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RESEARCH ARTICLE

Drivers of plasticity in freeze tolerance in the intertidal mussel *Mytilus trossulus*

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ABSTRACT

Freezing is an extreme stress to living cells, and so freeze-tolerant animals often accumulate protective molecules (termed cryoprotectants) to prevent the cellular damage caused by freezing. The bay mussel, *Mytilus trossulus*, is an ecologically important intertidal invertebrate that can survive freezing. Although much is known about the biochemical correlates of freeze tolerance in insects and vertebrates, the cryoprotectants that are used by intertidal invertebrates are not well characterized. Previous work has proposed two possible groups of low-molecular weight cryoprotectants in intertidal invertebrates: osmolytes and anaerobic byproducts. In our study, we examined which group of candidate cryoprotectants correlate with plasticity in freeze tolerance in mussels using ^{1}H NMR metabolomics. We found that the freeze tolerance of *M. trossulus* varies on a seasonal basis, along an intertidal shore-level gradient, and with changing salinity. Acclimation to increased salinity (30 ppt compared with 15 ppt) increased freeze tolerance, and mussels were significantly more freeze tolerant during the winter. Mussel freeze tolerance also increased with increasing shore level. There was limited evidence that anaerobic byproduct accumulation was associated with increased freeze tolerance. However, osmolyte accumulation was correlated with increased freeze tolerance after high salinity acclimation and in the winter. The concentration of most low molecular weight metabolites did not vary with shore level, indicating that another mechanism is likely responsible for this pattern of variation in freeze tolerance. By identifying osmolytes as a group of molecules that assist in freezing tolerance, we have expanded the known biochemical repertoire of the mechanisms of freeze tolerance.

KEY WORDS: Cryoinjury, Salinity, Cryoprotectant, Plasticity, Osmolyte, Anaerobic by-product

INTRODUCTION

Exposure to sub-zero temperatures is a common physiological stressor in many habitat types, both aquatic and terrestrial. Only a small subset of ectotherms are able to survive tissue ice formation (Sinclair et al., 2003). These organisms include overwintering insects (Lee, 2010), intertidal invertebrates (Aarset, 1982), as well as a few amphibians and lizards (Storey and Storey, 1988). This functionally critical tolerance is remarkable because freezing can inflict a wide variety of cellular damages, termed cryoinjuries. Potential cryoinjuries include damage from the osmotic stress associated with freezing, mechanical damage to cell membranes owing to extracellular ice crystal formation, protein denaturation, destabilization of cell membranes and oxidative damage (Storey and Storey, 2013).

Cryoprotectants are molecules utilized by freeze-tolerant species to cope with cryoinjuries (Storey and Storey, 2002). These can be separated into two broad categories by molecular weight: high molecular weight (on the kg mol^{-1} scale) cryoprotectants such as antifreeze proteins (Duman, 2001) and ice-nucleating agents (Zachariassen, 1992), and low molecular weight (on the g mol^{-1} scale) cryoprotectants. Low molecular weight cryoprotectants are metabolites that can function cytoprotectively in a variety of ways: by protecting against the osmotic stress associated with freezing, stabilizing macromolecules through interactions with their hydration shell, maintaining cell membrane permeability and/or functioning as antioxidants (Storey and Storey, 2002; Toxopeus and Sinclair, 2018). The low molecular weight cryoprotectants that have been identified in insects are primarily polyhydric alcohols (e.g. glycerol), sugars and amino acids (Lee, 2010). These low molecular weight cryoprotectants may be interchangeable and function on a colligative basis, or they may function non-colligatively wherein each cryoprotectant molecule has a unique cryoprotective function, e.g. through membrane and/or protein stabilization (Lee, 2010). Recent evidence supports the hypothesis that low molecular weight cryoprotectants have non-colligative cryoprotective properties (Toxopeus et al., 2019; Yancey, 2005).

When exposed to sufficiently cold air temperatures during low tides, it becomes very difficult for intertidal invertebrates to avoid freezing owing to the constant presence of water and algal particles in the intertidal zone, which can act as ice nucleators (Kanji et al., 2017). As a result, a wide variety of temperate and polar intertidal invertebrates are freeze tolerant, and among molluscs it is notable when an intertidal species is not (Aarset, 1982; Sinclair et al., 2004). However, the cryoprotective mechanisms that have been identified in insects and vertebrates are not used by intertidal invertebrates (Storey and Storey, 2002), and the biochemical adaptations that enable intertidal invertebrates to be freeze tolerant remain poorly understood.

Numerous freeze-tolerant intertidal invertebrates exhibit seasonal plasticity in their freeze tolerance, with maximum freeze tolerance in the winter (Bourget, 1982; Murphy, 1979; Murphy and Pierce, 1975; Stickle et al., 2010; Vallière et al., 1990). The mechanism responsible for this seasonal change in freeze tolerance is unknown. In addition, intertidal organisms' tolerance to temperature stress can vary significantly across the intertidal shore-level gradient (Davenport and Davenport, 2005; Somero, 2002). Intertidal organisms located higher on the shore are typically more tolerant to extreme temperatures because they are exposed to air for longer during low tides, meaning that they are more likely to experience more prolonged and extreme temperature stress on a daily basis (Stickle et al., 2017).

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Although little is known about the mechanisms of freeze tolerance in intertidal invertebrates, two previously proposed hypotheses for the identity of low molecular weight cryoprotectants are: (1) byproducts of anaerobic metabolism, which accumulate in intertidal bivalve tissues during low tides as they are unable to maintain aerobic respiration in air (Loomis et al., 1988), and (2) osmolytes, which are used to cope with osmotic stress (Yancey, 2005). Several laboratory studies have identified potential cryoprotective roles for anaerobic byproducts. For example, the intertidal mussel *Mytilus edulis* becomes more freeze tolerant after being exposed to air or to oxygen-deficient water (Theede, 1972). Anaerobic byproducts have membrane-stabilizing and enzyme-protecting effects during freeze–thaw cycles *in vitro* (Loomis et al., 1989). In addition, intertidal invertebrates survive both freezing stress and hypoxic stress using similar biochemical adaptations: through the use of antioxidants and chaperone proteins, and through metabolic rate depression (Storey et al., 2013). A broad array of anaerobic byproducts accumulate in intertidal invertebrates during anaerobic respiration: strombine, alanine and octopine accumulate preferentially in early phase anaerobiosis, whereas succinate, propionate and acetate accumulate after prolonged anaerobiosis (de Zwaan et al., 1982; Muller et al., 2012).

