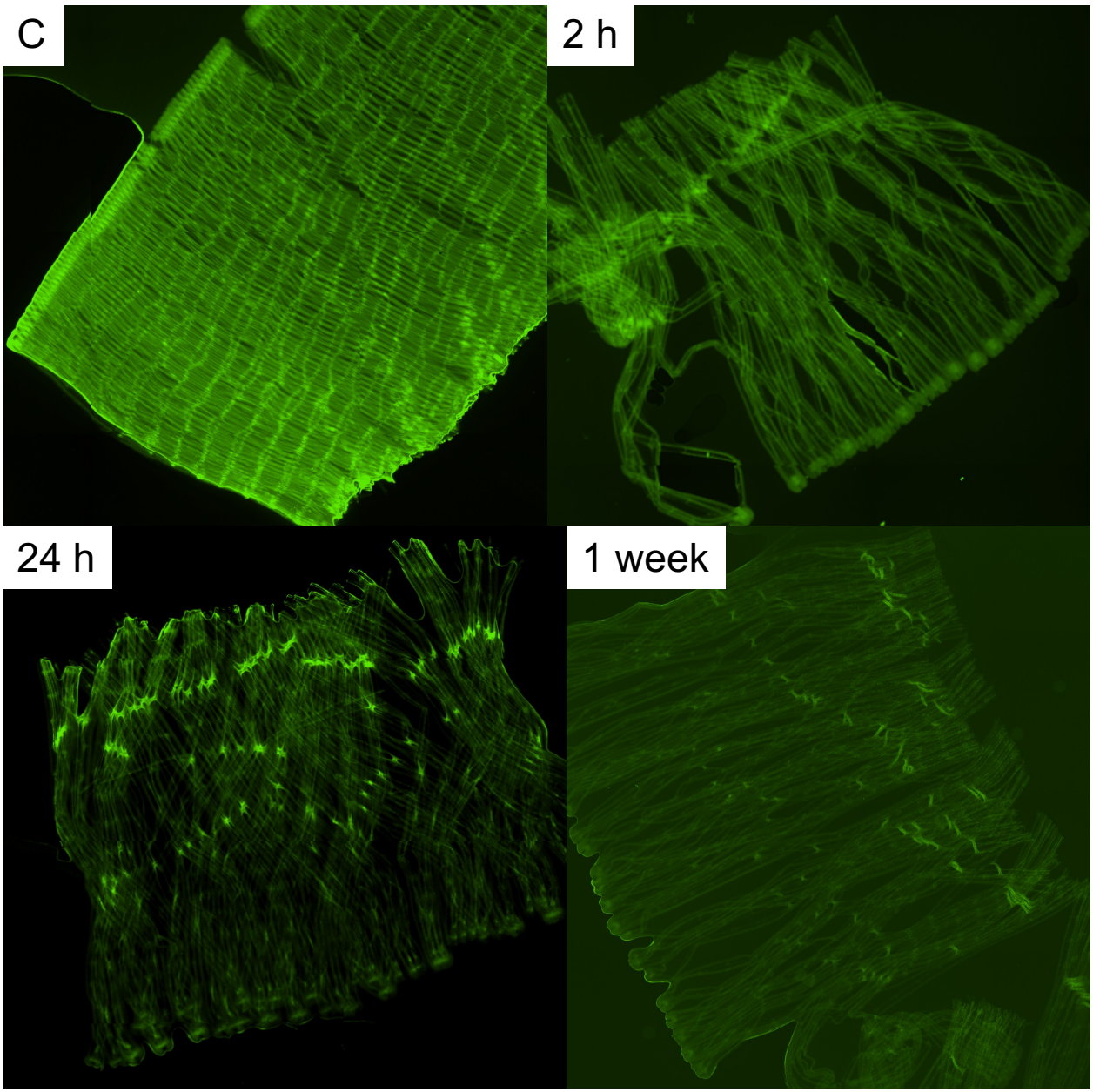

Figure 3.2 *Mytilus trossulus* gill tissues visualized under 4× magnification and stained for actin. Top left panel shows a control, un-frozen gill, top right panel shows a gill 2 hours after freezing, bottom left is a gill 24-hours post-freeze, bottom right is a gill 1-week post-freeze. General dis-organization of parallel gill filaments can be observed after freezing, particularly 2 hours after freezing. Mussels were collected from Tower Beach, Vancouver, BC and mussels were all frozen at -10 ºC for 3 hours.

3.3.3 Impacts of Freezing on Posterior Adductor Strength

3.3.3.1 December 2020 Trials

For Instron trials completed using mussels collected on December 12, 2020, I used a zero inflated negative binomial model to test if freezing treatment and mussel shell length impacted mussel posterior adductor muscle strength, measured as time to open under a 4 N pulling force. All freezing treatments resulted in a significant decrease in mussel posterior adductor muscle strength as measured by time to open (Table 3.4; Fig 3.3). Mussel shell length did not have a significant impact on mussel posterior adductor strength ($z=-0.064$, $p=0.949$).

Table 3.4 The time it takes *Mytilus trossulus* to open under a constant 4 N pulling force changes after mussels receive different freezing treatments and/or recovery time post-freeze. Results are from a zero-inflated negative binomial model; each treatment group is compared against control mussels. Significant p values are bolded. Mussels were frozen at -8 ºC for two hours in all treatments. In the "7 h Post (2 Freezes)" treatment group mussels were at -8 °C frozen twice (with 24 hours between freezes). Mussels were collected from Tower Beach, Vancouver, BC. *n*= 18-23 per treatment.

Treatment	**z value**	**p value**	**Mean Time to Open (s) ± Standard Error**
Control			147.9 ± 28.9
7 h Post Freeze	-2.61	**0.00918**	72.12 ± 15.2
7 h Post (2 Freezes)	-2.28	**0.0224**	79.45 ± 20.7
15 h Post Freeze	-2.69	**0.00723**	84.61 ± 15.1
24 h Post Freeze	-1.78	0.0746	79.89 ± 11.3

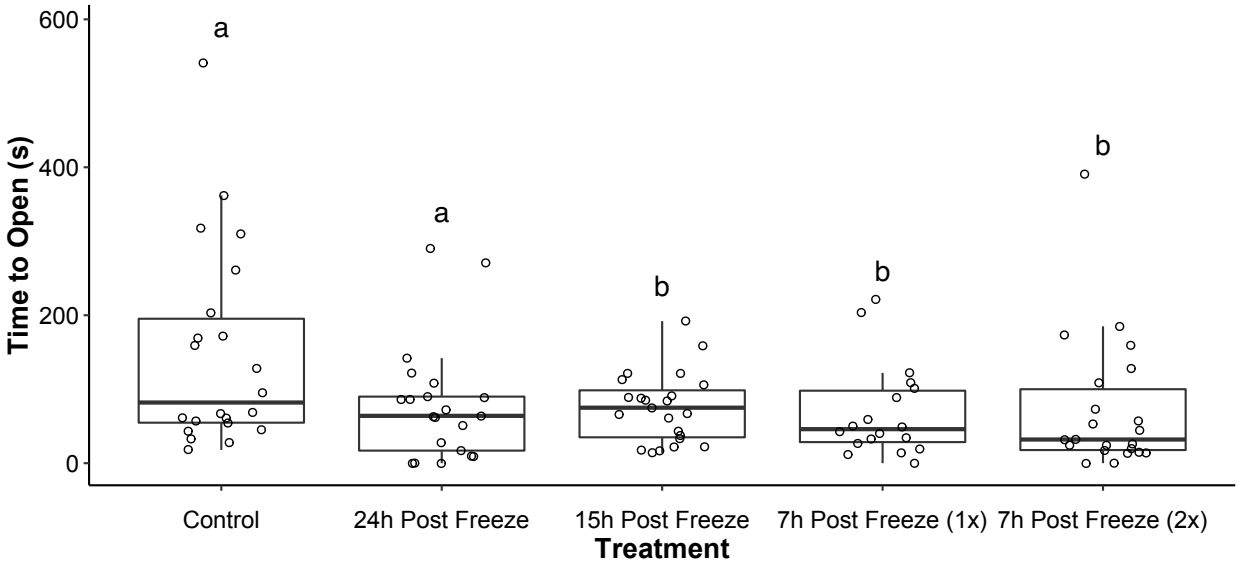

Figure 3.3 Freezing significantly lowers *Mytilus trossulus* posterior adductor muscle strength. Mussels were frozen at -8 ºC for two hours. In the "7 h Post × 2" treatment group mussels were at -8 °C frozen twice with 24 hours between freezes. Posterior adductor muscle strength is reported as the time it took mussels to open under a constant 4 N pulling force exerted by an Instron. Letters denote significant differences between groups, from a zero-inflated negative binomial model. Mussels were collected from Tower Beach, Vancouver, BC. *n*= 18-23 per treatment.

3.3.3.2 March 2021 Trials

For the March Instron trials, a zero-inflated negative binomial model was used to determine if freezing treatment, posterior adductor muscle diameter, or mussel shell length was a significant predictor of mussel time to open. Mussels frozen at -8 ºC twice opened significantly faster than control mussels ($z=-2.69$, $p=0.00715$, $df=9$; Fig 3.4; -8 ºC × 2 mean time to open = 26.29 ± 4.89 s), mussels frozen at -10 ºC once opened marginally-significantly faster than control mussels ($z=-1.87$, $p=0.0622$; -10 ºC mean = 39.27 ± 10.4 s), and mussels frozen at -8 ºC once did not have significantly different opening times, as compared to the control ($z=-1.27$, $p=0.203$; -8 ºC × 1 mean = 49.56 ± 10.5 s). Control mussels had a mean time to open of 70.17 ± 21.1 seconds. Mussels with larger posterior adductor muscles opened marginally-insignificantly faster ($z=-1.87$, $p=0.0610$, $df=11$; Fig C.3). Mussel shell length did not have a significant effect on time to open ($z=1.50$, $p=0.133$).

After three hours of recovery post-freeze, 2/20 mussels appeared to be “dead” (i.e., unable to hold their shells shut on removal from seawater) in the -8 ºC × 2 treatment and 4/20 mussels were categorized as “dead” in the -10 ºC treatment (Fig 3.5). These mussels were excluded from testing with the Instron.

