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Proteostasis in ice: the role of heat shock proteins and ubiquitin in the freeze tolerance of the intertidal mussel, *Mytilus trossulus*

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Abstract
The bay mussel, *Mytilus trossulus*, is an animal that can survive extracellular ice formation. Depending on air and ocean temperatures, freeze tolerant intertidal organisms, like *M. trossulus*, may freeze and thaw many times during the winter. Freezing can cause protein denaturation, leading to an induction of the heat shock response with expression of chaperone proteins like the 70 kDa heat shock protein (HSP70), and an increase in ubiquitin-conjugated proteins. There has been little work on the mechanisms of freeze tolerance in intertidal species, limiting our understanding of this survival strategy. Additionally, this limited research has focused solely on the effects of single freezing events, but the act of repeatedly crossing the freezing threshold may present novel physiological or biochemical stressors that have yet to be discovered. *Mytilus* are important ecosystem engineers and provide habitat for other intertidal species, thus understanding their physiology under thermal extremes is important for preserving shoreline health. We predicted that repeated freeze exposures would increase mortality, upregulate HSP70 expression, and increase ubiquitin conjugates in mussels, relative to single, prolonged freeze exposures. *Mytilus trossulus* from Vancouver, Canada were repeatedly frozen for a combination of 1 × 8 h, 2 × 4 h, or 4 × 2 h. We then compared mortality, HSP70 expression, and the quantity of ubiquitin-conjugated proteins across experimental groups. We found a single 8-h freeze caused significantly more mortality than repeated freeze–thaw cycles. We also found that HSP70 and ubiquitinated protein was upregulated exclusively after freeze–thaw cycles, suggesting that freeze–thaw cycles offer a period of damage repair between freezes. This indicates that freeze–thaw cycles, which happen naturally in the intertidal, are crucial for *M. trossulus* survival in sub-zero temperatures.

Keywords Freeze tolerance · Mussel · Intertidal · Protein damage

Introduction
Animals living in the intertidal zone—the area where the ocean meets the land between high and low tides—must contend with both marine and terrestrial conditions. Intertidal animals must survive intense UV radiation, hydrodynamic forces, desiccation stress, and extreme fluctuations in temperature, salinity, and pH (Carrington et al. 2009; Jensen and Denny 2016; Pulgar et al. 2017). Thermal extremes are an especially relevant abiotic factor in the rocky intertidal, as they determine both distribution and physiological performance of organisms (Helmuth and Hofmann 2001; Dong et al. 2021). During the winter, sub-zero temperatures often induce ice formation in intertidal animals due to the constant wet conditions of the intertidal zone and the presence of food particles in their bodies which can act as ice nucleators in the haemocoel (Storey and Storey 1996). However, ice formation in intertidal ectotherms can inflict cellular and protein damage (Aarset 1982). Despite this, intertidal invertebrates inhabit virtually all rocky coastlines in temperate and polar regions, and many are freeze tolerant, allowing them to survive the frigid conditions that occur during the winter months. Although there is a growing body of literature focusing on thermal tolerances of intertidal organisms, it focuses primarily on tolerance to heat stress, meaning that we are just beginning to understand the physiological mechanisms behind survival at low temperatures and how these mechanisms drive ecology.

Investigations into the freeze tolerance of intertidal animals are further complicated by the fact that these animals will often experience multiple freeze–thaw cycles.
throughout the winter. Repeated freezing in the intertidal zone is caused primarily by cyclic tidal regimes. When animals are immersed in sea water during winter, they are protected from freezing air temperatures and experience more moderate conditions (Kennedy et al. 2020). Sub-zero air temperatures can then cause organisms to freeze when the tide recedes, and then thaw upon immersion as the tide returns. Organisms that are located higher in the intertidal zone spend longer amounts of time out of water compared to animals located lower down on the shore, and thus must tolerate more extreme abiotic pressures and more severe sub-zero body temperatures during daily tide cycles in the winter (Davenport and Davenport 2005; Finke et al. 2007; Williams and Somero 1996). This time spent out of water means a cessation of feeding in suspension feeding animals, a decrease in metabolic rate, increased chance of desiccation, and other stressors (Storey and Storey 1988). This suggests that since high intertidal animals will be exposed to freezing conditions for longer, they may be better acclimated to freeze exposures, as compared to their low intertidal counterparts.