The second hypothesized set of low molecular weight cryoprotectants are osmolytes, which may be cryoprotective because they may aid in coping with the osmotic shock owing to freezing. Many intertidal invertebrate species are osmoconformers, matching the osmolarity of their cells to their environment by accumulating pools of intracellular osmolytes (Willmer, 1978). During freezing, extracellular water crystallizes into ice, which concentrates the extracellular fluid and creates an osmotic gradient (Storey and Storey, 2002). Thus, these intracellular osmolyte pools may play a role in cryoprotection by preventing cellular dehydration owing to extracellular ice formation. Several intertidal invertebrates increase their freeze tolerance after acclimation to high salinity (Murphy, 1979; Theede and Lassig, 1967; Williams, 1970). Because high salinity acclimation increases the intracellular osmolyte pool concentration, intracellular osmolyte pools may have a cryoprotective role, explaining why high salinity acclimation results in increased freeze tolerance in intertidal invertebrates. Important osmolytes in intertidal invertebrates include the amino acids taurine, betaine, glycine and alanine, as well as trimethylamine *N*-oxide (TMAO; Cappello et al., 2018).

To investigate the mechanisms underlying plasticity in freeze tolerance in intertidal invertebrates, we used the model organism *Mytilus trossulus*, the bay mussel. This mussel species is found in temperate and Arctic climates, and cannot behaviourally avoid freezing during aerial exposure coinciding with winter low tides because it is sessile. In its congener, *Mytilus edulis*, freezing tolerance is well documented (Bourget, 1982; Kanwisher, 1955; Vallière et al., 1990; Williams, 1970; reviewed in Aarset, 1982). Survivable equilibrium ice content in intertidal invertebrates varies from ∼57% to 67%, with *Mytilus* species averaging approximately 62–64% (Kanwisher, 1955; Williams, 1970).

The first main objective of our study was to investigate the drivers of freeze-tolerance plasticity in *M. trossulus*. We predicted that freeze tolerance would increase in winter, with high salinity acclimation, and at higher intertidal shore levels. The second main objective of our study was to investigate the potential mechanisms that underlie this plasticity. We did this by directly measuring a wide range of metabolites simultaneously using ^{1}H NMR metabolomics, and correlating osmolyte and anaerobic byproduct concentrations with freeze tolerance to evaluate the link between freeze tolerance and low molecular weight metabolite concentrations to test the hypotheses that osmolytes and anaerobic byproducts are important cryoprotectants in intertidal invertebrates.

MATERIALS AND METHODS

Mytilus trossulus collection

All *Mytilus trossulus* Gould 1850 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). Mussel collections took place during low tides, when tidal height was <1 m above the chart datum. Vancouver has relatively mild winters given its latitude; however, winter temperatures can reach lows of −5°C to −10°C (Fig. S1). 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 occurs around midnight, meaning that intertidal invertebrates are exposed to some of the coldest air temperatures of the year during winter low tides. Additionally, significant seasonal changes in salinity at Tower Beach occur owing to seasonal changes in freshwater outflow levels of the nearby Fraser River (Covernton and Harley, 2020; Fig. S1). When the Fraser River outflow is highest in the spring and summer, the salinity at Tower Beach is brackish, at roughly 10–15 ppt, with a yearly minimum of ∼5 ppt (Covernton and Harley, 2020). In the winter, when Fraser River outflow is low, the water at Tower Beach reaches salinities of approximately 20–25 ppt (Covernton and Harley, 2020; Fig. S1).

Mussels were collected at various dates throughout the period of July 2019–January 2020 (Table 1) in order to capture potential seasonal variation in mussel freeze tolerance. Mussels were not likely reproductive during any of the sampling dates since the peak spawning period for mytilid mussels from Puget Sound, Washington, USA, is in April and May (Kagley et al., 2003). 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 (diameter=2.2 cm) for experimental cold exposures. The size range of

Table 1. Summary of all low-temperature exposure experiments and environmental measurements taken throughout the study

	17 July 2019	2 August 2019	25 September 2019	29 November 2019	22 January 2020
Site salinity (ppt)	11.7	17	13.8	27.4	22.5
Site air temperature (°C)	21.3	22	14.2	1.5	7.2
Site water temperature (°C)	20.5	15.9	14.5	5	8.1
Low tide	0.7 m at 13:00 h	0.2 m at 13:30 h	1.0 m at 09:30 h	0.4 m at 01:00 h	0.6 m at 22:23 h
Laboratory salinity acclimation treatments (ppt)	15	0, 5, 10, 15, 20, 25, 30	15	15, 30	15, 30
Test temperatures (°C)	0, −2, −4, −6, −8, −10	−6, −8 (just 15 ppt)	−4, −6, −8	−6, −8, −10, −12	−6, −8, −10, −12, −14
Gill samples taken for metabolomics?		Yes		Yes	

The dates listed in the top row represent the date when those mussels were collected. Site conditions were measured at Tower Beach, Vancouver, BC, Canada. Mussels were acclimated to various salinities for 1 week and then exposed to one of the various test temperatures for 3 h. Both high and low intertidal mussels were tested in all treatments. *n*=12 per shore level per temperature per collection date (apart from January, where *n*=15 per group).

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the entire population of mussels at Tower Beach is slightly greater than the size range of those that were included in this study, with the mean shell length of *M. trossulus* at Tower Beach equal to 2.67± 0.687 cm (only considering mussels >1 cm in shell length) and a maximum shell length of approximately 4.5 cm.

Mussels were collected from the same outcropping of rocks on each 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 the lowermost edge of the mussel bed during low tide (approximately 1 m above chart datum). This was done in order to capture potential variation in freeze tolerance along an intertidal shore height gradient. Both collection areas are located directly in front of the abandoned searchlight tower at Tower Beach. Site salinity, water temperature and air temperature were measured with a YSI handheld salinity and temperature meter (Pro 30 series with a PRO 30 COND-T probe) at approximately 25–50 cm water depth on all collection days. All mussel collections were completed under a Scientific Licence, Management of Contaminated Fisheries Regulations from the Department of Fisheries and Oceans Canada (licence number: XMCFR 22 2019).

Laboratory acclimation and low temperature exposures

Mussels were acclimated to laboratory conditions for 1 week before any low temperature exposures were conducted. Mussels were kept in 20 litre aquaria, maintained at 15°C, and water was aerated using air stones. Tank water was changed every 48 h. Tank salinity and temperature were monitored every 48 h using a YSI handheld meter (Pro 30 series with a PRO 30 COND-T probe). Mussels were kept in a mixture of natural seawater (sourced from the Vancouver Aquarium) and dechlorinated fresh water in various ratios to achieve the desired salinity, which varied no more than ±1 ppt from the desired level. Mussels were not fed during acclimation. 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 their aquaria and their shells were dried with a paper towel. Mussel shells were then measured with calipers to the nearest 0.5 mm. Shell length, width and height were all measured from the longest, widest and highest part of the shell, and then mussels were labelled with an ID number using nail polish. Next, a 16 gauge Type T thermocouple was taped to each mussel's shell and connected to Picolog TC-08 thermocouple interfaces, allowing all mussel body temperatures to be recorded continuously during freeze exposures using PicoLog 6 beta software for Windows (Pico Technology, Cambridge, UK). Thermocouple sampling rate was once per second. Except July trials (when incubators were used to freeze mussels), all other mussels were exposed using refrigerated circulating baths, which cooled at a rate of −1.5°C min^{-1}. 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).