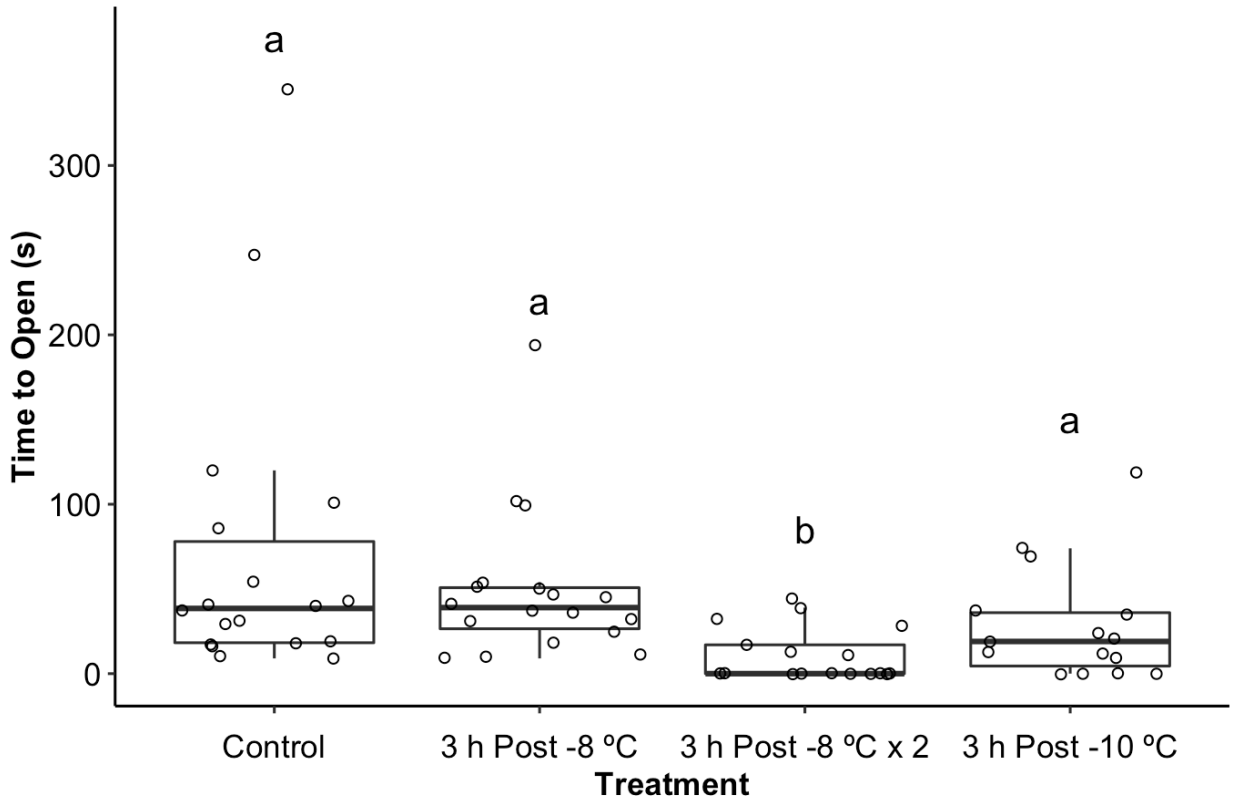

Figure 3.4 Experiencing freezing reduces the time that *Mytilus trossulus* can resist opening their shells under a constant 4 N pulling force exerted by an Instron, but only after being frozen at -8 ºC for 3 h twice. Mussels were collected from Tower Beach, Vancouver, BC. Letters refer to significant differences between treatments, as detected through a zero-inflated negative binomial model. Sample sizes were: 18 for the control, 18 for the -8 ºC × 1 treatment, 17 for the -8 ºC × 2 treatment, and 15 for the -10 ºC treatment.

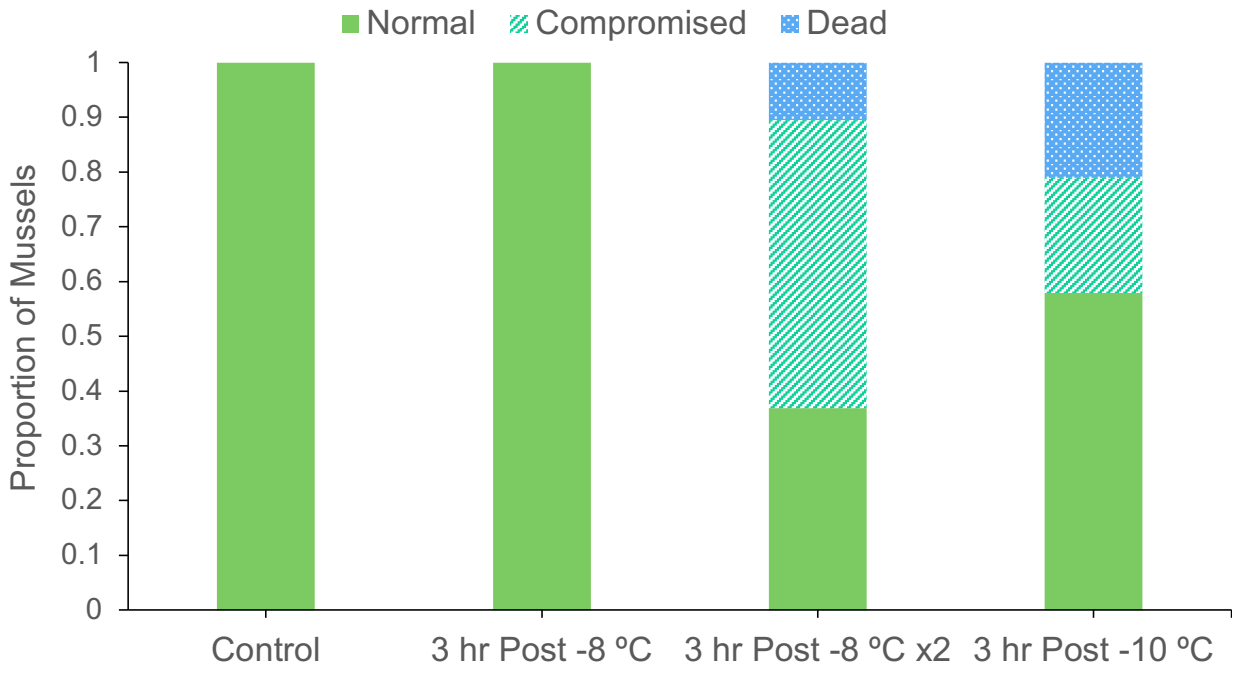

Figure 3.5 Upon removal from the seawater after freezing, a significant portion of *Mytilus trossulus* mussels were either compromised (i.e., they opened before the Instron machine reached the 4 N pulling force) or they appeared to be "dead" (i.e., the mussel immediately gaped upon removal from the seawater), but just in the two most extreme freezing treatments. "Normal" refers to mussels that held their shells shut when removed from seawater and resisted the pulling force of the Instron, at least until it reached a pulling force of 4 N. Mussels were collected from Tower Beach, Vancouver, BC.

3.3.4 How Freezing Impacts Sea Star Feeding Rate on Mussels

An ANOVA revealed that the number of mussels eaten per sea star per day was not dependent on whether the mussels that the sea stars were fed had been recently frozen ($F_{1,22}$=0.0010, p=0.98; Fig 3.6), and it was also not significantly impacted by the sea star's arm lengths ($F_{1,22}$=0.0040, p=0.95). Daily feeding rate was significantly impacted by experiment day ($F_{3,22}$=4.9, p=0.0097) with feeding rate on day 2 being significantly higher than day 3 and day 4 (day 2 vs. day 3 p=0.0225; day 2 vs. day 4 p=0.0487). The interaction term between treatment and day was insignificant ($F_{3,22}$=0.57, p=0.64).

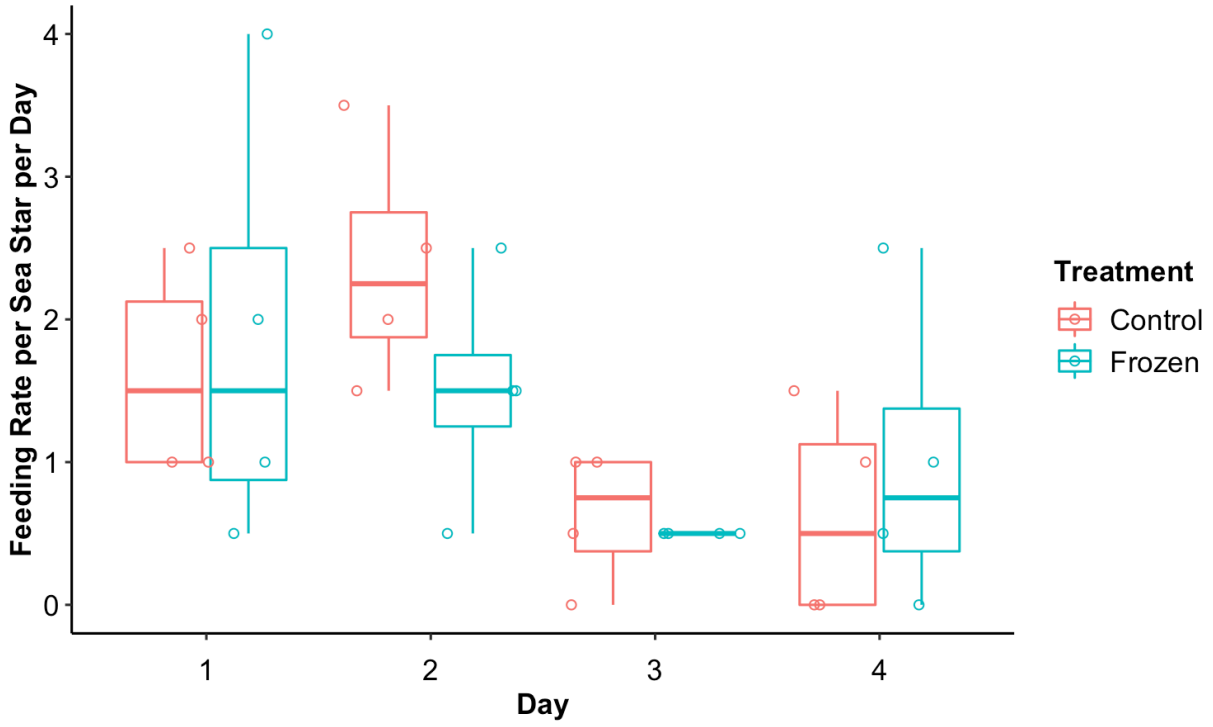

Figure 3.6 *Evasterias troschelii* feeding rate does not significantly change based on whether the stars are fed *Mytilus trossulus* mussels that had been recently frozen at -10 ºC for three hours or fed control, un-frozen mussels. Feeding rate was measured each day for four days and is reported as the number of consumed mussels per sea star per day (n=4). Sea stars were collected near the Girl in a Wetsuit statue in Stanley Park and mussels were collected from Jericho Beach, both sites are in Vancouver, BC.

3.3.5 Mussel Survival After Repeated Freezes

All mussels that were used in these repeated freezing experiments froze, as evidenced by the characteristic supercooling point or exothermic release of energy indicating that ice formation has occurred. The average supercooling point was -3.3 ± 0.15 ºC (n=60). In order to determine how the number of repeated freezing exposures impacts survival, with total time frozen held constant at 8 hours, I compared the following three repeated freezing treatments: a single exposure for 8 hours, two 4 hour freezes and four 2 hour freezes. I compared these three freezing treatments amongst mussels frozen at -8 °C and -10 °C separately.

For the -8 °C group, freezing treatment was a significant predictor of survival (df=2, deviance=24.95, $p<0.001$; Fig 3.7), however intertidal shore position and shell length were not (df=1, deviance=2.95, p=0.086; df=1, deviance=0.035, p=0.85). A Tukey post-hoc test revealed that survival after a single 8-hour freeze was significantly lower than both other treatments: the two 4 hour freezes (p=0.024) and four 2 hour freezes ($p<0.001$). Survival was not significantly different between the 2 × 4-hour freeze treatment and the 4 × 2-hour freeze treatments (p=0.077).

In the -10 °C group, freezing treatment was a significant predictor of survival (df=2, deviance=40.01, $p<0.001$; Fig 3.7); however, intertidal shore position and shell length were not (df=1, deviance=0.51, p=0.47; df=1, deviance=1.14, p=0.29). A Tukey post-hoc test revealed that mussels frozen for two hours four times survived significantly better than those in the single eight-hour freeze treatment ($p<0.001$) and mussels frozen for four hours twice ($p<0.001$). Mussels frozen in the single 8-hour freeze and 2 × 4-hour freezes did not have statistically significant different survival proportions (p= 0.207).