To survive freezing, intertidal animals must be able to survive the formation of ice and then regain natural function once thawed (Storey and Storey 1988). Ice formation begins in the haemocoel of invertebrates, spreading through the extracellular spaces of the tissues. The formation of extracellular ice crystals causes osmotic dehydration of cells, which in turn prevents intracellular freezing which is lethal in most animals (Sinclair and Renault 2010). However, irrespective of ice formation, animals exposed to low temperatures can still experience considerable damage from the cold (Ramløv 2000). Low temperatures can cause a decrease in cellular reaction rates, a suppression of protein synthesis, and promote destabilizing, hydrophobic interactions among protein residues rendering them non-functional (Al-Fageeh and Smales 2006; Privalov 1990; Ramløv 2000). In addition, when exposed to sufficiently cold temperatures, extracellular ice formation can occur imposing mechanical and osmotic stress on the organism (Ramløv 2000). As extracellular water is formed into ice, solutes are excluded, causing the surrounding fluids to become increasingly solute rich (Lee 2010), which in turn, may lead to cellular protein denaturation (Baust 1973). Finally, once the frozen animal begins to thaw and recover, damaging reactive oxygen species are generated, and potential further mechanical damage can occur due to ice recrystallization (Doelling et al. 2014; Mazur 2010). Overall, three distinct processes will impose challenges on intertidal organisms during freeze–thaw cycles: cooling, freezing, and thawing (Toxopeus and Sinclair 2018).

To withstand damage due to freezing, animals can accumulate cryoprotectants, which function to protect against the effects of low temperature and ice formation (Storey and Storey 2013). These can be classified into two main categories: high molecular weight such as antifreeze proteins (Duman 2001) and low molecular weight such as sugars (Lee 2010). Furthermore, cryoprotectants can function on either a colligative basis, where molecules act as a pool and are interchangeable or a non-colligative basis, where each cryoprotectant has a unique function (Lee 2010). Low molecular weight cryoprotectants have non-colligative properties in the freeze-tolerant cricket Gryllus veletis (Toxopeus et al. 2019), but it is still unclear whether they function on a colligative or non-colligative basis in intertidal animals such as the mussel M. trossulus (Kennedy et al. 2020).

Although not typically regarded as a cryoprotectant, heat shock proteins (HSPs), are a potential mechanism by which organisms can reduce the impacts of protein denaturation due to freezing. Heat shock protein 70 (HSP70) is a highly conserved, 70 kDa chaperone protein that acts as a chaperone to ensure cellular proteins are folded correctly in their native state (Boroda et al. 2020). In an environment with cold-induced protein denaturation, HSP70 also plays a role in re-folding misfolded proteins to regain their native states and can also target denatured proteins to remove them from the cell (Todgham et al. 2007). This removal can prevent the irreversible formation of cytotoxic protein aggregates (Venetianer et al. 1994).

Most previous work on HSP70’s role in thermal tolerance has focused on upper thermal limits and heat shocks. Increased expression of HSP70 as a result of elevated temperature stress has been widely observed in intertidal organisms, such as Mytilus galloprovincialis mussels, Lotia gigantea limpets, and snail species of the genus Tegula (Miller et al. 2009; Tomanek and Sanford 2003; Toyohara et al. 2005). However, much less is known about the extent of HSP70’s role in relation to freezing stress. HSP70 is upregulated in Epiblema scudderiana larvae in response to winter declines in temperature, and plays a significant role in their winter survival (Zhang et al. 2018). HSP70 expression is also upregulated in fruit flies in response to acute cold shocks (Štětina et al. 2015). Larvae of the Antarctic midge, Belgica antarctica, similarly expressed increased levels of HSP70 after repeated freezing, indicating more protein damage (Teets et al. 2011). In response to cold shock, the marine crab Carcinus maenas exhibited no change in HSP70 protein content (Kelley et al. 2013) and the Antarctic barnacle Nacella concinna downregulated the expression of HSP70 genes (Clark and Peck 2009). Overall, it appears that the upregulation of HSP70 is often observed in response to cold and freezing stress in insects but has mixed effects in marine species in response to cold shock. To date, this has not been investigated in an intertidal species in response to freezing specifically.