All mussels were emersed during our laboratory cold exposures to mimic a low tide, which is when freezing and/or cold stress would occur in nature. The duration of all experimental low temperature exposures was 3 h. 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. We chose to expose mussels for 3 h periods in an attempt to remain as ecologically relevant as possible. Because the upper limit of the mussel bed at Tower Beach is approximately 3.5 m above chart datum (C.D.G.H., unpublished data), the highest intertidal *M. trossulus* at Tower Beach would only rarely be exposed to air for more than ∼9 consecutive hours, and even then only if wave action was minimal. Because the time length of exposure to air varies with intertidal shore height and low intertidal mussels would more commonly be exposed to air for only a few hours, we chose 3 h as a representative exposure duration.

Our choice to use rapid cooling rates of −1.5°C min^{-1} in our low temperature exposure trials also reflects the conditions that *M. trossulus* would experience in the field. Intertidal mussels are suddenly exposed to sub-zero air temperatures when the tide goes out during the winter and would experience rapid cooling rates because the magnitude of difference between water and air temperatures can be quite large. For instance, during a low tide in January 2020, air temperature was −8°C and water temperature was 4.4°C at Tower Beach (J.R.K., unpublished data). In addition, over the winter of 2014–2015, iButton temperature loggers placed in the intertidal zone at nearby Stanley Park, Vancouver, showed maximum rock temperature cooling rates of approximately −0.1°C min^{-1} during low tides, and we would expect mussels to cool at comparatively much faster rates owing to their significantly smaller mass (C.D.G.H., unpublished data). In addition, our decision to rapidly thaw our tested mussels by placing them back in seawater is representative of what they would experience in the field as the tide returns.

Each mussel was only used once in cold exposure trials. Mussels were haphazardly assigned to be exposed to one of a variety of sub-zero temperatures (from −2°C to −14°C) in order to calculate a population-wide LLT_{50} (lower lethal temperature, the low temperature that causes 50% mortality in a population of organisms; Table 1). In order to obtain a robust estimate for LLT_{50}, sub-zero temperatures were chosen such that we observed a range between 0% and 100% survival (apart from November freezing trials, where logistical constraints prevented us from freezing mussels at temperatures lower than −12°C). Sample sizes were chosen based on previously conducted pilot studies that could reliably detect a minimum 1°C shift in LLT_{50}.

Continuously monitoring all mussel body temperatures throughout all cold exposures allowed us to 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 owing to ice formation, so the presence of a SCP proves that internal ice formation occurred (Lee, 2010). The value of the SCP was recorded for all mussels that had a SCP (see Fig. S2 for an example of a mussel temperature trace during a cold exposure, with the SCP highlighted).

Immediately after the low temperature exposure, mussels were placed in a 500 ml plastic container filled with seawater (matching the salinity that they had been acclimated to) at 15°C, returned to aerated aquaria within 10 min, and separated by treatment using 150 ml plastic containers with mesh windows to allow water flow. Survival was assessed daily after the cold exposure, and the proportion of mussels alive after one week was used to calculate LLT_{50}. Mussels were considered dead when they were unable to hold their shells shut after removing them from the seawater. Mussels were not used in low temperature exposure experiments if they had been kept in the laboratory for longer than 2.5 weeks.

Mussels collected in July were cooled in a Panasonic MIR-154 cooled incubator. The mussels were laid out flat on a tray placed on the incubator shelf so that they were not touching one another. The cooling rate in the incubator was approximately −1.5°C min^{-1}. Mussels from this collection date were held at a constant 15 ppt salinity level, as mussels from this collection date were a part of a

pilot study to solely explore the effects of temperature on low-temperature survival.

Mussels collected in August, November and January were acclimated to varying salinities to test for changes in freeze/low temperature tolerance owing to changes in salinity. Mussels collected in August were acclimated for 1 week to one of seven different salinities between 0 and 30 ppt (outlined in Table 1). For the August low-temperature exposures, which served as a pilot study to investigate the extent of salinity-induced changes in low-temperature survival, all mussels were exposed to −6°C for 3 h using circulating baths following salinity acclimation. Next, to test for salinity-induced changes in freeze tolerance in mussels collected in November and January, mussels were acclimated to either 'low salinity' at 15 ppt or 'high salinity' at 30 ppt for 1 week, and then exposed to various sub-zero temperatures to determine LLT_{50}. These two salinity levels were chosen as they are representative of conditions observed through time at Tower Beach (Fig. S1) and nearby shorelines occupied by *M. trossulus* (Covernton and Harley, 2020). One-week acclimation periods were used to allow enough time for mussel osmoregulation, as maximal/minimal osmolyte accumulation/dissipation occurred within 6 days in the mussel *Modiolus demissus* (Baginski and Pierce, 1977).

^{1}H NMR metabolomics

One-dimensional, 600 MHz proton nuclear magnetic resonance spectroscopy (^{1}H NMR) was used to measure the metabolite profiles of mussel gill tissue. This technique is ideal for low molecular weight polar metabolites such as the potential cryoprotectants of interest (osmolytes and anaerobic byproducts). We focused on gills because they play an important role in osmoregulation in mussels, and ice formation in *M. trossulus* occurs primarily in the mantle cavity (Fig. S2), meaning that gills are directly exposed to ice.

Mussels used in metabolomics measurements were collected on 29 November 2019 (Table 1). To determine how shore level, salinity and −6°C cold exposure impact mussel gill metabolite concentrations, both high intertidal and low intertidal mussels were acclimated for 1 week to 15 or 30 ppt salinity. Of these mussels, half were exposed to −6°C for 3 h using circulating baths, and then returned to their aquaria (at 15°C water temperature) for 24 h, after which their gills were excised and frozen at −80°C. The other half of the mussels used in metabolomics were not exposed to −6°C. The 24 h recovery period after the −6°C exposure was used to give mussels enough time to potentially accumulate cryoprotective molecules. Additionally, to make a seasonal comparison between summer and winter metabolite profiles, we measured metabolites in four mussels collected on 2 August 2019. These mussels were from the low intertidal zone and were acclimated to 30 ppt salinity for 2 weeks. Sample sizes for metabolomic analyses were five mussels per intertidal zone per salinity, apart from the following treatment groups that had a sample size of four mussels: 30 ppt acclimated low intertidal mussels exposed to −6°C, 15 ppt acclimated high intertidal mussels exposed to −6°C, and mussels collected in August 2019. Mussels used in metabolomics analyses were not a part of the LLT_{50} determination work (e.g. they had not been previously manipulated and/or tested in any way). Sample sizes were chosen based on previously conducted pilot studies that could reliably detect a minimum 10 mmol difference in metabolite concentration.

Sample preparation was based on Cappello et al. (2013). A sample 100 mg of mussel gill tissue was excised, dried with a Kimwipe to remove excess water, weighed and frozen at −80°C. Frozen tissue was homogenized in 400 µl cold methanol and 85 µl cold water 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 ***g*** at 4°C. 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. Alternatively, the methanol layer was dried by leaving it in the fume hood overnight, and then stored at −80°C. Immediately prior to NMR analysis, the dried polar extracts were resuspended in 500 µl of 0.1 mol l^{-1} sodium phosphate buffer (pH 7.0, 50% deuterium oxide, Sigma-Aldrich) containing 1 mmol l^{-1} 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; Sigma-Aldrich) as internal reference. The mixture was then vortexed and transferred to a 5 mm NMR tube for ^{1}H NMR.