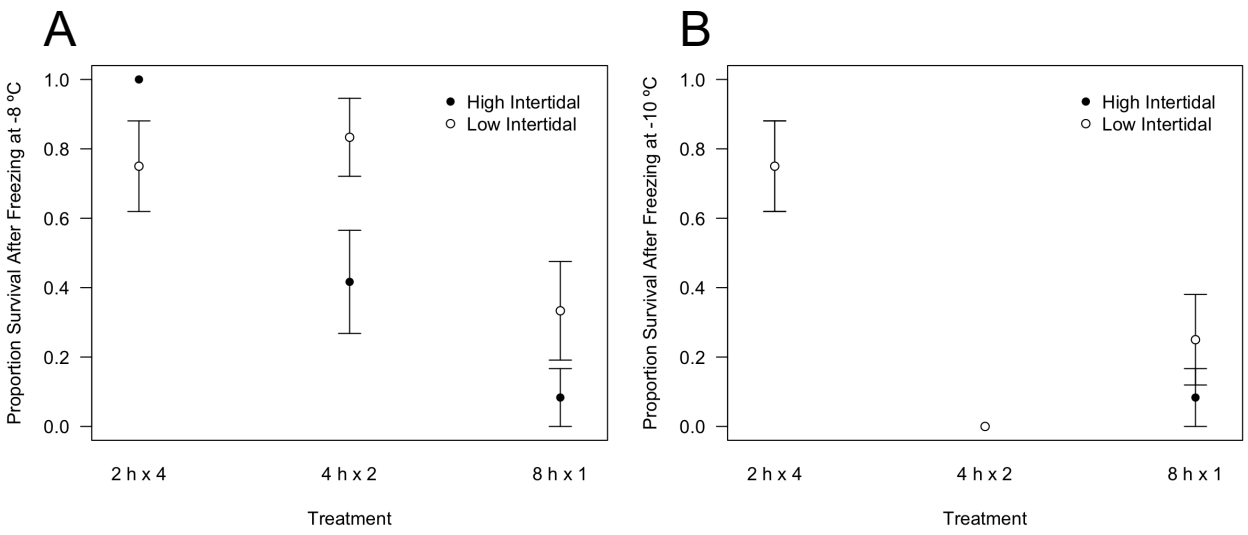

Figure 3.7 *Mytilus trossulus* survive better after a set of repeated 2 h freezing exposures (with 24 h for recovery between each freeze), as compared to more prolonged freezing exposures of 4 h × 2 and 8 h × 1. Panel A shows mussel survival after exposure to -8 ºC and Panel B shows mussel survival after exposure to -10 ºC. *n*=12 per treatment. Mussels were collected from Tower Beach, Vancouver, BC. Error bars are standard error of the proportion.

When considering only mussels frozen at -8 °C for two hours once, twice, three times or four times, cumulative freeze number did not significantly impact survival (df=3, deviance=5.12, p=0.16; Fig 3.8), neither did intertidal shore height (df=1, deviance=0.89, p=0.16) nor shell length (df=1, deviance=0.46, p=0.50). Considering mussels frozen at -10 °C for two hours once, twice, three times or four times, cumulative freeze number was a significant predictor of survival (df=3, deviance=15.47, p=0.0015; Fig 3.8), but intertidal shore position and shell length were not (df=1, deviance=0.39, p=0.53; df=1, deviance=0.25, p=0.61). A Tukey post-hoc test showed that mussels frozen three times at -10 ºC for 2 h survived significantly less than those frozen only once (p=0.018) and mussels frozen four times survived less than those frozen once, although this difference was only marginally insignificant (p=0.0556). Mussels frozen twice also had marginally significantly higher survival than those frozen three times (p=0.0556). All other comparisons were insignificant.

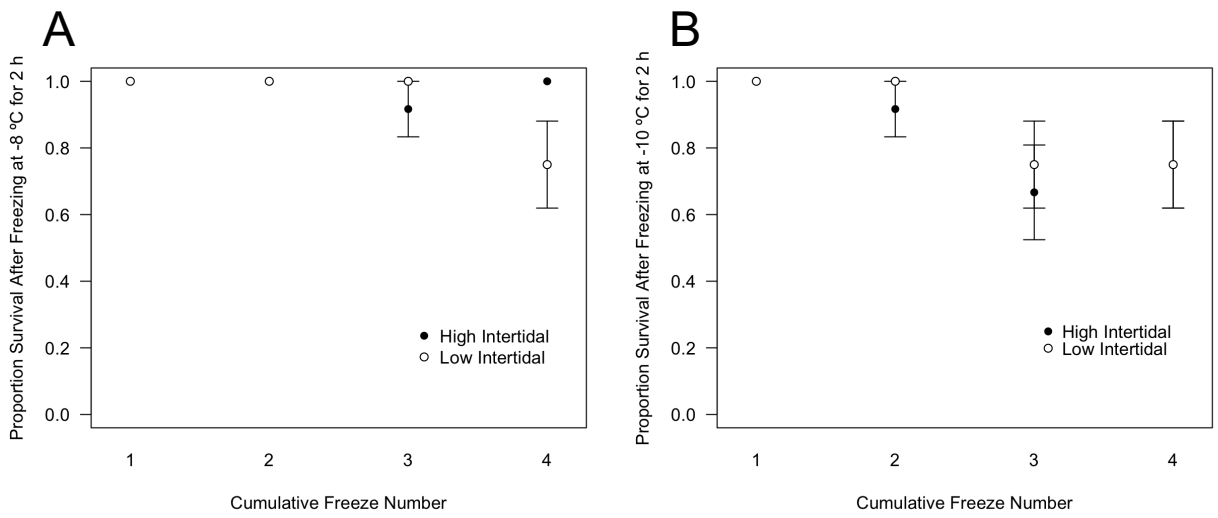

Figure 3.8 *Mytilus trossulus* survival decreases slightly when they are frozen for 2 h repeatedly, with decreasing survival as cumulative freeze number increases. This pattern was insignificant for the -8 ºC treatment (Panel A) but was significant for the × 3 and × 4 groups in the -10 ºC treatment, compared to the control (Panel B). Mussels were given 24 hours in seawater to recover between freezes. Mussels were collected from Tower Beach, Vancouver, BC. Error bars are standard error of the proportion.

Mussels frozen for four hours only one time survived better than mussels frozen for four hours twice at both test temperatures: -8 °C (df=1, deviance=4.14, p= 0.0548; Fig 3.9) and -10 °C (df=1, deviance= 42.4, p<0.001). Low intertidal mussels survived better than high intertidal mussels, but only at the -8 ºC test temperature (df=1, deviance=8.37, p=0.00381). There was no significant effect of intertidal shore height on mussel survival after freezing at -10 ºC (df=1, deviance=1.87, p=0.171).

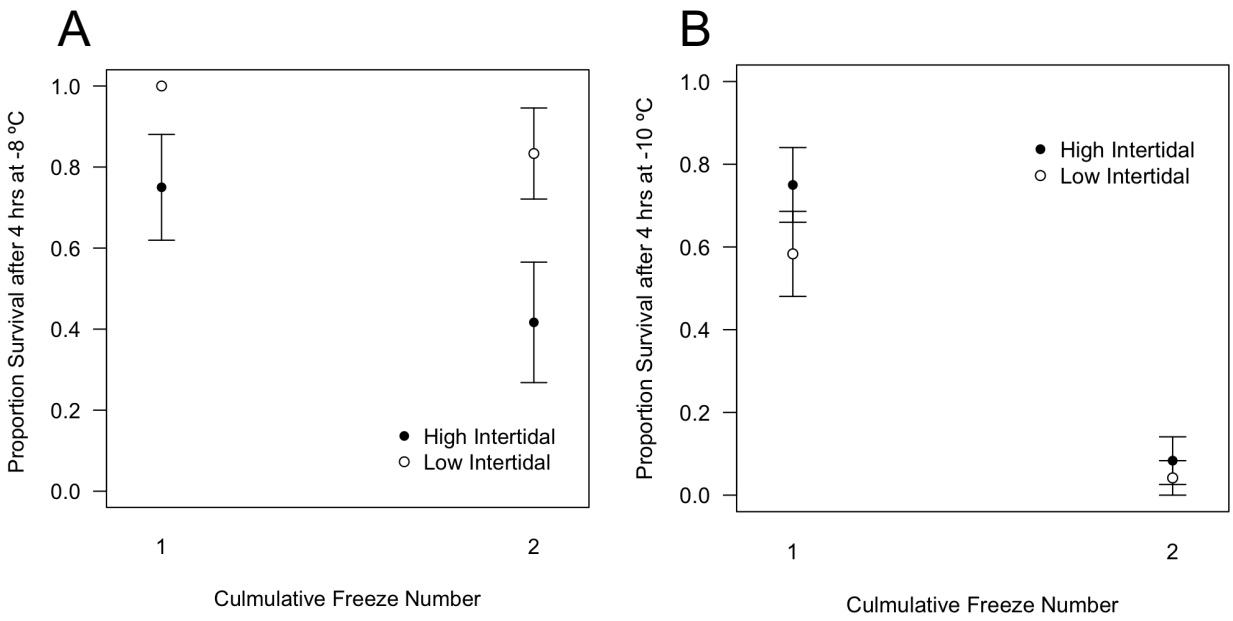

Figure 3.9 *Mytilus trossulus* survival decreases when they are frozen for 4 h twice, compared to just once. This pattern was significant for the -8 ºC treatment (Panel A) and -10 ºC treatment (Panel B). Mussels were given 24 hours in seawater to recover between freezes. Mussels were collected from Tower Beach, Vancouver, BC. Error bars are standard error of the proportion.

3.4 Discussion

Mytilus trossulus experiences freezing in its habitat during winter low tides; however, little is known about the impacts of freezing on their ecological functioning. Here I have provided some of the very first data on how sublethal freezing stress impacts mussel performance. First, I showed that mussels do not filter feed for the first four hours after a sublethal -10 ºC freeze, but after 24 hours of recovery post-freeze, mussels did resume filter feeding, albeit at a slower rate than control mussels. Relatedly, I demonstrated that mussel gill tissues are detectably damaged after freezing. I also showed that mussel posterior adductor strength does significantly weaken after sublethal freezing. However, sea star feeding rates were not higher on frozen mussels as compared to control mussels. Additionally, repeated freezing is not nearly as damaging as more prolonged freezes are, in terms of mussel survival rates. Although, I was able to show that repeated freezes do negatively impact mussel posterior adductor muscle strength. Taken together, I have shown that there are negative impacts of sublethal freezing on mussel performance, which may have downstream impacts on intertidal ecosystem.

3.4.1 Impacts of Freezing on Mussel Filtration Rate

Mussels do not filter feed for the first 4 hours post sublethal freeze (-10 ºC for three hours). Mussel filtration resumed 24 hours after freezing, however, recently frozen mussel filtration rates were still significantly slower in the recently-frozen group even after this 24-hour recovery period. Thus, mussels resume filter feeding 24 hours after sublethal freezing, but at a reduced rate.

This reduction in filter feeding may be a result of direct gill damage from freezing, which can be visualized in Figure 3.2 as increased gill filament disorganization, which may be due to

cytoskeletal damage, more specifically damage to actin. Since the latero-frontal cilia of neighbouring gill filaments form a lattice which enables the mussel to "catch" plankton cells from the water column, the proper, parallel positioning of gill filaments in relation to one another is key for filter feeding (Riisgård et al., 2015), so the disorganization of gill filaments in the frozen gills shown in Figure 3.2 may be why mussels were unable to filter feed for the first few hours after freezing. These qualitative observations are supported by previous research that indicates that cytoskeletal damage is often caused by freezing in various freeze tolerant insect species (Cottam et al., 2006; Des Marteaux et al., 2018; Kayukawa and Ishikawa, 2009).

Furthermore, visual, qualitative examinations of microscopy images of gill tissues indicate that mussel gills tissues are able to repair themselves after freezing damage, with some repair happening as soon as 24 hours post-freeze, which suggests that the temporary cessation in mussel filtration rate immediately after freezing may be caused by gill damage. The possibility that mussels are repairing freeze-inflicted gill damage is further supported by another study which found that zebrafish gill tissues are able to repair and regenerate after cryoinjury (Ramel et al., 2021). This study found that it took 3-4 weeks for zebrafish gills to fully repair, however the severity of cryoinjury was much higher in this experiment (experimental cryoinjury was performed *via* touching gills with a steel rod cooled with liquid nitrogen) than in my study, which may explain why I observed relatively faster gill recovery (Ramel et al., 2021).