While molecular chaperones like HSP70 help to mitigate protein damage, freezing can still induce irreversible protein damage and denaturation. In this case, ubiquitin, a
low-molecular-mass protein, marks the damaged proteins to be degraded by proteases (Hochstrasser 1995). Elevated levels of ubiquitin-conjugated proteins accumulating within the cell indicate increased levels of protein damage (Hofmann and Somero 1995). Elevated levels of ubiquitin conjugates have been observed in cold-adapted notothenioid fish (Todgham et al. 2007), indicating that cold temperatures affect protein integrity at an organismal level. In response to elevated summer temperatures, *M. trossulus* displayed increased levels of ubiquitin conjugates (Hofmann and Somero 1995). This increase in ubiquitin conjugates was also correlated with increased levels of HSP70, suggesting that chaperone proteins may not sufficiently mitigate protein damage during high temperature stress (Hofmann and Somero 1995). Taken together, these findings suggest that freezing may induce protein damage, leading to elevated levels of ubiquitin conjugates, and may also induce an increase in molecular chaperone expression.

The freeze tolerance of intertidal animals has only been investigated through single freezing events, which does not account for the repeated freeze thaw cycles that the animal would experience in nature. While there has been no prior work on the effects of repeated freeze–thaw (RFT) in intertidal species, repeated freezing has several important effects in insects, which are distinct from the effects of single, prolonged freezing events. With repeated freezing events, both a species of hoverfly (Brown et al. 2004) and caterpillar (Marshall and Sinclair 2011), had decreased survival compared to single sustained freezes of the same length. Similarly freeze thaw cycles induced oxidative stress on the freeze tolerant goldenrod gall fly, and reduced survival compared to a single freeze (Doelling et al. 2014). In response to RFT, goldenrod gall flies also upregulate their sorbitol (a cryoprotectant) content as a cost to subsequent egg production (Marshall and Sinclair 2018). By contrast, there was no significant difference in survival for *B. antarctica* larvae exposed to repeated freezing events compared to those that received a prolonged freeze exposure of equal length (Teets et al. 2011). It is evident that RFT has important effects on some insects, although it is unclear how this may translate to intertidal invertebrates.

Here we examine the impacts of repeated freeze thaw on HSP70 expression in the bay mussel *Mytilus trossulus*, which is a freeze tolerant invertebrate that inhabits rocky intertidal zones along the west coast of North America, that experiences stressful freezing temperatures throughout the winters. When mussels are exposed to the air during low tides, they tightly close their valves, trapping seawater within their shell. When this seawater freezes, this causes animal tissues to come into direct contact with ice, which initiates ice formation in the mantle cavity, which then likely propagates through tissues (Storey and Storey 1996). When mussels finish freezing, over 60% of their total body water can convert to ice (Kanwisher 1955). Research on cryoprotection in *M. trossulus* in response to freeze exposure is limited. Low molecular weight cryoprotectants, like amino acids, are thought to play an important role in preventing osmotic shock in response to freezing in *M. trossulus* (Kennedy et al. 2020). Additionally, the amino acid taurine has been demonstrated to be cryoprotective in *M. edulis* by preserving membrane integrity in vitro (Loomis et al. 1988). However, these previous studies were conducted under constant temperature conditions, but fluctuating temperatures (such as RFT) may produce undiscovered effects on *M. trossulus* (Marshall et al. 2021).