A Bruker Advance 600 (with Cryoprobe) spectrometer was used to perform ^{1}H NMR data acquisition. One-dimensional ^{1}H NMR spectra were acquired at a frequency of 600.15 MHz at an 8.4 kHz spectral width, with 64 scans at 300 K, using TopSpin software (v 2.1, Bruker), requiring 10 min of acquisition time. Final peak identification of the NMR spectra was performed with Chenomx NMR Suite 8.5 (Chenomx, Edmonton, Alberta, Canada) that uses the Human Metabolome Database compound spectral reference library (Wishart et al., 2018). Determination of metabolite concentrations was performed using Chenomx Profiler (within the Chenomx NMR Suite 8.5 software), which determines the concentrations of individual metabolites using the concentration of a known DSS signal (see Fig. S3 for a representative ^{1}H NMR spectrum). The online NMR spectra predictor from nmrdb.org (Banfi and Patiny, 2008) was used to qualitatively compare the predicted spectra of anaerobic byproducts specific to bivalves that were not included in the HMDB library (such as strombine and octopamine). Metabolite concentrations are reported as mmol 100 mg^{-1} gill wet mass.

Statistical methods

Statistical analyses were performed using R (v. 3.5.1; https://www.r-project.org/). The dose.p command from the 'MASS' R package was used to interpolate the temperature at which 50% of individuals died (LLT_{50}) and the associated standard errors of this estimate from binomial generalized linear models (Venables and Ripley, 2002). Generalized linear models were used to determine which factors significantly predicted mussel survival after exposure to sub-zero temperatures.

Two one-way ANOVAs were used to determine first how season and then how salinity impacted mussel SCP. This analysis was performed only considering mussels frozen at −8°C and collected in September, November and January. The SCPs were normally distributed and homoscedastic.

To test the interactive effects of time of year and shore level on freeze tolerance, we first examined only mussels that were acclimated to 15 ppt salinity (collected in July, September, November and January) using a generalized linear model. We used test temperature and shell length as covariates. Next, to test for the effects of shore level and salinity on freeze tolerance, separate generalized linear models considering mussels from each individual collection date were used. We also used test temperature and shell length as covariates here. To test for changes in survival owing to altered salinity and shore level after low temperature exposures in August, we used a generalized linear model. Then, to examine how salinity acclimation interacted with shore level and time of year, we used generalized linear models to test for differences

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in freeze tolerance in mussels acclimated to 15 or 30 ppt from both high and low shore positions, collected in November and January. Here, we also used test temperature and shell length as covariates.

Lastly, generalized linear models were used to determine which metabolites differed significantly in response to shore level, −6°C cold exposure and salinity acclimation, using a separate model for each metabolite.

RESULTS

Supercooling points

All mussels exposed to −8°C and below froze, as indicated by the presence of the characteristic thermal inflection point representing energy release during ice formation, visualized by the temperature trace graph of each individual mussel during all freezing trials (Fig. S2). A total of 78.2% of all mussels exposed to −6°C throughout this study froze. The mean SCP across mussels acclimated to 15 ppt salinity and exposed to −8°C in September, November and January was −5.50±0.06°C.

We determined whether season and/or salinity acclimation had an effect on mussel SCPs from mussels collected in September, November and January. An ANOVA indicated that mussel collection date significantly affected mussel SCP ($F_{2,93}$=15.69, P<0.001), and a Tukey *post hoc* test showed that mussels collected in September had a significantly lower SCP (by approximately 1°C) than mussels collected in January (P=0.0018) and November (P<0.001). In addition, a separate ANOVA showed that high salinity acclimation depressed mussel SCPs significantly by approximately 1°C, compared with mussels acclimated to 15 ppt salinity ($F_{1,112}$=27.75, P<0.001).

Interactive effects of season and shore level on lower lethal temperature

To examine the effect of time of year and shore level on mussel survival, the survival of mussels acclimated to 15 ppt salinity was compared using a generalized linear model incorporating survival data from mussels collected in July, September, November and January (Fig. 1). Mussel survival after freezing decreased at lower test temperatures (d.f.=371, deviance=202.7, P<0.001), varied significantly through time, with the highest survival in winter-collected mussels (deviance=11.47, P<0.001), and increased if mussels were collected from the high shore position (deviance=13.46, P<0.001). There was no significant interaction between the effects of shore level and collection month (deviance=2.70, P=0.10), and the effect of shell length was insignificant (deviance=0.324, P=0.57).

Seasonal changes in lower lethal temperature

Using separate logistic regression models for each individual collection time point, we examined the effect of shore level, salinity acclimation, test temperature and body size on survival of mussels (see Table 1 for a description of all test temperatures and laboratory salinity acclimation treatments used across the study period).

For mussels collected in July, lower test temperatures significantly decreased survival (d.f.=136, z=5.76, P<0.001), but there was no effect of shore level (z=1.01, P=0.310). Mussel shell length was not measured in these freezing experiments.

For mussels collected in September, lower test temperatures significantly decreased survival (d.f.=119, z=5.95, P<0.001), but shell length (z=1.57, P=0.118) and shore level (z=−1.39, P=0.163) did not significantly affect mussel survival.

Mussels collected in November 2019 and January 2020 were exposed to factorial combinations of low temperatures and altered salinity (see Results, Salinity effects on freeze tolerance, for a full analysis of salinity×shore level×date). When analyzed separately, November-collected mussels exhibited significantly lower survival following exposure to lower temperatures (d.f.=137, z=5.86, P<0.001). High salinity acclimation (30 ppt) significantly increased survival, relative to 15 ppt salinity acclimation (z=5.97, P<0.001). Mussels from the high intertidal survived significantly better than mussels from the low intertidal (z=−2.74, P=0.0061). There was a trend towards larger mussels surviving better (z=0.093, P=0.093). The interaction between shore level and salinity was not significant (z=0.22, P=0.8299).