Since most of the freeze tolerance literature utilizes insect model organisms, and most insects cease feeding during the winter months (Sinclair, 2015), there is limited literature with which to compare my results to. One previous study tested how cold shocks impacted the feeding rate of intertidal predators and found that the intertidal sea stars *Evasterias troschelii* and *Pisaster ochraceus* and the intertidal dogwhelk *Nucella lamellose* all showed significantly lower

feeding rates for two weeks after they were exposed to a sublethal yet stressful cold exposure of -0.5 ºC air temperature for three hours, which aligns with my finding that low temperature stress reduces feeding rate in *M. trossulus* (Currie-Olsen, 2021). Another relevant previous study found that *Mytilus edulis* filtration rates significantly decrease when seawater temperatures are between 4-7 ºC, as compared to warmer water temperatures, which corresponds to lower mussel growth and metabolic rates, and supports my finding that mussel filtration rates are negatively impacted by cold temperatures (Daigle and Herbinger, 2009; Hawkins and Bayne, 1992). Here I've shown that mussels have reduced feeding rates after sublethal freeze exposures, yet the effect of freezing on intertidal mussel feeding rates is much shorter lasting than in the effect of cold stress on intertidal predator feeding rates.

Mussel filter feeding is important on a more broad ecological scale since the filter feeding activity of mussels has been shown to significantly improve water quality (Al-Mamun and Khan, 2011; Jansen et al., 2018) and is a key component of benthic-pelagic ecosystem coupling (Menge et al., 1997). It is not entirely clear how much local water quality in the Burrard Inlet would be negatively affected if an entire mussel bed ceased feeding after a freezing low tide, particularly since phytoplankton concentrations are typically low during the winter (Venrick, 1993)**,** and because it remains unclear exactly when mussels resume filter feeding after freezing. In addition, the effect of freezing on filter feeding rates likely depends on the severity of the freeze exposure. Perhaps if a cold snap were to occur during a low tide series and mussels were repeatedly exposed to sublethal cold and/or freezing stress for several consecutive days this may lead to a longer-term cessation of feeding in mussels. Further studies investigating how repeated freezing impacts filtration rate would be illuminating to better understand the potential ecological impacts of freezing in the intertidal. This future direction would be particularly interesting since up-

regulating cryoprotective mechanisms and repairing freeze-related damages are known to be energetically costly in insects (Marshall and Sinclair, 2018), and so if mussels are unable to feed for a significant amount of time post-freezing then they may become severely energetically limited, perhaps compounding the negative impacts of freezing even further.

3.4.2 Impacts of Freezing on Mussel Susceptibility to Sea Star Predation

While freezing does significantly lower posterior adductor strength in mussels, this effect does not lead to a significant increase in the number of mussels are eaten by the sea star *Evasterias troschelli* across a four-day period. This matches my prediction that freezing would weaken mussels' posterior adductor muscle strength, presumably due to the physiological stress inflicted by freezing. These findings align with a previous study which found that after being exposed to pH stress (i.e., after being kept in acidic seawater), *Mytilus trossulus'* posterior adductor muscles weakened (Flynn, 2016). Additionally, when mussels only had 3 hours to recover from freezing, a proportion of mussels were unable to hold their shells shut on removal from seawater, absent of any external pulling force, which would make them susceptible both to desiccation and to sea star predation (Fig 3.5, Fig C.5). To better understand the mechanism behind this finding, one further direction would be to determine if glycogen content in mussel posterior adductor muscles decreases post-freeze which would indicate that energy stores post-freeze may be depleted as more energy is devoted to recovering from freezing. Alternately, mussels' posterior adductor muscles might be directly damaged from freezing which might be the reason behind the decrease in posterior adductor muscle functionality that I observed.

Interestingly, mussel body size (measured as shell length) and mussel posterior adductor muscle diameter did not have a significant effect on mussel posterior adductor strength. Although, this finding may be due to the fact that I only tested mussels with a narrow range of

body sizes. One previous study found that *Myiltus edulis* specimens with heavier posterior adductor muscles were handled by the sea star *Asterias rubens* for significantly longer, indicating that these mussels were better able to resist being opened by the stars (Freeman, 2007). Another study found that *M. edulis* mussels from a population with wider mean posterior adductor muscle diameters were eaten at a slower rate by the sea star *Asterias rubens*, compared to mussels from a population with smaller posterior adductor muscle diameters, again indicating that posterior adductor muscle diameter is important in determining how easily a sea star can pry open and consume a mussel (Hancock, 1965), but contrary to my findings that posterior adductor muscle diameter had no significant effect on mussels' ability to resist being opened.

Although posterior adductor strength does significantly weaken after mussels are frozen at -10 ºC for three hours, sea stars did not exhibit increased feeding rates when fed mussels that have been frozen at -10 ºC for three hours, as compared to sea stars that are fed control, un-frozen mussels. This result is contrary to my prediction that if mussels' posterior adductor muscles weaken due to freezing, this would make them more susceptible to being eaten at a faster rate by sea stars. Previous work has established that mussels which take a longer time to open under the 4 N force exerted by the Instron also take longer to be opened by sea stars of the species *Pisaster ochraceus* (Flynn, 2016), but this study did not actually look at the number of mussels consumed by sea stars over an extended period, as I did in my study. One reason why sea star feeding rate does not increase when the stars are fed frozen mussels might be because the effect size of freezing on posterior adductor muscle strength is relatively small; for instance, in March-collected mussels, the control group mean time to open 70.2 ± 21 seconds while the -10 ºC treatment group mean time to open was 28.8 ± 8.8 seconds. Thus, since the time it took mussels to open only changed by around one minute, this effect might not make a measurable

impact on overall sea star feeding rate since the rate that sea stars can find and attach to new mussels and/or their digestive rates might be more important factors in determining the rate at which sea stars can consume mussels. Further, since the sea star feeding trial went on over the course of four days, mussels may have had time to recover from freezing before they were attacked by a sea star, since mussels posterior adductor muscle strength returned to normal 24 hours post-freeze (Fig 3.3). Additionally, sea star predation might increase if mussels are frozen repeatedly before they are fed to sea stars, so future studies should consider this possibility.

One important caveat is that sea stars are not very common in the estuarine sites used in this study. Since sea stars are relatively intolerant to low salinity (Held and Harley, 2009) they are rarely found at the low-salinity Tower Beach site (Fig A.1). However, sea stars can be found at considerably high densities at the Jericho Beach site (a few hundred sea stars across ~50 m of shore), but sea star abundance drops dramatically at this site in the summer in accordance with a seasonal drop in salinity (Kay et al., 2019). Since mussels upregulate their predatory defence through increasing their posterior adductor muscle size and strength when reared in close proximity to sea stars (Freeman, 2007; Reimer and Tedengren, 1996), the mussels in this study may all just have low posterior adductor strength to begin with, which may account for the small effect size of freezing on posterior adductor muscle strength that I measured. Thus, more studies measuring changes in mussel posterior adductor strength after freezing using mussels from populations with more sea star predation would be a worthwhile future direction.

In conclusion, while there is a slight, yet statistically significant, decrease in mussel posterior adductor muscle strength after freezing, a relatively mild freezing low tide is not likely to have discernable impacts on the overall predation pressure by sea stars on intertidal mussels, at least at the study sites used in my thesis.

3.4.3 Mussel Survival After Repeated Freezes

As expected, mussel survival decreased as the number of cumulative freezes increased for the 2 hour and 4-hour freeze-length treatment groups. However, unexpectedly, mussel survival was significantly higher after 4 repeated 2-h freezes, as compared to one single, prolonged 8-h freeze event, for both the -8 and -10 ºC test temperatures. This result was contrary to my predictions since I expected that the added stressors of repeated cooling, freezing, and thawing would've resulted in lowered survival for the repeatedly frozen mussel group due to the unique physiological stressors involved with each of these three stages.

There are a few potential explanations to account for the trend I observed. First, perhaps the 24-hour recovery period between freezes is key for mussels to up-regulate cryoprotective mechanisms in preparation for subsequent freezes and/or to repair freeze-related damages. This possibility is supported by the fact that *Mytilus trossulus* up-regulated the heat shock protein HSP70 (which is thought to be cryoprotective due to its protein stabilizing capacity) 20 hours after being repeatedly frozen at -6 ºC for two hours four times, but not after a continuous 8 h freeze at -6 ºC (Gill et al., 2022). This suggests that the recovery periods between freezes give mussels the opportunity to upregulate cryoprotective mechanisms such as the HSP70 response, and so further study on the processes which occur during high tide recovery periods should be investigated further.

Furthermore, while an animal is frozen, oxygen transport and other essential metabolic processes either greatly slow or entirely cease (Storey and Storey, 1988), and so perhaps the extended lack of oxygen supply which occurs during these prolonged freezing treatments is what is causing the increased mortality. Additionally, freezing in live organisms is a dynamic process that continues for hours, wherein the percent of an organisms' body which is frozen increases

throughout the freezing exposure, and so in the shorter freezing exposures mussels likely experienced lower proportions of their body water turning to ice, as compared to the more prolonged freezing treatments (Layne and Lee, 1987). This is supported by qualitative visual examinations of mussels bodies throughout a two hour freeze exposure which showed that the visible precent of the mussels' body which was frozen increased through time, and it appeared as though the entire mussel body does not completely freeze until approximately one hour and 30 minutes into the freezing exposure (Fig C.4). I did confirm that mussels in the short two-hour freeze exposures experienced internal ice formation (as evidenced by the supercooling points, and the presence of ice in the mantle cavity in Fig C.4), but likely a lower percentage of their body water was converted to ice during the two hour exposures, as compared to the more prolonged four and eight hour freezing events which probably resulted in a significantly higher ice fractions. Higher proportions of body ice content are known to be more physiologically damaging to freeze tolerant organisms (Claussen and Costanzo, 1990), and so mussels might have experienced higher rates of mortality after prolonged freezing *vs.* repeated freezing for this reason (when total time frozen is held constant). Thus, analysing how total body ice content varies over time in mussels more precisely through calorimetry is worth exploring in future studies, since studies on freeze tolerant frogs showed that there is a significant delay (~ 20 hours) between a frog's supercooling point and the point that equilibrium (i.e., maximum) ice content is reached (Layne and Lee, 1987; Layne and Lee, 1989).

Previous studies comparing the effects of repeated freezes vs. prolonged freezes in insects have yielded contradictory results: in some cases repeated freezes results in higher survival compared to prolonged freezes of the same cumulative length (Teets et al., 2011) and in others repeated freezing lowers survival (Doelling et al., 2014; Marshall and Sinclair, 2011).

This was the first study to compare repeated vs. prolonged freezing survival rates in intertidal invertebrates, and so more study on other intertidal invertebrate species will be useful to better understand how freezing duration relates to survival across a broader taxonomic range of freeze tolerant species.

Additionally, I compared the survival of high and low intertidal mussels since intertidal organisms which are found higher up on the intertidal shore are exposed to air for longer periods, as compared to organisms lower down on the shore which spend more time submerged under seawater, meaning that high intertidal mussels would be exposed to longer and perhaps more severe cold and freeze exposures during low tide emersion. Therefore, one would expect that mussels inhabiting areas higher up on the intertidal shore would be more tolerant to extreme cold stress and/or freezing, as compared to mussels found in lower intertidal areas, since high intertidal mussels would more regularly be exposed to longer and potentially more severe freezing temperatures *in situ* (Reid and Harley, 2021)**.** However, in this study I found that shore height did not have a significant effect on mussel survival after freezing, apart from in one set of data where low intertidal mussels survived better than high intertidal mussels (in the comparison between survival after a -8 ºC freeze for 4 h either once or twice). Overall, I did not find that high intertidal mussels were significantly more tolerant of freezing in this study, as I had expected, which contradicts some of my previous work wherein high intertidal mussels were found to be more freeze tolerant than low intertidal mussels (Kennedy et al., 2020). However, since only two test temperatures were used in this study, and since the effect of shore height that was reported in my 2020 paper was quite small, perhaps we were unable to detect a shore height effect since we only used two test temperatures.

Thus, shorter, repeated freezes result in higher mussel survival, as compared to more prolonged freezes, when total time frozen remains constant. However, as cumulative number of repeated freezes increases, mussel survival decreases, which was expected. This likely means that the recovery period between freezes is important increasing survival after freezing, which makes sense since *in situ*, mussels get a reprieve from freezing when they are submerged under seawater during the high tide. However, if a multi-day cold snap were to align with a set of very low tides, this may be detrimental to mussels, if the cumulative length of time that mussels are frozen for exceeds a certain threshold.