There are many unknowns surrounding the physiological and biochemical impacts of freezing: two examples being the unknown effects of freeze–thaw cycles on survival compared to prolonged freezing periods, and the poorly understood role of HSP70 in mediating protein damage in response to cold and freezing stress. How those effects are impacted by an organism’s intertidal shore position or the amount of recovery time an organism has post-freeze is also unclear. Here we hypothesize that repeated freeze–thaw cycles are more stressful to mussels, as compared to single, prolonged freezing events. We predict that freeze–thaw cycles will reduce *M. trossulus* survival relative to single freezes, since mussels will need to survive and cope with various stressors during multiple rounds of the cool/freeze/thaw cycle, as opposed to prolonged-freeze-exposed mussels that only experience one round of cool/freeze/thaw. We expect RFT will particularly reduce survival in low shore mussels, since they are not as well acclimated to freeze exposures. Alternatively, since mussels experience RFT in their natural conditions, we may expect that they will have higher survival during RFT compared to single prolonged freezes. We also predict that HSP70 and ubiquitin conjugates will be upregulated in response to freeze–thaw cycles, as the more strenuous freeze exposures will result in elevated levels of protein damage that will need to be repaired. We expect this trend to be most pronounced in low shore mussels.

**Methods**

*Mytilus trossulus* collection

Specimens of *Mytilus trossulus* were collected from Tower Beach, Vancouver, British Columbia, Canada (49.2733° N, 123.2578° W) on Oct. 14, Nov. 14, Dec. 13, 2020, and then on May 25, 2021 for a follow up experiment. Mussel collections were completed using a Scientific License, Management of Contaminated Fisheries Regulations from the Department of Fisheries and Oceans Canada (License number: XMCFR 22 2019). In Vancouver, sea water...
temperatures usually hover between 7 and 12 °C year-round, while winter air temperatures are relatively mild but can reach lows of −5 °C to −15 °C (Figure S1). During the winter, the lowest daily tide happens at night, meaning invertebrates are exposed to the coldest yearly air temperatures for 1–2 h during winter low tides.

On each sampling day, mussels were retrieved from the same outcropping of rocks at the lowest tide daily. High intertidal mussels were located from the uppermost edge of the mussel bed, and low intertidal mussels were collected from the lower edge of the mussel bed, at approximately 1 m shore height above Canadian chart datum. Neither sex nor age were controlled for during collections and experiments, though mussels were selected for size which is a proxy for age (Richardson et al. 1990). Animals collected were selected for an approximate size of 2–3 cm. Within 1 h of collection, mussels were placed in aerated 20 L aquaria in natural seawater (27.5 ppt salinity) sourced from seawater taps in the UBC Zoology Aquatics Facility. The aquaria were placed in 7 °C incubators (MIR-154, Sanyo, Bensenville, USA) in the Biosciences Building at the University of British Columbia, Canada. Approximately 100 mussels were stored in each aquarium. A full seawater change was performed every two days. Once collected, mussels were haphazardly assigned to experimental groups.

Freeze exposures

To conduct the freeze exposures, *M. trossulus* were removed from the aquaria, dried with paper towel and labelled with an identification number on the shell with nail polish. Mussel shell length was measured at the longest length of the valves using manual calipers and recorded. Then a copper-constantan type-T thermocouple (OMEGA Engineering, St. Eustache, Quebec) was attached to each mussel’s shell with adhesive putty or small pieces of masking tape. Thermocouples were connected to computers using PicoLog 6 beta software for Windows through Picolog TC-08 interfaces (Pico Technology, Cambridge, UK) throughout the freeze exposure to track mussel body temperatures and record freezing events. This was demonstrated as a rapid release of heat and dramatic rise in body temperature, which happens immediately after the supercooling point is reached (which is the point where body fluids begin turning to ice; Lee 2010). Mussels were stacked in 25 mm *Drosophila* vials, with individuals separated by Styrofoam to avoid inter-individual ice nucleation. The mussels were placed in a cooling bath with an initial temperature of 7 °C (to represent the temperature of the seawater) that then cooled to the desired temperature with a rate of −1 °C/min or −1.5 °C/min (in the survival assay) and −1.5 °C/min for the HSP70 and protein aggregation assays, based on the cooling rate used by Kennedy et al. (2020). This relatively fast rate of cooling mimics the process that occurs during a low tide event, when mussels would be rapidly exposed to much colder temperatures when the tide recedes.