Mussels collected in January exhibited similar patterns to November-collected mussels when analyzed alone. Lower test temperatures significantly decreased survival (d.f.=196, z=7.13, P<0.001). High salinity acclimation (30 ppt) significantly increased

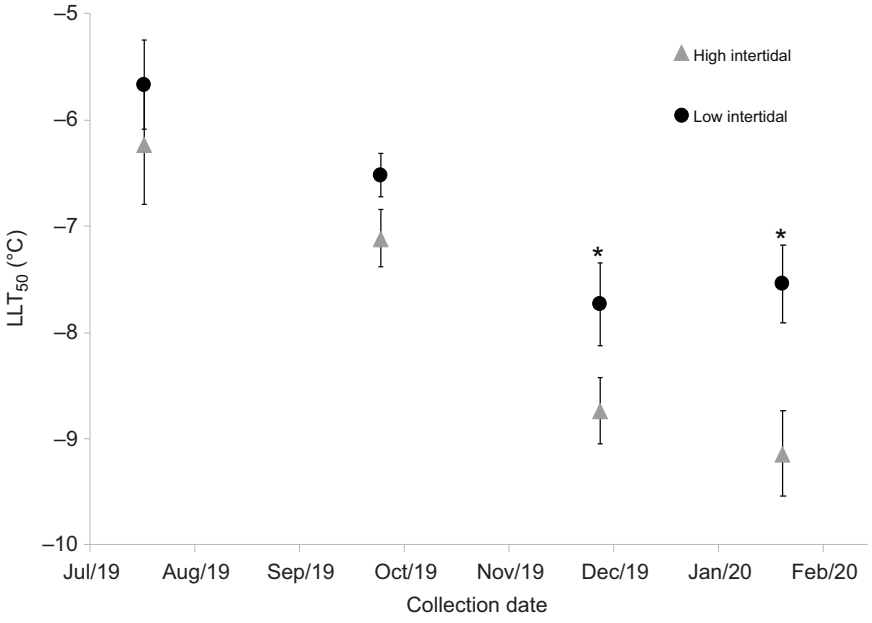

Fig. 1. *Mytilus trossulus* lower lethal temperature (LLT_{50}) changes seasonally and with shore level. Mussels became significantly more freeze tolerant in winter, and high intertidal mussels were more freeze tolerant than low intertidal mussels (asterisks denote collection dates where intertidal zone was a significant predictor of survival after freezing in univariate tests). Triangles indicate mussels collected from the high intertidal; circles indicate mussels collected from the low intertidal. Error bars are standard error of the LLT_{50} estimate. Mussel collection dates are plotted on the *x*-axis (month/year). All mussels were kept for a 1-week acclimation period in aerated aquaria with natural seawater at 15 ppt before being exposed to experimental freezing at temperatures ranging from −2 to −14°C. n=12–15 mussels per test temperature (3–5 test temperatures per timepoint per intertidal zone). LLT_{50} is defined as the temperature that causes 50% mortality and is derived from generalized linear models.

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survival, relative to 15 ppt salinity acclimation (z=6.37, P<0.001). High intertidal mussels survived significantly better than low intertidal mussels (z=–3.53, P<0.001). Shell length was not a significant predictor of survival (z=0.98, P=0.33), nor was the interaction between shore level and salinity (z=0.052, P=0.96).

Salinity effects on freeze tolerance

We conducted salinity acclimation experiments in August 2019 where we acclimated mussels to seven different salinity levels (0, 5, 10, 15, 20, 25 or 30 ppt) and exposed them all to −6°C. A generalized linear model demonstrated that acclimation to higher salinity significantly increased survival after exposure to −6°C (d.f.=158, z=4.05, P<0.001). Shore level was not a significant predictor of survival (z=0.492, P=0.623), nor was shell length (z=–0.448, P=0.655). The interaction between salinity and intertidal zone was insignificant (z=−1.504, P=0.132). Mussels were able to survive in salinities from 30 ppt to as low as 5 ppt; however, mussels were only able to survive for 1 week in fresh water (0 ppt).

Using a generalized linear model, we examined the effect of salinity acclimation (either 30 or 15 ppt), shore level and time of year on survival using mussels collected in November and January (Fig. 2). Shell length and test temperature were significant covariates; mussels were less likely to survive exposures to lower temperatures (P<0.001) and smaller mussels were less likely to survive than larger mussels (P<0.001; Table S1). However, the effect size of shell size on survival was relatively small, with only a 0.6% increase in survival probability with an increase in mussel shell length of 1 cm (the approximate range of values in our dataset). Mussels acclimated to higher salinity (30 ppt) survived better than those acclimated to lower salinity (15 ppt, P<0.001), and mussels from the high intertidal zone survived better than those from the low intertidal zone (P<0.001). None of the interaction terms were significant (P>0.05 in all cases), and there was no difference in survival between the two collection dates (P=0.81).

Metabolomics

The most predominant metabolites found in *M. trossulus* gill tissues were taurine, glycine, TMAO and betaine (see Table 2 for a list of all detected metabolites; metabolites were only compared among treatments if they were found in >20% of measured samples).

High salinity acclimation, season, exposure to −6°C for 3 h, and increasing shore level caused several metabolites to significantly change in concentration (Fig. 3). The three-way interaction between salinity, −6°C cold exposure and shore position was tested, but was not significant for any metabolite. Thus, in the results included in Table S1, shore level, salinity and −6°C cold exposure were incorporated into the generalized linear models without interactions. The only metabolites with significant interaction terms among −6°C cold exposure, salinity acclimation and shore level were alanine (salinity×cold exposure), glycine (salinity×shore level and cold exposure×shore level), and acetate (salinity×cold exposure; Table S3).

Salinity accounted for the largest relative change in metabolite concentrations, characterized by an accumulation of osmolytes (Table S2). Mussels acclimated to 30 ppt salinity had a mean gill organic metabolite pool concentration (calculated by summing all of the concentrations of the metabolites that were detected in NMR analysis) of 84.86±6.05 mmol l^{-1}, which is approximately double that of 15 ppt acclimated mussels, which had a mean gill organic metabolite pool concentration of 41.79±2.63 mmol l^{-1}. The following metabolites increased in concentration in mussel gills after acclimation to 30 ppt salinity: glycine, β-alanine, TMAO, alanine, malonate, betaine, taurine and glutamate. Conversely, the concentration of AMP, acetoacetate and acetate lowered in 30 ppt salinity acclimated mussels, as compared with 15 ppt salinity acclimated mussels.

Total organic metabolite pool concentration increased approximately threefold in November (mean=83.64±6.98 mmol l^{-1}) as compared with August (mean=28.39±5.46 mmol l^{-1}). This increase was primarily driven by accumulations of the most prevalent osmolytes: betaine, TMAO, alanine, taurine and glycine, as well as malonate and β-alanine (Table S2). However, a few metabolites did decrease in concentration in November (trimethylamine, aspartate and acetate).