3.5 Conclusion

Here I have shown that while mussels can survive relatively short freezing exposures, there are significant negative effects on mussel performance caused by sublethal freezing that have previously been overlooked. First, mussel filer feeding entirely ceases for the first 4 hours after being frozen, however mussels regain their capacity to filter feed after a day of post-freeze recovery, albeit at a slightly slower rate. The ecological significance of this remains unclear since a 24-hour suspension in filter feeding across an entire mussel bed may or may not lead to a significant drop in local water quality. Although, if mussels experience multiple freeze exposures over the course of subsequent daily low tides this may lead to more drastic impacts on water quality and organism energy supply. Additionally, through qualitative assessments I showed that mussel gills are damaged due to freezing, and it appears as though mussels are able to repair their gills after freezing. Mussel posterior adductor strength significantly decreases after freezing, but not by a large margin. This effect does not translate into increased susceptibility to sea star predation, meaning that mussel posterior adductor muscle weakening post-freeze might not be an ecologically relevant effect. I have found that sublethal freezing does have significant, negative

impacts on mussel performance, but it does not seem as though these impacts are so detrimental as to cause severe negative impacts on mussel populations, and/or the wider intertidal ecosystem. Overall, my finding that freezing negatively impacts mussel performance aligns with previous literature on the effects of heat stress on the intertidal mussel *Mytilus californianus*, where heat stress reduces mussel performance by lowering mussels' gonadosomatic indices (i.e., reproductive capacity; Waite and Sorte, 2022) and growth rates (Fitzgerald-Dehoog et al., 2012). Additionally, one previous study showed that the intertidal snail *Littorina scutulata* had reduced performance (as evidenced by a decreased capacity for snails to right themselves) following two hour cold exposures to -6 or -9 ºC air (Reid and Harley, 2021). Finally, mussels survive shorter, repeated freezes better than more prolonged freezes, even when total time frozen is held constant, perhaps indicating that the recovery periods between freezes are important for repairing freeze damage and/or upregulating cryoprotective mechanisms.

Chapter 4: Conclusion

In my thesis I investigated the mechanisms and consequences of surviving freezing in the intertidal mussel, *Mytilus trossulus*. First, to better understand the biochemical mechanisms underlying freeze tolerance in mussels, I explored the role of osmolytes as cryoprotectants in mussels by artificially manipulating mussel osmolyte concentrations, and then testing how mussel freeze tolerance changed. Then, I determined if sublethal freezing has ecologically relevant, negative consequences on mussel performance. Specifically, I determined if mussels are negatively impacted by sublethal freezing by measuring a) how mussel filtration rate changes post-sublethal-freeze and b) how mussel susceptibility to predation changes post-sublethal-freeze. Lastly, to shed further light on how freezing impacts mussels in an ecologically relevant way, I tested how well mussels survive repeated freeze exposures which were separated by recovery periods to mimic what mussels would experience *in situ* during a low tide series and compared this to mussel survival after more prolonged freezes of the same length. Overall, I found that mussels are well-equipped to survive freezing and repair freeze-related damages; however, there are sublethal effects of freezing exposure, and some of these effects may translate into negative consequences to the intertidal ecosystem.

4.1 Chapter 2 Summary

I found that mussel gills readily take up osmolytes when incubated in osmolyte-enriched seawater (apart from the osmolyte TMAO). However, to maintain osmotic balance, as mussels took up the osmolyte that they were incubated in, other osmolyte concentrations decreased, meaning that incubating mussels in osmolyte-enriched seawater disrupted the normal relative proportions of various osmolytes in mussel tissues. Thus, in hopes of enriching osmolyte concentrations without modifying the overall composition of the osmolyte pool I then incubated

mussels in seawater enriched with a combination of all the five most prominent osmolytes, relative to the proportions in which they are typically found in mussel gill tissues, which still resulted in significant changes to osmolyte pool composition, although not as pronounced changes as in the single osmolyte-incubation treatments. Modifying osmolyte pool composition either had no effect on freeze tolerance (as in the taurine- and betaine- incubation treatments) or the treatment resulted in decreased freeze tolerance (as in the alanine, glycine and the osmolyte cocktail treatments). Importantly, I found that TMAO was the only metabolite which was a significant predictor of freeze tolerance in mussels. Overall, my findings lend support to the hypothesis that osmolytes are non-colligative cryoprotectants since a) disrupting the normal proportions of osmolytes in mussel tissues decreases freeze tolerance, at least in some cases, and b) TMAO concentration alone predicts mussel survival after freezing, indicating that it is a uniquely cryoprotective osmolyte.

This thesis adds to a growing body of literature which is beginning to shed light on the physiological mechanisms which enable mussels, and by extension other intertidal invertebrates, to survive freezing. Here I've shown that mussels likely use osmolytes as non-colligative cryoprotectants, and that the osmolyte TMAO is significantly correlated with mussel freeze tolerance and is therefore a good candidate cryoprotectant. These findings are supported by previous studies which have suggested that osmolytes are important cryoprotectants in intertidal invertebrates, in accordance with the observation that high salinity acclimation increases freeze tolerance in intertidal invertebrates (Kennedy et al., 2020; Loomis et al., 1988; Murphy, 1979). However, there are very likely many other cryoprotective mechanisms which contribute to mussel freeze tolerance, not just osmolyte accumulation. One such mechanism is the use of heat shock proteins, which are upregulated in *M. trossulus* following repeated freezing stress. Thus,

heat shock proteins are likely another important group of cryoprotective molecules that help mussels repair and avoid freeze-related protein damage, particularly when mussels exposed to multiple, subsequent freezes, as would be the case during a winter low tide series (Gill et al., 2022). In addition, putative ice binding proteins (IBPs) have been identified in *M. trossulus* (Box, 2021), and a BLAST search found that IBPs are over-represented in intertidal invertebrates, compared to other habitats, indicating that IBPs might be another important, but currently understudied, group of cryoprotectants in intertidal invertebrates (Box et al., 2022).

4.2 Chapter 3 Summary

Here I presented some of the very first data on how sublethal freezing impacts the performance of intertidal invertebrates and was able to demonstrate that mussels are negatively impacted by freezing, even if they survive the freezing exposure. I found that mussel filtration rate significantly decreases after sublethal freezing stress, and that mussel gill tissues appear visually damaged after freezing. I also found that mussel posterior adductor muscles weakened post-freeze, but this weakening effect did not correspond to an increase in predation by sea stars in the lab. Thus, mussel performance does decrease after sublethal freezing, but these effects are relatively short-lived, and the ecological relevance of these effects are unclear. Additionally, I also showed that mussels survived several repeated freezes better than prolonged freezes of the same total duration, which might mean that the recovery periods that mussels in the repeated freezing treatments received are important for mussels to upregulate cryoprotective mechanisms and/or repair damage from freezing.

This was the first study to test how extreme low temperature stress impacts intertidal mussel performance. My findings align previous research that found that extremely cold temperature exposures reduce intertidal invertebrates' performance. In the intertidal snail

Littorina scutulata, sub-zero air temperatures caused lower mobility (Reid and Harley, 2021). Also, in the intertidal sea stars *Pisaster ochraceus* and *Evasterias troschelii* and the dogwhelk *Nucella lamellosa* cold shocks led to reduced feeding rates (Currie-Olsen, 2021). Furthermore, my findings are supported by previous studies which showed that high temperature stress negatively affects intertidal mussel performance; in *Mytilus californianus*, heat stress caused mussels to have lower gonadosomatic indices (i.e., reproductive capacity; Waite and Sorte, 2022) and lower growth rates (Fitzgerald-Dehoog et al., 2012). Thus, although intertidal mussels are able to tolerate a wide range of temperatures, their performance is negatively impacted by both extreme high and low temperature stress. My study was also the first to investigate the effects of repeated freezes in intertidal invertebrates, but in the insect freeze tolerance literature the effects of repeated freezing on survival are relatively more well documented, although the effects of repeated vs. prolonged freezes on survival vary across different insect species (Doelling et al., 2014; Marshall and Sinclair, 2011; Teets et al., 2011).

4.3 Implications

In the second chapter of my thesis, I studied the role of osmolytes in mussel freeze tolerance and found that osmolytes likely act as cryoprotectants *via* non-colligative mechanisms, rather than just by increasing intracellular osmolarity. This means that different osmolytes likely play unique physiological roles in intertidal mussel freeze tolerance, and/or perhaps the specific relative concentrations of various osmolytes in mussel gill tissues is important for cryoprotection. Since I was unable to increase mussel freeze tolerance by enriching mussels with osmolytes, this suggests that many different mechanisms contribute to increasing mussel freeze tolerance, not just osmolyte accumulation, indicating that intertidal invertebrate freeze tolerance mechanisms are likely much more complex than is currently understood. Furthermore, TMAO

has been identified in my thesis and in other studies to be an important cryoprotectant (Catalá et al., 2021; Raymond and DeVries, 1998), and so perhaps TMAO may be a useful molecule for applications such as cryopreservation.

Investigating how mussel performance is impacted by freezing stress is an important avenue of study, particularly considering that mussels are important members of the intertidal ecosystem, as ecosystem engineers (Buschbaum et al., 2009). Since freezing negatively impacted mussel filtration rate, this would decrease mussel energy supply, which might lead to further negative consequences for mussel performance and/or survival after freezing. This may be particularly harmful if it takes mussels a full 24 hours to recover the ability to filter feed post-freeze, since this would align with subsequent low tide air exposures (during which time mussels are unable to feed) which might lead to an even larger period of starvation. In addition, mussel filter feeding has been shown to significantly improve the surrounding water quality (Jansen et al., 2018), particularly when mussels are found in dense aggregations, as in intertidal mussel beds. Therefore, a significant decrease in mussel filter feeding caused by freezing would be expected to decrease local water quality, although it is not clear to what degree.

Sea star predation is a key biotic factor shaping mussel density and distribution in the intertidal, and so changes in predation pressure by sea stars are very important to understand when trying to predict how mussel populations may be impacted by freezing. Since I found no effect of freezing on mussel susceptibility to sea star predation in the sea star feeding rate experiment, the weakening effect of freezing on mussel posterior adductor strength that I measured using the Instron apparatus probably does not translate into a change in sea star feeding rates on a recently-frozen mussel bed in the field. Therefore, sublethal freezing likely

won't impact intertidal mussel density and/or distribution *via* increased sea star predation *in situ*, at least not at my study sites and not after a single -10 ºC freeze for three hours.

Lastly, the fact that mussels survived repeated freezes better than prolonged freezes when total time frozen was held constant could mean that the recovery periods between repeated freezes are important for mussels to repair freeze related damage and/or upregulate cryoprotective mechanisms. It could also mean that there is a threshold maximum proportion of a mussels' body water that can be frozen before the freezing treatment becomes lethal, since body ice content is likely higher in mussels frozen for more prolonged periods (Claussen and Costanzo, 1990).

4.4 Limitations

In my second data chapter, I was unable to measure the concentrations of some of the key anaerobic by-products that have been previously proposed to be important low-molecular weight cryoprotectants in intertidal invertebrates (namely, octopine, propionate, and strombine). The effect of these metabolites on mussel freeze tolerance therefore remains unclear. Also, there are a few peaks in the mussel gill ^{1}H NMR spectra that I was not able to match to any of the compounds in the Chenomx metabolite library, and so further work to characterize these peaks would be useful to fully describe mussel's metabolite profiles. Lastly, I was unable to measure both survival after freezing and the metabolite profile of each individual mussel, and so in my analyses comparing mussel metabolite concentrations to freeze tolerance I had to use group survival proportions and group mean metabolite concentrations, which takes away statistical power from my analyses.