Mussels used in survival trials were collected from the high and low edges of the mussel bed on November 14, 2020 and kept in seawater aquaria at 27.5 ppt salinity for 2–10 days before freezing. Mussels were then frozen at −8 °C for different allotments of time: one continuous exposure for 8 h (1 × 8 h), two exposures that were 4 h each (2 × 4 h) or four separate exposures that were 2 h each (4 × 2 h). The chosen experimental temperature of −8 °C was based on a pilot study that produced 90% mussel survival after a 2 h freeze. After freeze exposure, mussels were taken immediately from freezing baths and placed into seawater aquaria set to 7 °C. Mussels that were repeatedly frozen were allowed to recover in the aquaria for 24 h between each freeze. After their last freezing exposure, mussels were monitored daily for viability for seven days and were determined to be dead if the valves did not close when the animals were removed from the water, or upon mechanical stimulation (light probing with a metal tool).

The 4 × 2 h treatment group was designed to represent a repeated freeze-thaw scenario that would occur in natural conditions, modelling the immersion/emersion times of a low intertidal mussel experiencing a mixed semi-diurnal tidal cycle with one aerial exposure per day. Mussels in this scenario would be exposed to “winter time” aerial conditions for 2 h, but then allowed to recover in seawater as if the tide had covered them once again. To compare the effects of repeated freeze–thaw with prolonged freezes, we then aggregated the time spent frozen into allotments of different lengths: 2 × 4 h and 1 × 8 h. This design allowed us to examine if *M. trossulus* was affected by the thawing/re-freezing process or simply by the total amount of time spent frozen.

Mussels used in HSP70 and ubiquitin-conjugated protein quantification were collected from the high and low edges of the mussel bed on December 12, 2020 and kept in 25 ppt, 7 °C aerated seawater aquaria for 24 h until freezing. To investigate how freeze thaw cycles affect HSP70 expression, mussels were frozen once at −6 °C for either 8 consecutive hours (1 × 8 h) or four repeated freeze times of 2 h (4 × 2 h) with 22 h of recovery (rather than the 24 h used in the survival experiments) between each freeze (n = 10). A temperature of −6 °C was chosen as the experimental temperature based on a pilot study done using mussels from this collecting trip that produced 90% mussel survival after a 2-h freeze. After the final freeze exposure for each treatment group, mussels were returned to their aquaria for 2 h or 20 h. The 2 h allowed for adequate time for protein expression (Miller et al. 2009), and the 20 h recovery time was used to investigate any possible differences in protein expression.
with longer recovery times. Additionally, since RFTs were separated by 22 h, 20 h was chosen as the longer recovery time—as this would be the time that they would be next frozen if they were to freeze again. As a control group for time of day, seven mussels that were not exposed to any freezing treatments were sacrificed at each same timepoint.

As part of a follow-up experiment, a new subset of *M. trossulus* were collected on May 25, 2021 and stored using the same methods as before but in 17.5 °C seawater (reflecting the temperature of seawater on date of collection). This follow up experiment was used to observe differences in HSP70 expression between single freezes of varying lengths, but not as a comparison to the mussels in the main experiment that were collected during the winter, as the lower lethal temperature tolerances of *M. trossulus* vary seasonally (Kennedy et al. 2020). To observe HSP70 expression in *M. trossulus* after a single freeze exposure, mussels were frozen only once at −6 °C for 2, 4, 6, or 8 h and then allowed to recover for 20 h in 17.5 °C seawater aquaria.