Shore level accounted for the smallest change in metabolite concentrations relative to the other factors (Table S2). Only β-alanine increased in concentration with increasing shore level, whereas

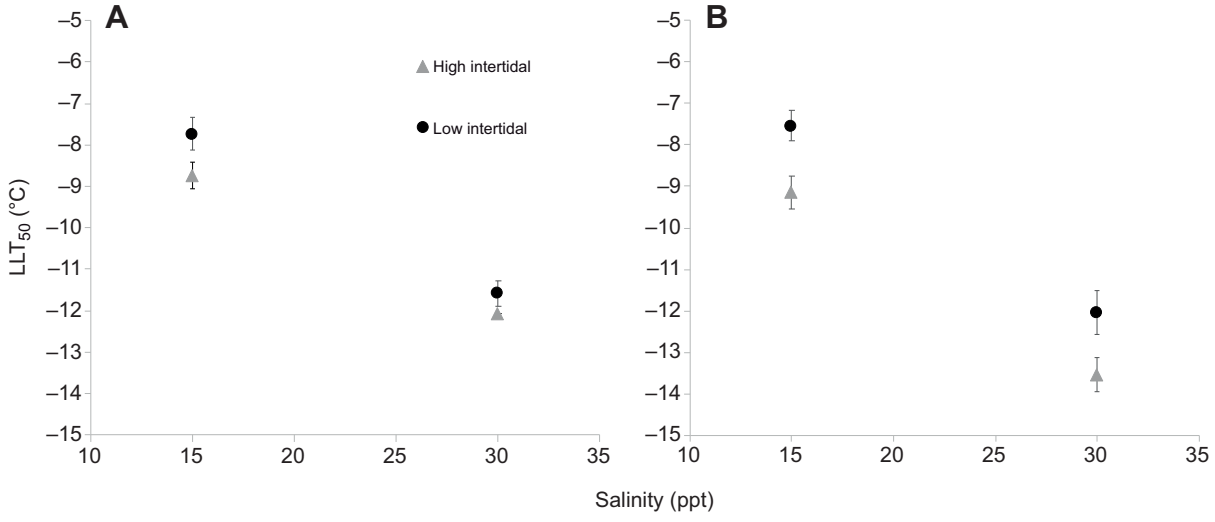

Fig. 2. High salinity acclimation decreases lower lethal temperature (LLT_{50}) in *M. trossulus*. (A) Mussels collected on 29 November 2019. (B) Mussels collected on 22 January 2020. Triangles indicate mussels collected from the high intertidal; circles indicate mussels collected from the low intertidal. Error bars are standard error of the LLT_{50} estimate. Prior to freezing, mussels were acclimated for 1 week at 15 or 30 ppt salinity. Mussels were frozen for 3 h at the following temperatures: –6, –8, –10, –12 and –14°C. n=12 mussels per test temperature/time point/salinity in November; n=15 per group in January. LLT_{50} is defined as the temperature that causes 50% mortality, as estimated from a logistic regression.

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Table 2. List of metabolites detected in >20% of gill tissue samples (*n*=42)

Osmolytes	Energy metabolites	Anaerobic byproducts
Taurine	Adenosine monophosphate (AMP)	Succinate
Trimethylamine *N*-oxide (TMAO)	Acetoacetate	Acetate
Betaine	Guanidinoacetate	Alanine
β-Alanine		Lactate
Trimethylamine (TMA)		Malate
Glycine		Malonate
Aspartate		
Glutamate		

Mussels were collected from Tower Beach, Vancouver, BC, Canada, and measured using ^{1}H NMR spectroscopy.

acetoacetate, malonate and betaine decreased in concentration with increasing shore level.

After mussels were exposed to −6°C for 3 h and given a 24 h recovery period, the anaerobic byproduct succinate, and the amino acids glutamate, β-alanine and alanine all decreased in concentration (Table S2). Aspartate was the only metabolite to increase in concentration after exposure to −6°C.

There was a clear correlation between osmolyte accumulation and increased freeze tolerance (i.e. LLT_{50} depression) in *M. trossulus* (Fig. 4). The five most prominent gill osmolytes all increased in concentration with increased freeze tolerance (i.e. LLT_{50} depression) to a similar degree, apart from glycine, which increased in concentration most drastically with LLT_{50} depression. High shore position reduced LLT_{50} independently of metabolite concentration.

DISCUSSION

We found that there is significant plasticity in the freeze tolerance of *M. trossulus*, and it is driven by three factors: salinity, seasonality and shore level, listed from largest to smallest relative effect size. Freeze tolerance was higher in mussels acclimated to high salinity, in mussels collected in the winter months and in mussels from higher shore positions. Osmolytes accumulate in correlation with increased freeze tolerance both seasonally and in response to high salinity, providing support for a potential cryoprotective role, while anaerobic byproducts were not correlated with increased freeze tolerance in *M. trossulus.* Taken together, these results suggest that osmolytes are important for modulating plasticity in mussel freeze tolerance; however, osmolytes are not sufficient to completely explain the presence of freeze tolerance nor all aspects of plasticity in freeze tolerance, because shore level effects remain unexplained by our data.

High salinity acclimation resulted in increased freeze tolerance by the most significant degree. Mussels acclimated to 30 ppt seawater depressed their LLT_{50} by 4–5°C, as compared with mussels acclimated to 15 ppt (Fig. 2). This finding is congruent with previous studies that found that intertidal invertebrate freeze tolerance increases markedly after high salinity acclimation (Murphy, 1979; Theede and Lassig, 1967; Williams, 1970). We found that high salinity acclimation is correlated with an accumulation of the osmolytes taurine, glycine, TMAO, betaine and alanine, which causes a significant increase in total osmolyte pool concentration in high salinity acclimated mussels. Because high salinity acclimation depressed mussel LLT_{50} by a relatively large degree, this supports the hypothesis that osmolytes are important low molecular weight cryoprotectants in *M. trossulus*.

Mussels also become significantly more freeze tolerant in the winter. We found the most significant increase in mussel freeze tolerance between the months of September and November, during which a 2°C decrease in LLT_{50} occurs (Fig. 1), corresponding to the time period with the greatest change in local air temperature (Fig. S1). This finding is congruent with previous studies that have found that intertidal invertebrate freeze tolerance increases in the winter months (Bourget, 1982; Murphy, 1979; Murphy and Pierce, 1975; Stickle et al., 2010; Vallière et al., 1990). The increased freeze tolerance that we observed is ecologically relevant, considering the natural temperature regime of our study area. High intertidal mussels acclimated to 30 ppt salinity had an LLT_{50} of −13.5±0.4°C in January, and the minimum air temperature that mussels would experience in Vancouver is approximately −10°C (Fig. S1). One caveat to our LLT_{50} estimates is that we acclimated all mussels to a uniform 15°C water temperature for 1 week in the laboratory before we measured mussel low temperature tolerance, and while 15°C is representative of water temperatures at Tower Beach for the majority of the year (Table 1), water temperatures drop below 15°C during the winter months at Tower Beach. Thus, our LLT_{50} estimates from laboratory-acclimated mussels may not be exactly equal to the 'true' LLT_{50} of mussels in the field.

We found a stark increase in total organic metabolite pool between August and November, which is also primarily driven by the same five prominent osmolytes that increased in concentration in high salinity acclimation (taurine, glycine, TMAO, betaine and alanine). A similar effect was found in European *Mytilus* spp., where the whole-body amino acid pool, particularly driven by taurine, significantly increases in winter (Kube et al., 2007). Taken together, this indicates that osmolyte concentrations are strongly correlated with seasonal and salinity plasticity of freeze tolerance in *M. trossulus*. As all mussels tested were acclimated to a common salinity (15 ppt) for 1 week prior to freezing, seasonal changes in environmental salinity are not driving our results. It is unclear whether salinity and seasonality effects are synergistic (where the combined effects of seasonality and salinity increase freeze tolerance by a larger magnitude than the sum of each individual factor's effect on freeze tolerance), additive or antagonistic (where the combined effects are less than the sum of the individual effects). We were unable to make seasonal comparisons about the effect of salinity on freeze tolerance because mussels collected in July and September were only acclimated to 15 ppt salinity.