In the third chapter of my thesis, I found that mussels did not filter feed for the first four hours post-freeze, and I showed that they resume filter feeding 24 hours after freezing, but I am

unable to say exactly when mussels would resume filter feeding post-sublethal-freeze, apart from it being somewhere between 4 and 24-hours post-freeze. Next, since I only characterized damage to mussel gill tissues through qualitative assessments, I cannot put much weight into the patterns of damage that I noticed since I did not quantitatively assess damage. Lastly, after the sea star feeding rate experiment was completed many of the sea stars began to show symptoms of sea star wasting disease around one week after the experiment, so some stars may have been infected with the disease during the experiment, which could represent an extraneous source of variation in this dataset.

4.5 Future Work

The field of intertidal invertebrate freeze tolerance is vastly understudied, and so many questions persist regarding both the mechanisms and consequences of freezing in this group of naturally freeze tolerant animals. My study identified TMAO as a potentially important cryoprotectant, and other recent work has highlighted the role of ice binding proteins (Box et al., 2022) and heat shock proteins (Gill et al., 2022) as mechanisms which enable intertidal invertebrate freeze tolerance. However how all of these cryoprotective mechanisms work together *in vivo* to enable freezing remains unclear. One promising avenue of future study would be to further investigate the role of TMAO in mussel freeze tolerance, since I was not able to artificially enrich mussel tissues with TMAO by incubating mussels in TMAO-enriched seawater, yet still found a significant effect of TMAO on mussel survival across all osmolyte-incubation treatments. Studying the cryoprotective role of TMAO *in vitro* is a worthwhile future direction, in addition to attempting to increase mussel TMAO concentrations *in vivo* through dietary supplementation rather than incubation in TMAO-enriched seawater (De Vooys, 2002), since it is not clear if bivalves have TMAO transporters. Also, ensuring that mussels incubated in

osmolyte-enriched seawater do not have higher baseline mortality levels than control mussels will verify that the decreased freeze tolerance that I measured after certain osmolyte incubation treatments is related to changes in mussel freeze tolerance and not because the osmolyte-incubation treatments were otherwise toxic to mussels.

Further research should be done on a wider array of intertidal invertebrate species to determine what the sublethal effects of freezing in other intertidal invertebrates may be. This would provide a more nuanced understanding how freezing affects the entire intertidal ecosystem. Furthermore, testing how different populations of intertidal invertebrates respond to sublethal freezing will be illuminating since perhaps organisms found in habitats with harsher winters do not experience similar decreases in performance as mussels from the relatively mild Vancouver area do. In addition, since both of my study sites have low sea star densities, mussels from my study sites perhaps are not well adapted to resist sea star predation, and so testing how mussel posterior adductor strength is affected by freezing using mussels from sites with higher levels of sea star predation would also be a worthwhile next step.

Pairing the repeated-freezing treatments more closely with experiments assessing sublethal impacts of freezing (i.e., posterior adductor muscle strength, sea star feeding rates, filtration rates, gill morphology) will lend more insights into how the intertidal ecosystem might be impacted after organisms are exposed to a set of sub-zero low tides during a bout of cold winter weather. In general, studying the effects of repeated freeze exposures (with ~24 recovery periods between freezes), not just single freezes, in future studies on intertidal invertebrate freeze tolerance will better replicate natural freezing regimes that intertidal organisms experience *in situ* in the lab.

My qualitative assessment of mussel gill damage after freezing indicates that further studying how mussel's gill cytoskeleton is damaged by freezing is a promising topic to delve further into, particularly considering that mussel gill tissues appear to be able to repair themselves after freezing. To further substantiate my findings in regards to freeze-related gill damage, future studies should use quantitative methods to assess of gill filament organization and/or cytoskeletal integrity after freezing. Also, staining mussel gills for tubulin, in addition to actin, is another worthwhile future direction.

Lastly, understanding how intertidal invertebrates repair freeze-related damage, and how this repair impacts energy stores and metabolic rate, is another important next step to better understand how organisms will be impacted by freezing, since energy limitation from repairing freeze-damage and/or up-regulating cryoprotective mechanisms combined with the decreased food intake that I measured may lead to higher rates of mortality due to freezing than we would otherwise expect.

Overall, my thesis provides many novel insights into the mechanisms and consequences of surviving freezing in intertidal invertebrates, which lay the groundwork for future research on this group of sorely understudied freeze tolerant organisms. My first chapter lends support to the hypothesis that osmolytes act as non-colligative (i.e., non-interchangeable) cryoprotectants in intertidal invertebrates and highlights TMAO as a potentially important low-molecular weight cryoprotectant that should be further investigated. My second chapter revealed that mussels are quite well-defended against mortality from short, repeated freezes, and although mussels are negatively impacted by sub-lethal freezing, these effects are fairly limited and the broader implications of these reductions in mussel performance to the rest of the intertidal ecosystem may not be very consequential.

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Appendices

Appendix A General Appendix

Figure A.1 Map of sites in the Burrard Inlet where intertidal organisms were collected for this study. *Mytilus trossulus* mussels were collected from Tower Beach and the dock pilings near the Royal Vancouver Yacht Club at Jericho Beach. *Evasterias troschelii* were collected at the Girl in a Wetsuit site which is in Stanley Park. Black stars highlight the nearby mouth of the Fraser River which inputs fresh water into the Burrard Inlet.

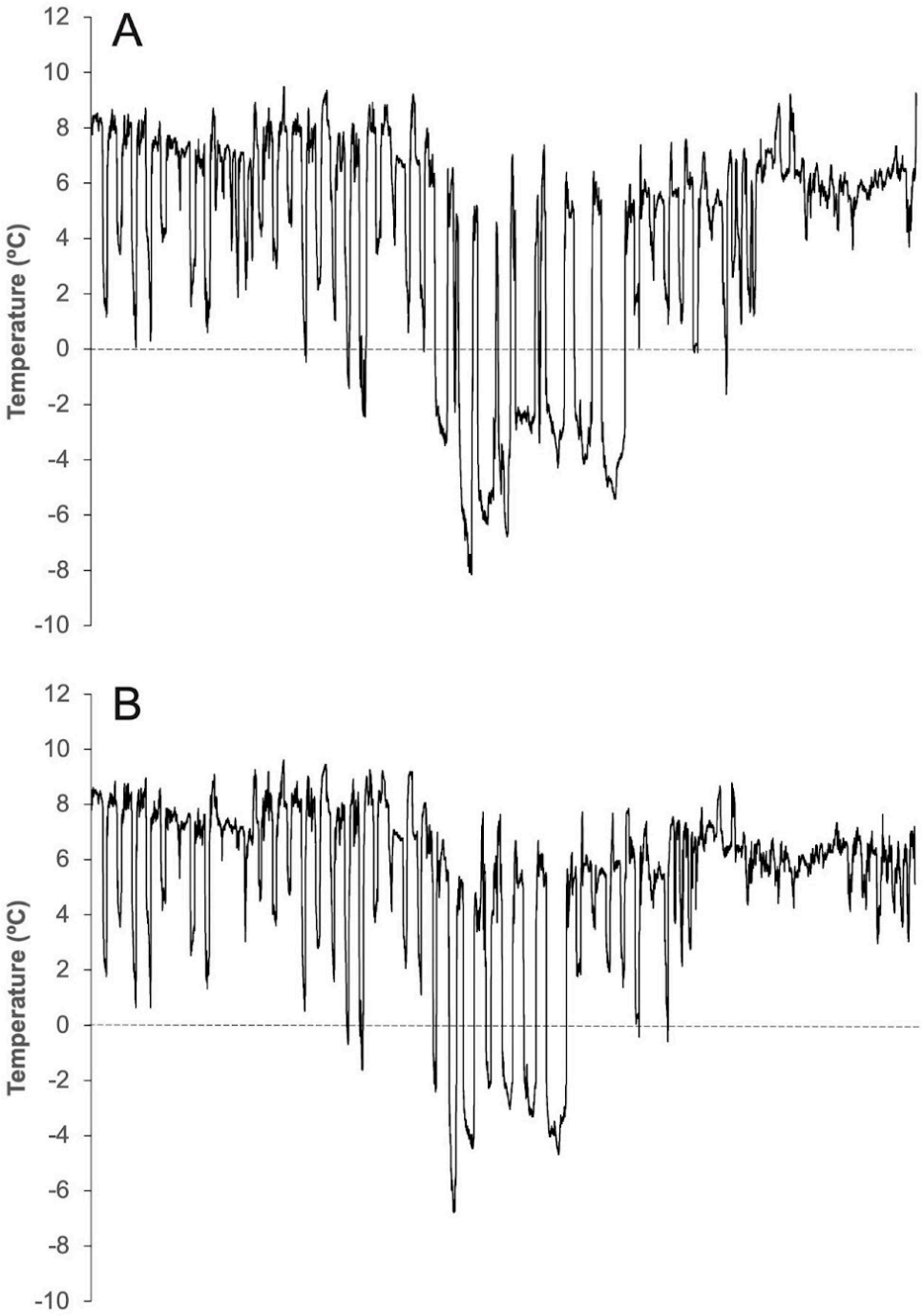

Figure A.2 Two representative temperature traces from the intertidal zone at Tower Beach, Vancouver BC, Canada. Panel A is data from a “high intertidal” temperature logger placed at approximately 3 m above chart datum, Panel B is from a logger placed in the “low intertidal" at approximately 1 m above chart datum. Data was collected every 45 seconds from December 1, 2021 to January 28, 2022.

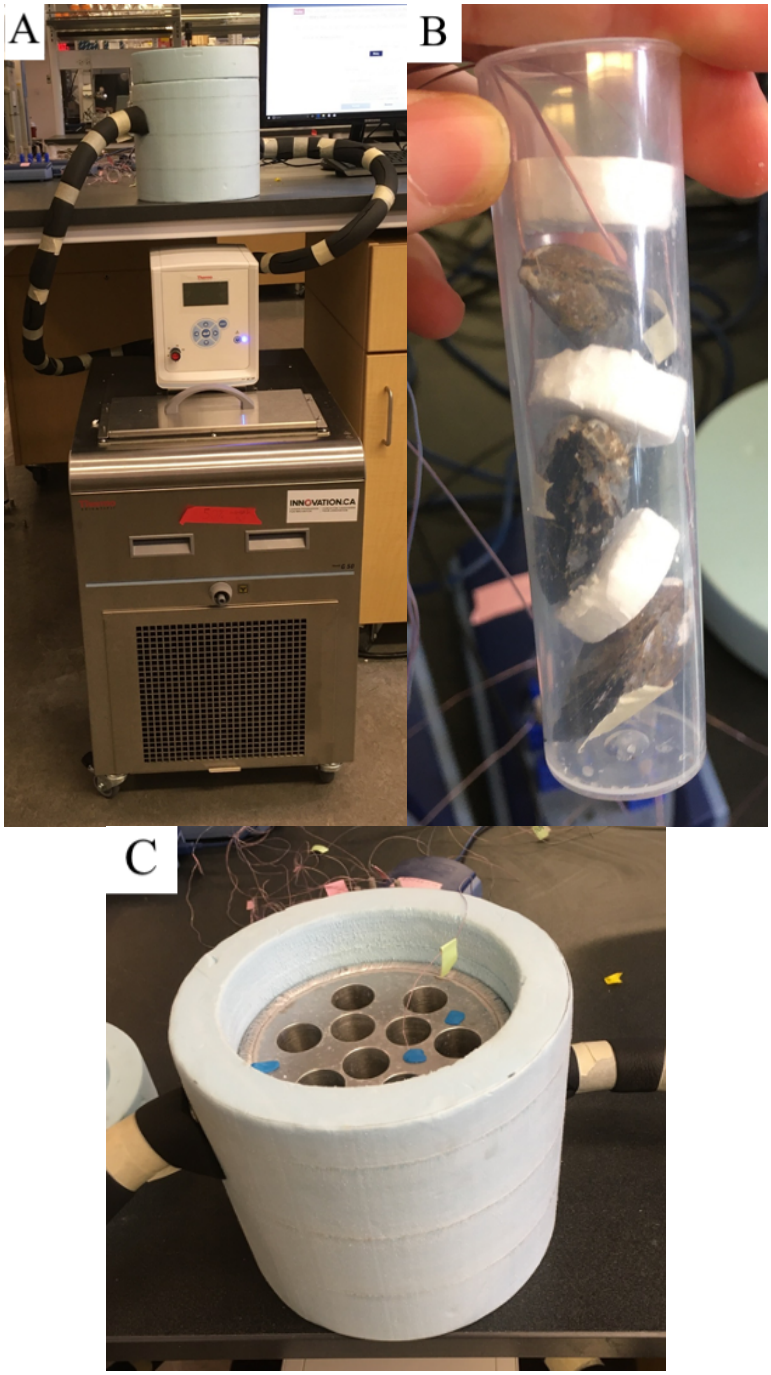

Figure A.3 Circulating bath freezing method. A) circulating bath which is filled with methanol and water (40:60). Coolant is pumped into the aluminum head that is surrounded by Styrofoam (C) through plastic piping that surrounded by piping insulation. B) *Drosophila* culture tube with 3 mussels inside, prior to being placed in the aluminum head. Each mussel is individually attached to a thermocouple, and all are separated by Styrofoam. C) *Drosophila* tubes containing mussels were placed into this aluminum head pictured here during freezing exposures.

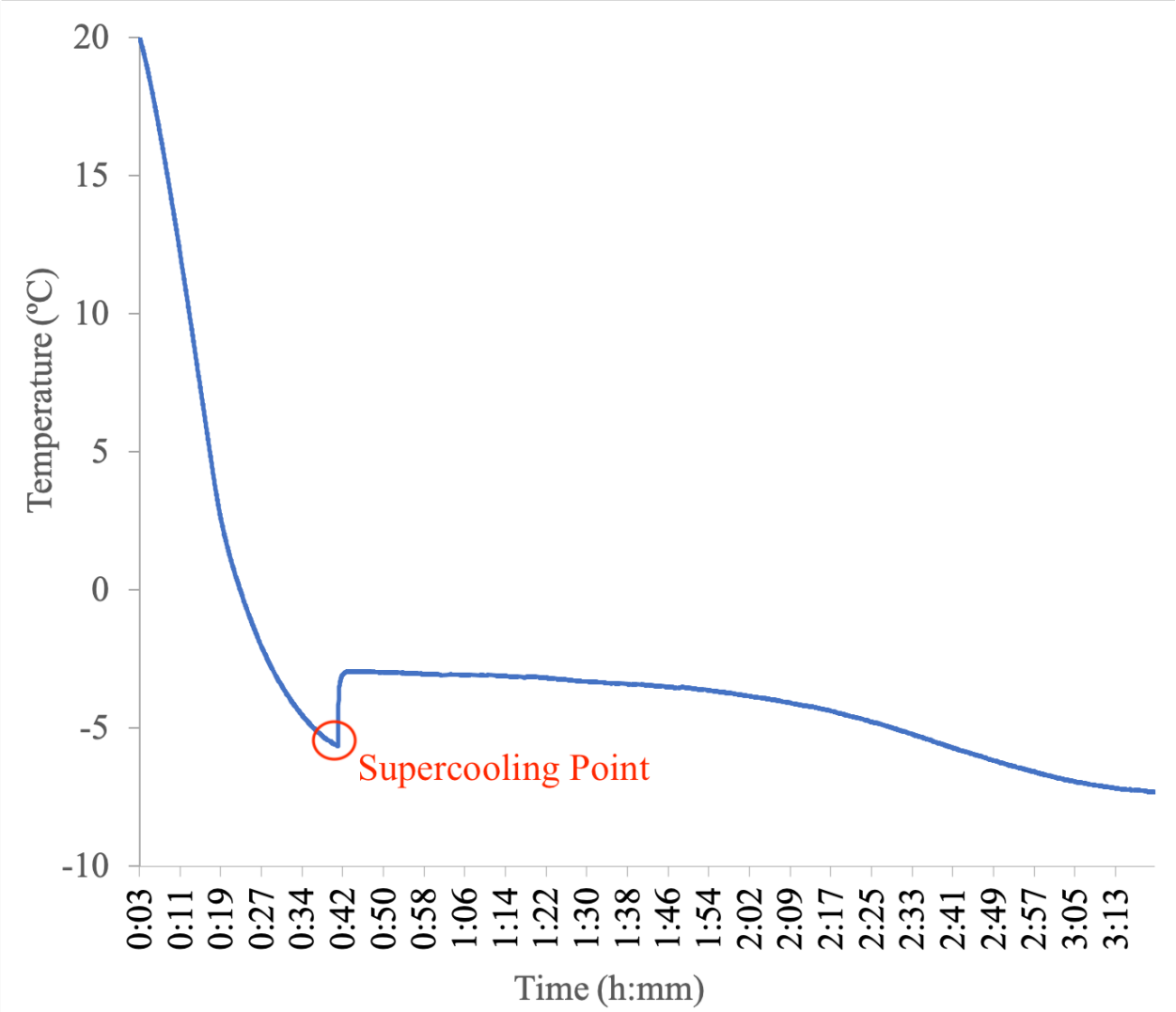

Figure A.4 A mussel's temperature trace during an experimental freezing exposure. Mussels were frozen using a refrigerated circulating bath. The sharp increase in temperature is the exothermic release of energy caused by ice formation. The x-axis represents the time after the freezing exposure began in hours:minutes.

Appendix B Chapter 2

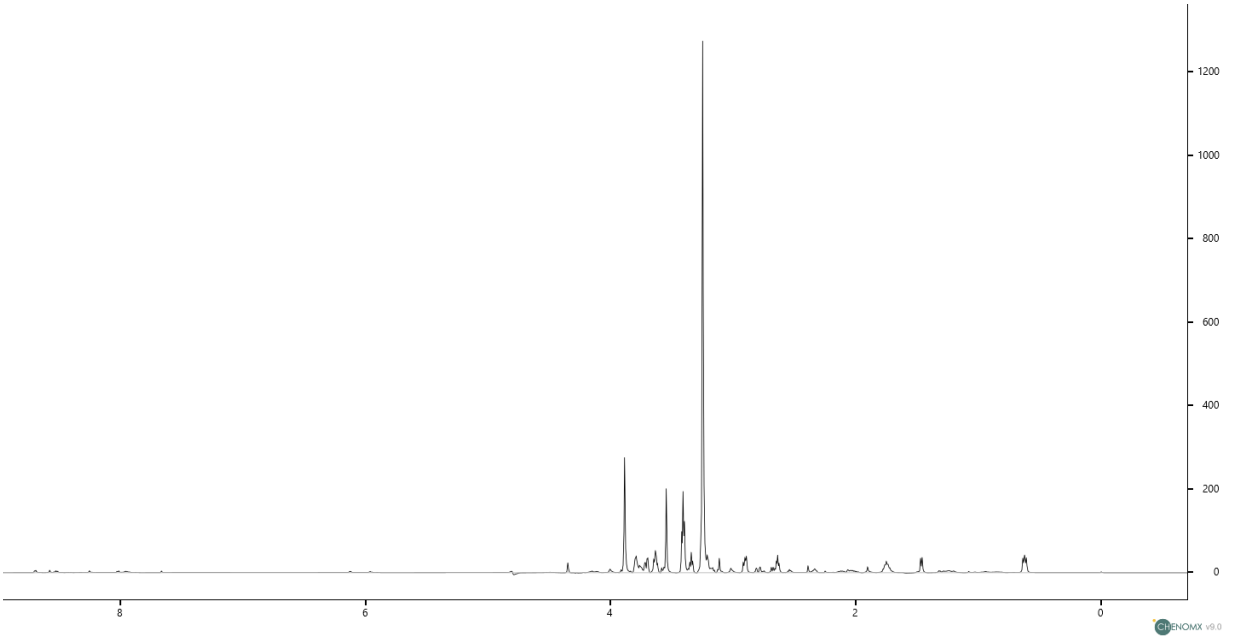

Figure B.1 Example ^{1}H NMR spectrum of *M. trossulus* gill tissue, visualized using the Chenomx Processor software.

Appendix C Chapter 3

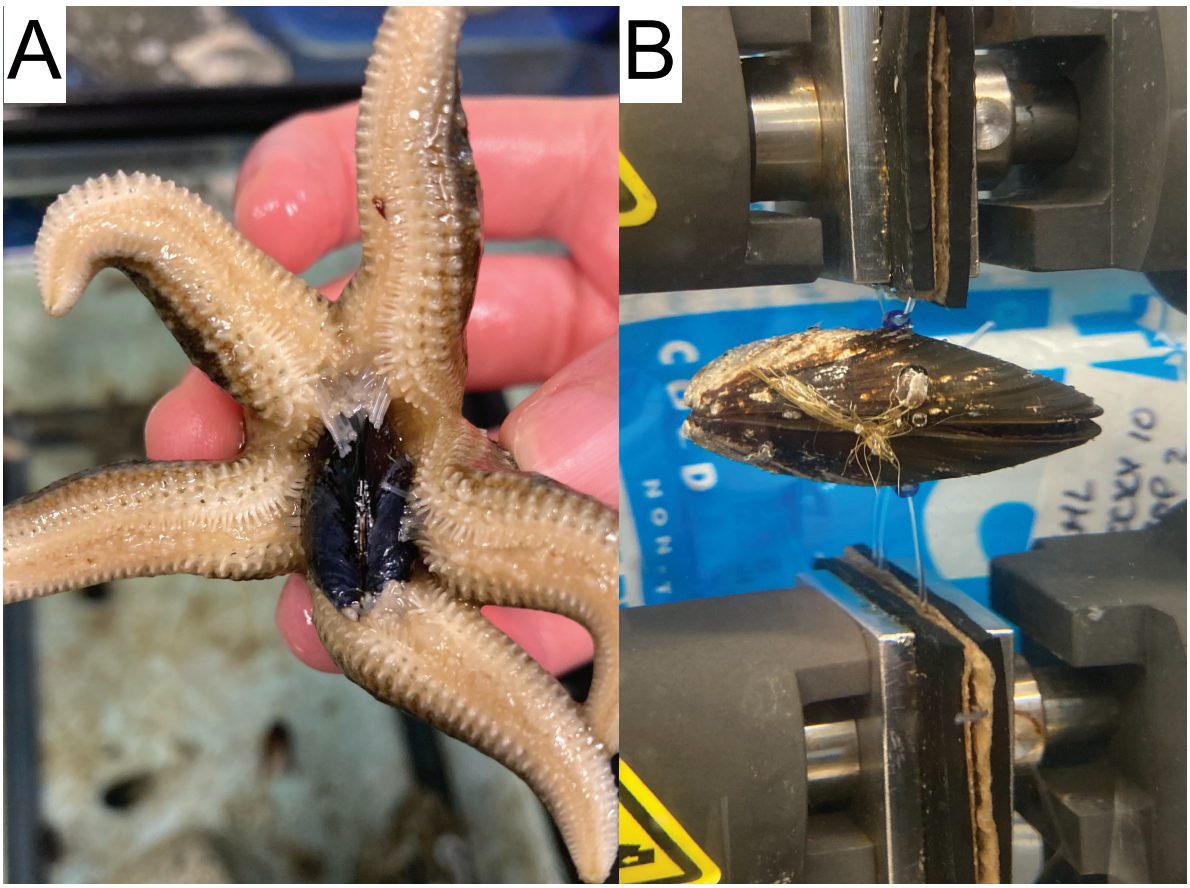

Figure C.1 A) Photograph of a sea star, *Evasterias troschelii*, in the process of consuming a mussel, exhibiting how the sea star uses its tube feet to suction on to the mussels shell's and pull them apart to access the mussel's flesh. B) A mussel being pulled open by the Instron apparatus used in this study.