In Vancouver, the natural increase in winter salinity works in the favour of freeze-tolerant intertidal invertebrates such as *M. trossulus*; however, this may not be the case in all areas. For instance, in the northern Gulf of Alaska, coastal sea surface salinity is lowest in the autumn, caused by increases in freshwater discharge and high precipitation levels owing to autumn storms (Royer, 2005). This could potentially pose challenges for the freezing survival of intertidal invertebrates if they are subjected to freezing conditions in the autumn owing to occasional katabatic winds.

Mussels from the high intertidal zone are generally more freeze tolerant than low intertidal mussels, but this pattern was more pronounced in the winter, and univariate tests were only significant for winter dates. The size of the effect of intertidal zone on mussel freeze tolerance is relatively small, with only a 1–1.5°C LLT_{50} depression in high intertidal mussels in November and January. This effect may be due to phenotypic plasticity, induced by the increased cold exposure that high intertidal mussels experience in the winter months. Or, this effect may be due to post-settlement selection, where mussels that are not adequately freeze tolerant are unable to survive overwintering in the relatively harsher high intertidal.

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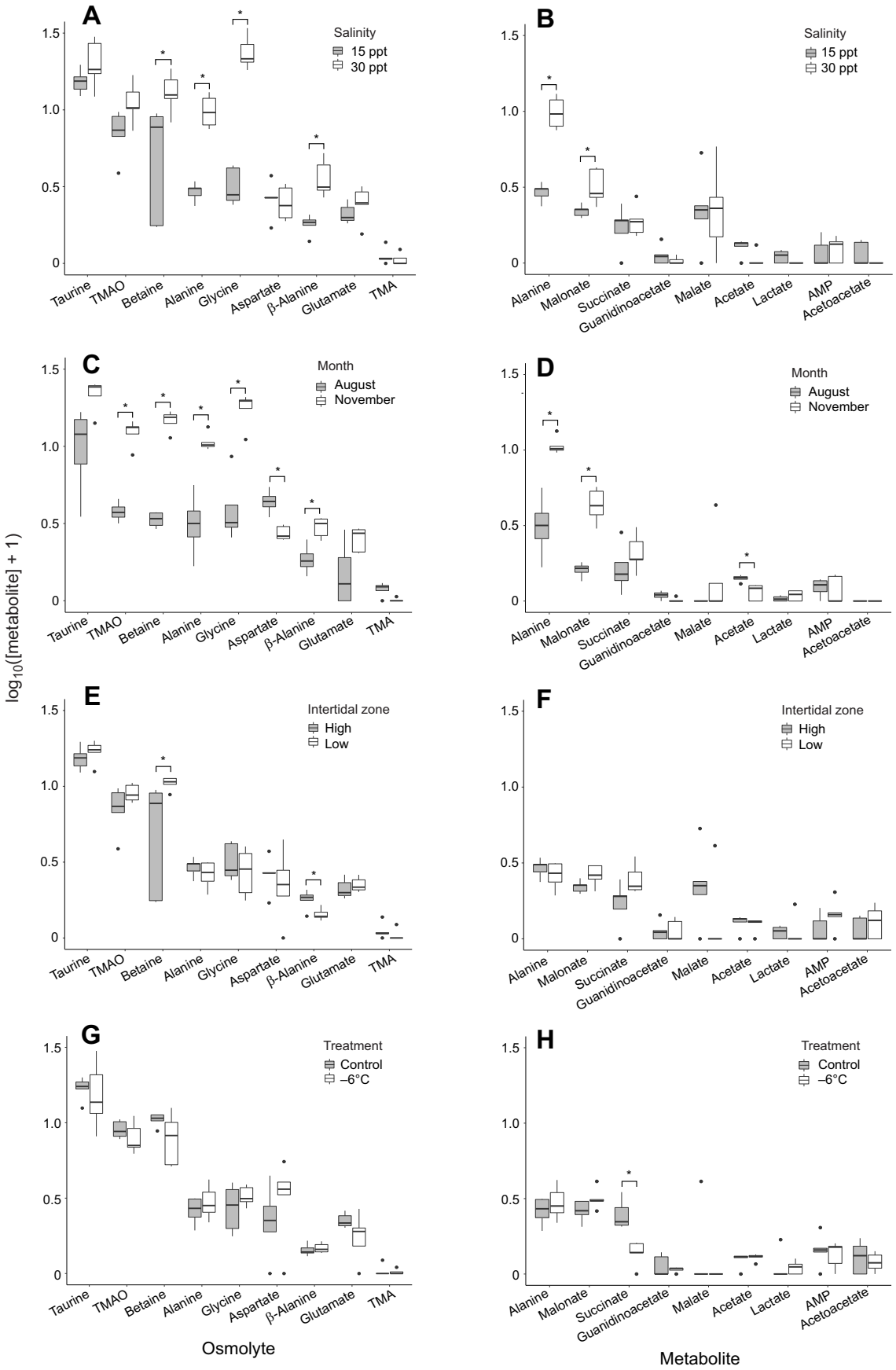

Fig. 3. See next page for legend.

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Fig. 3. The impact of shore level, seasonality, salinity acclimation and freezing exposure on metabolite concentrations in *M. trossulus* gill tissue. All data were transformed as follows: $\log_{10}$([metabolite]+1). Asterisks denote metabolites that changed significantly within that binary comparison, as indicated by Wilcoxon tests (P<0.05). The left column indicates osmolytes; the right column indicates anaerobic byproducts. Alanine appears on both sides as it can function as both an osmolyte and anaerobic byproduct. (A,B) Effects of salinity acclimation on metabolite concentrations; data shown are only high shore mussels (n=5 per group). (C,D) Effects of seasonality on metabolite concentrations, only considering low intertidal mussels acclimated to 30 ppt salinity (n=4 for August, 5 for November). (E,F) Effects of shore level on metabolite concentrations, only considering mussels acclimated to 15 ppt salinity (n=5 per group). (G,H) Effects of exposure to −6°C for 3 h on metabolite concentrations, only considering low intertidal mussels acclimated to 15 ppt salinity (n=5 per group).

Furthermore, no clear change in metabolite concentrations were found with increasing shore level. Instead, this change in freeze tolerance owing to shore level may be driven by high molecular weight cryoprotectants that we did not measure, such as heat shock proteins or antifreeze proteins. Although there is robust evidence that increasing shore level correlates with increased heat tolerance in intertidal invertebrates (see Somero 2002 for a review), very few studies have focused on this pattern on low temperature tolerance, so our study gives important insight into this facet of the temperature tolerance of intertidal organisms.

Mussel SCPs did not change seasonally in a clear way and most likely not in a biologically significant way, as the change was within 1°C, which is within the error of type T thermocouples (thermocoupleinfo.com). Salinity acclimation depresses SCP by approximately 1°C, which again may not be biologically significant because the magnitude of change is small. Increased SCPs are associated with the accumulation of ice nucleators in freeze-tolerant animals (Toxopeus and Sinclair, 2018), and so the lack of strong shift in SCP in our *M. trossulus* population suggests that an accumulation of ice nucleators may not explain the plasticity of freeze tolerance that we observed.