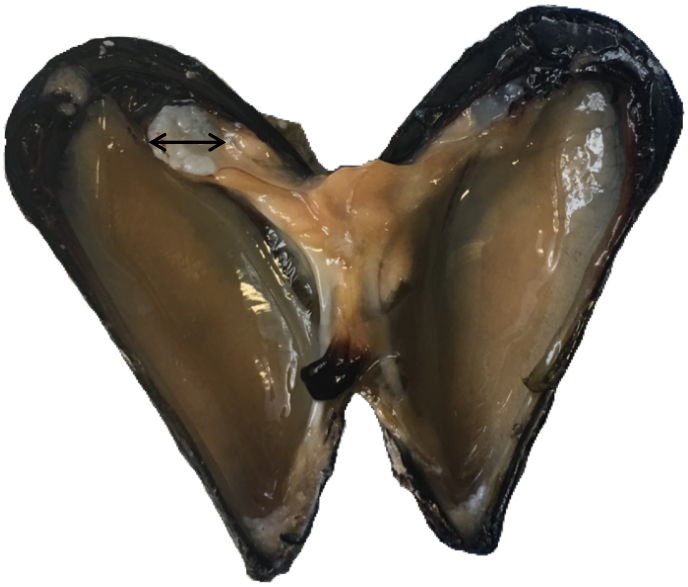

Figure C.2 A dissected mussel, *Mytilus trossulus*, with the black arrow indicating the posterior adductor muscle diameter.

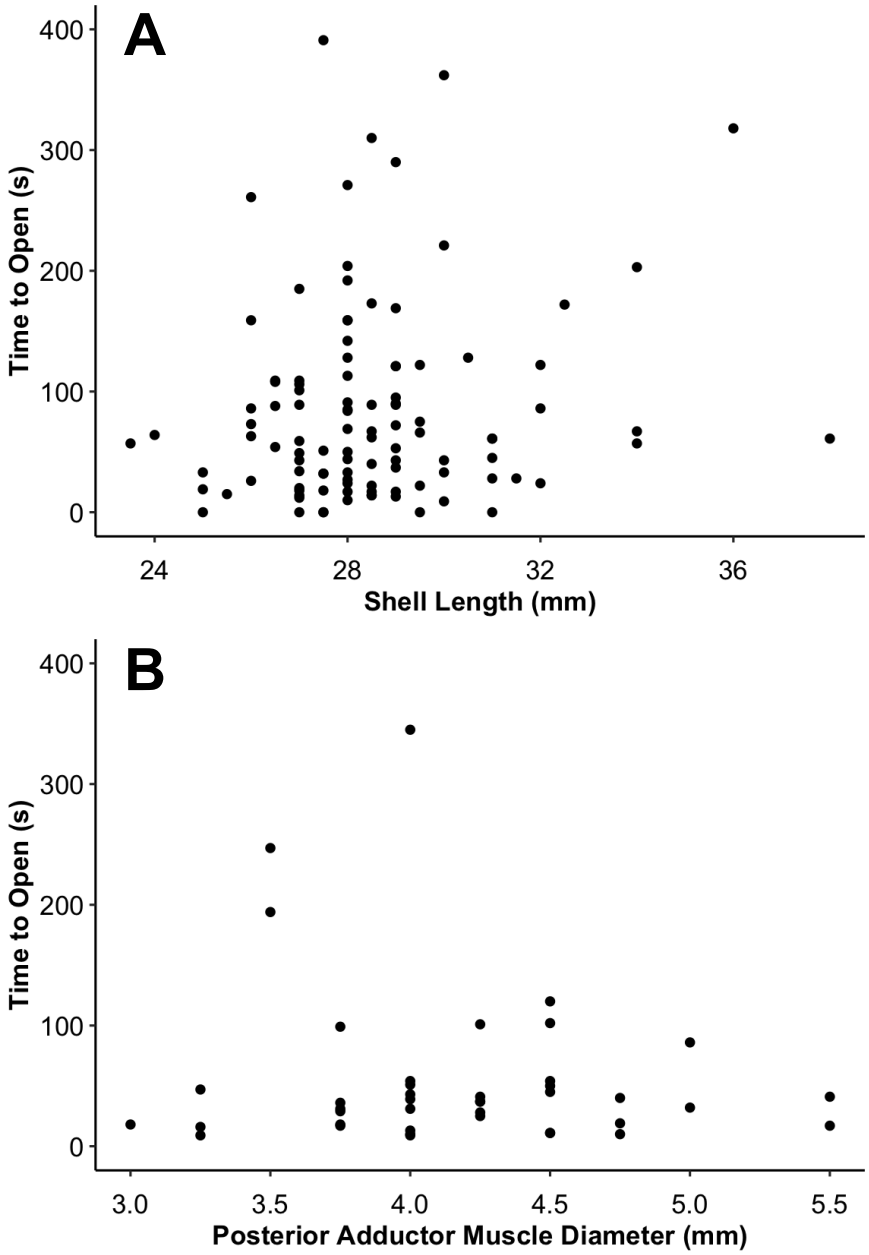

Figure C.3 A) Mussel shell length also does not have a significant impact on how long it takes mussels to open under a constant 4 N pulling force. This plot displays data from mussels collected from Tower Beach on December 12, 2020 and March 8, 2021. B) *Mytilus trossulus* posterior adductor muscle diameter does not have a significant effect on how long it takes mussels to open under a constant 4 N pulling force. Data in this plot is from mussels which were collected from Tower Beach on March 8, 2021.

C.1 Time Course of Ice Formation During Freezing

My qualitative assessment of the amount of body water frozen in mussels during a 2 hour exposure to -8 ºC revealed that almost all of the body water was frozen after 1 hour and 30 minutes of exposure to -8 ºC (Fig C.4). Mussels exposed to -8 ºC reach their supercooling point in, on average, 23.8 minutes (± 1.51 min, n=14) after the circulating bath reaches the final temperature of -8 ºC (example mussel body temperature trace highlighting the SCP can be found in Fig A.4).

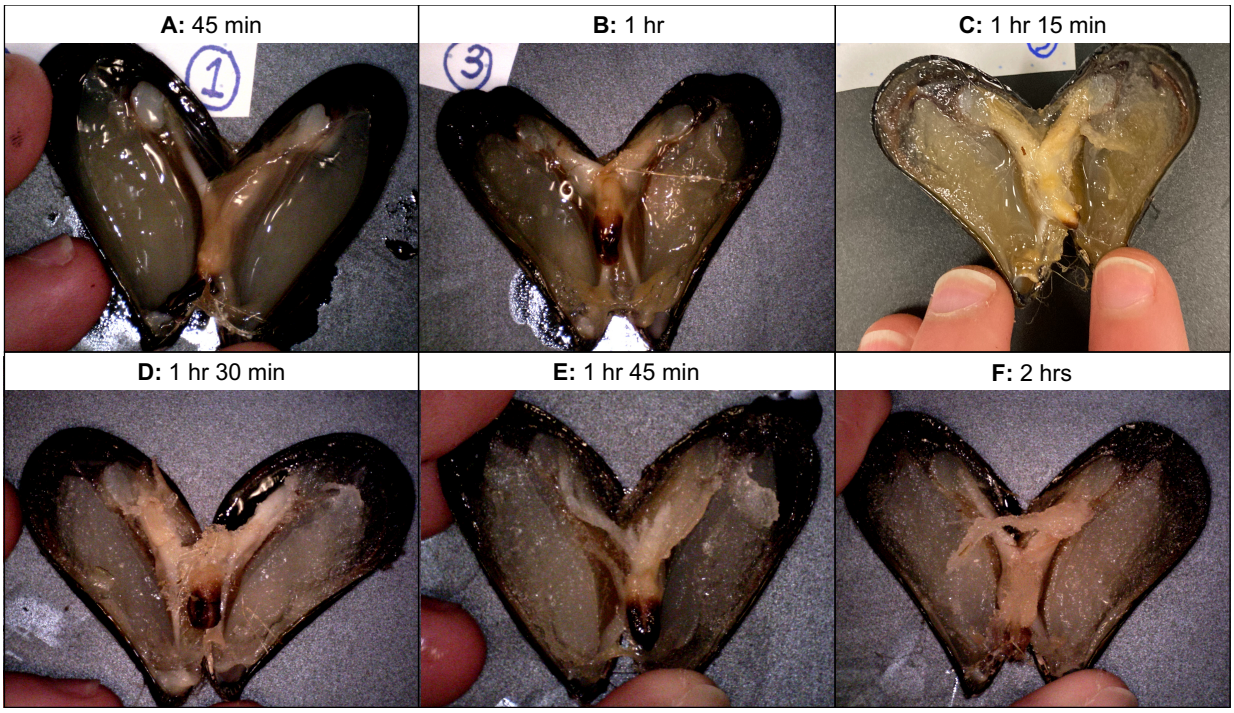

Figure C.4 Photographs of dissected mussels following exposure to -8 ºC for various lengths of time, as indicated in the top panel. Partial freezing can be observed in panels A-C, with ice formation beginning in the posterior region of the mantle cavity. Apparent complete freezing of the mussels' bodies can be seen in panels D-F.

C.2 Post-Freeze Recovery Time Course

The proportion of mussels which recover post-freezing (as assessed as whether they can hold their shells shut when removed from seawater) does not significantly change over time for the first day post-freeze ($F_{3,71}$=2.21, p=0.094). Although, a slight, albeit insignificant, increase in proportion of mussels recovered can be observed when comparing data from the first two hours post-freeze and 25 hours post-freeze (Figure C.5).

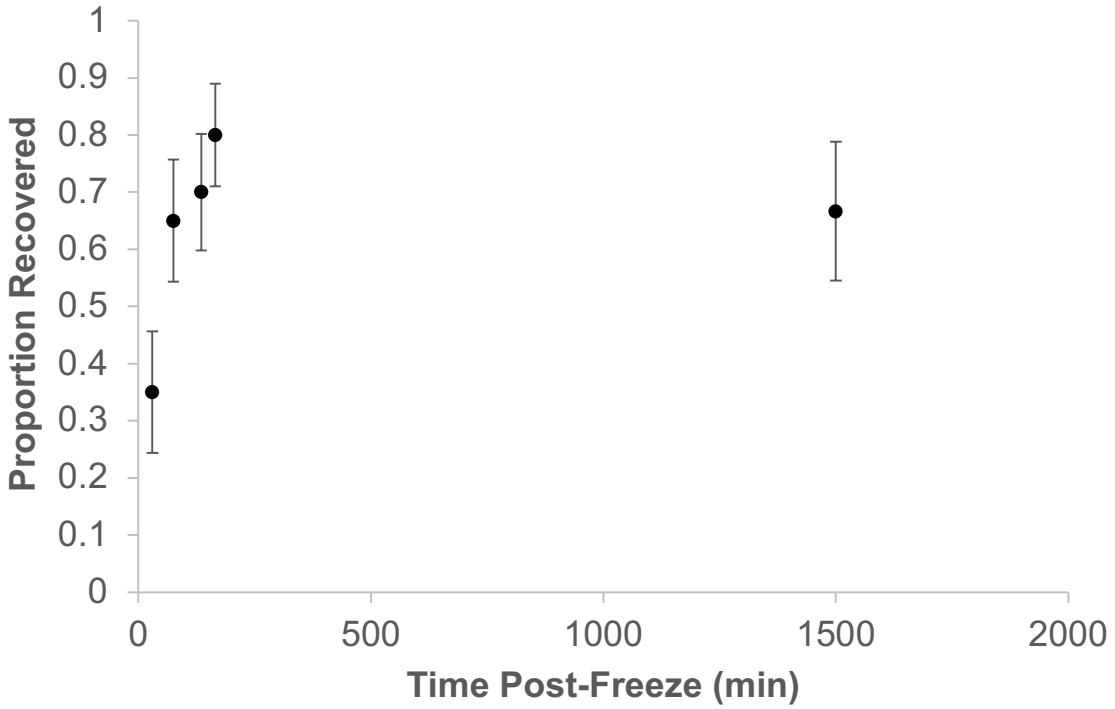

Figure C.5 After freezing at -12 ºC for 3 h, some mussels temporarily gape upon removal from seawater, particularly for the first few hours after freezing, after which most recover their ability to hold their shells shut in air. Error bars are standard error of the proportion.