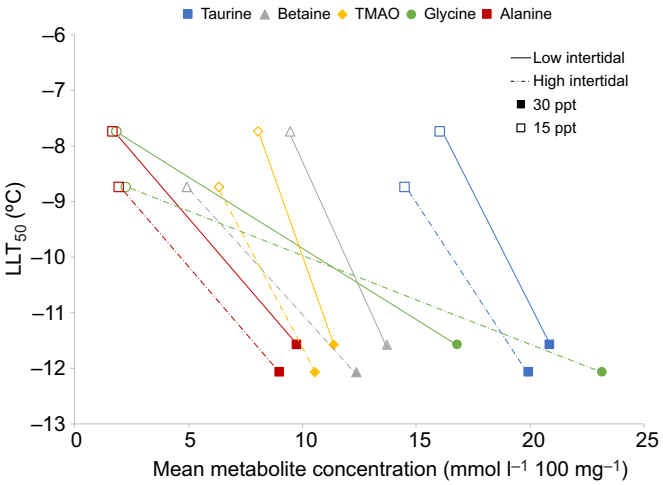

Fig. 4. Changes in prominent gill osmolyte concentrations in relation to changes in lower lethal temperature (LLT_{50}) in *M. trossulus*. High salinity acclimation (30 ppt) results in LLT_{50} depression and osmolyte accumulation, but this pattern does not hold for shore level LLT_{50} depression. Open symbols represent mussels acclimated to 15 ppt salinity seawater, closed symbols indicate mussels acclimated to 30 ppt. Solid lines indicate mussels collected from the low intertidal zone; dashed lines indicate high intertidal mussels. Each point represents the mean metabolite concentration from five mussel gill samples. Error bars are removed for clarity. All mussels were collected from Tower Beach, Vancouver, BC, Canada, on 29 November 2019. LLT_{50} is defined as the temperature that causes 50% mortality and is derived from generalized linear models.

The effect of exposure to −6°C air on subsequently analysed mussel gill metabolite concentrations remains unclear. We might expect that if metabolites were cryoprotective, they should increase in concentration after exposure to sub-zero temperatures; however, the overall effect of −6°C exposure on metabolite concentrations was quite small and most of the metabolites that were affected by the −6°C treatment decreased in concentration post-cold exposure. These decreases in metabolite concentrations may be caused by the energetic stress associated with cold and potential freezing stress. In a similar study, Storey and Churchill (1995) measured changes in metabolite concentrations in the freeze-tolerant intertidal bivalve *Geukensia demissus* after 2 or 12 h of exposure to −6°C. Storey and Churchill (1995) reported that the cumulative amino acid pool in *G. demissus* gills decreased in concentration following −6°C exposure, primarily driven by a decrease in glycine and taurine concentrations. They also noted that in gill tissues, almost no change in anaerobic byproduct concentrations were observed after −6°C exposure.

We found mixed evidence at best for a cryoprotective role for anaerobic byproducts in *M. trossulus*. The anaerobic byproducts succinate and acetate do not accumulate in gill tissues in correlation with increased freeze tolerance. However, alanine, which is both an osmolyte and anaerobic byproduct, does correlate with increased freeze tolerance seasonally and with high salinity acclimation in our work. The metabolomic response in mussels is tissue-specific (Cappello et al., 2018; Storey and Churchill, 1995), so in future studies a full atlas of mussel tissue metabolomic responses could assess whether anaerobic byproducts are perhaps accumulating in other mussel tissue types to aid in increasing freeze tolerance.

Our study identified osmolytes as a candidate class of cryoprotectants in intertidal invertebrates. However, it seems unlikely that the putative cryoprotectants we have identified are solely responsible for conferring freeze tolerance in mussels. This is because mussels were still somewhat freeze tolerant during the summer, when concentrations of osmolytes were quite low, and osmolyte concentrations did not explain the difference in freeze tolerance between shore levels. Thus, there are likely other cryoprotective mechanisms that enable freeze tolerance in *M. trossulus*. In addition, it is still unclear whether osmolytes function cryoprotectively on a colligative basis, where the increased osmolyte pool concentration increases mussel freeze tolerance, or whether they function non-colligatively, wherein each osmolyte has a unique cryoprotective function. Osmolytes may be non-colligatively cryoprotective because the osmolytes taurine, glycine and alanine have been shown to be membrane stabilizing (Anchordoguy et al., 1988; Schaffer et al., 2003), and the osmolytes TMAO and betaine (Street et al., 2006) have protein-stabilizing effects. Recently, it has been shown that the non-colligative properties of low molecular weight cryoprotectants are important in freeze tolerance in the cricket *Gryllus veletis* (Toxopeus et al., 2019). Further studies of freeze tolerance in intertidal species should include this possibility.

Our study contributes to the field of freeze-tolerance physiology, shedding light on the physiological mechanisms underlying freeze tolerance in an understudied group of organisms. By investigating the relationships between plasticity in freeze tolerance and changes in the concentrations of a wide range of low molecular weight metabolites, we have shown that acclimation to high salinity and winter acclimatization both increase mussel freeze tolerance and are correlated with an accumulation of intracellular osmolytes. This indicates that osmolytes are strongly correlated with increased

freeze tolerance and are strong candidate cryoprotectants. This adds to the limited body of knowledge on the physiological mechanisms responsible for freeze tolerance in intertidal invertebrates. Better understanding of natural freeze tolerance is an important pursuit as the threat of freezing will remain in the future for intertidal invertebrates, despite a warming world. Freeze-associated mortality events have been documented in a number of intertidal invertebrates, including *M. trossulus* (Carroll and Highsmith, 1996). More generally, lower thermal limits are often approached or even exceeded by environmental temperatures for many ectotherms (Sunday et al., 2014), and the degree to which this limits the distribution and abundance of species is especially important for taxa such as intertidal mussels, which are foundational members of the intertidal ecosystem (Buschbaum et al., 2009) and may be the ecological leverage point through which environmental change acts (Sunday et al., 2017). Lastly, understanding which cryoprotectants mussels use to survive freezing can help us to choose potential cryoprotective molecules for biomedical purposes, such as the cryopreservation of human cells, tissues and organs (Jang et al., 2017). Future work on freeze tolerance, particularly in under-studied taxa such as intertidal invertebrates, may yield novel and important physiological and ecological insights.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.R.K., C.D.G.H., K.E.M.; Methodology: J.R.K., K.E.M.; Formal analysis: J.R.K., K.E.M.; Investigation: J.R.K.; Resources: C.D.G.H., K.E.M.; Data curation: J.R.K.; Writing - original draft: J.R.K.; Writing - review & editing: C.D.G.H., K.E.M.; Visualization: J.R.K.; Supervision: K.E.M.; Project administration: K.E.M.; Funding acquisition: C.D.G.H., K.E.M.

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