### Sample preparation

After recovery in the aquaria, mussels were removed, and the gill tissue was dissected from the mussels (approximately 100 mg). Gill tissue was chosen as it is the most sensitive to temperature shifts, thus more likely to upregulate HSP70 expression (Aleng et al. 2015). After being blotted with a Kimwipe to remove excess water, gill tissue was weighed and immediately frozen in microcentrifuge tubes at −80 °C and stored until further use. The HSP70 assays were carried out using a protocol similar to that of Sagarin and Somero (2006). Frozen gills were placed in 400 μL of pH 7.1 lysis buffer [32 mM Tris–HCl (pH 7.1), 1 mM ethylendiaminetetraacetic acid (EDTA), 1 mM sodium dodecyl sulfate (SDS), 0.25 mg/mL phenylmethylsulphonyl fluoride (PMSF), and 10 μg/mL leupeptin]. Samples were twice boiled in a dry bath for five minutes to denature the proteins, and then homogenized using a bullet blender (Bullet Blender 50 Blue, next advance) with 200 μL of 3.2 mm round beads for 10 min at setting 8 in 1.8 mL microcentrifuge vials (Eppendorf safe-lock). Homogenates were transferred to a new microcentrifuge vial and briefly placed on ice for phase separation before centrifugation at 14,000×g.

**Western blots**

Protein separation was conducted by electrophoresis in a Mini Gel Tank on 10% Tris–Glycine pre-cast gels (Invitrogen™ Novex™ Value™ 10%, Tris–Glycine, 1.0 mm, Mini Protein Gel, 15-well). In each gel, one lane was dedicated to the prestained marker (Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, 10–250 kDa), one lane was loaded with 5 μg BSA as a negative control, and another was loaded with 10 ng of human HSP70 (His tagged human HSP70, SRP5190, Millipore Sigma), which allowed comparisons to be made among separate gels. The band quality of the human HSP70 control on the gels used was highly consistent, and therefore a single control lane for each gel was sufficient. Four μL of NuPAGE™ Reducing Agent (10X) and 20 μL of Tris–Glycine SDS Sample Buffer (2X) were added to 5 μg of protein in the samples and topped up with deionized water to reach a final volume of 40 μL. Samples were then heated to 85 °C for 2 min. The 13 remaining lanes on the gel were dedicated to these protein samples, and 10 μL of sample was loaded into each well. Each protein sample was loaded three times, in separate gels—for a total of three technical replicates per protein sample. Loaded gels were submerged in running buffer (Invitrogen™ Novex™ Tris–Glycine SDS Running Buffer) and run for approximately 45 min at 225 V.

Western blotting is considered a semi-quantitative process because the resulting densitometric values represent a relative comparison of protein content, rather than an absolute measure of protein quantity (Mahmood and Yang 2012). Western blots were used to quantify HSP70 expression. Gels were immediately transferred to 0.45 μm nitrocellulose membranes (BioRad) in a Trans Blot chamber (BioRad) for 6 h or overnight at 30 V. After equilibration in transfer buffer, gel was sandwiched with nitrocellulose membrane between two pieces of filter paper, and two blotting pads submerged in transfer buffer (Tris–glycine and 20% methanol) during the transfer. After the transfer, the membrane was stained with Ponceau Red for three minutes and then destained with water to visualize the protein transfer. Membranes were air dried and stored between filter paper in Ziploc bags at 4 °C before the start of the immunoblot.

Immunoblotting was conducted using a protocol similar to Miller, Harley, and Denny (2018). Membranes were incubated in blocking buffer [1X Tris-buffered saline (TBS), 3% BSA, 0.1% Tween-20] for 1 h on an orbital shaker.
Membranes were then washed four times in TBS with 0.1% Tween-20 (TBST) for 5 min each. Then, membranes were incubated in a 1:5000 dilution of the primary antibody in PBS with 2.5% BSA (antibody MA3-007, clone 5A5, Mouse Monoclonal Antibody, FisherScientific). The membranes were washed four times in TBST for 5 min each before incubation in secondary antibody (A1418, Anti-mouse IgG (Fc specific) alkaline phosphatase antibody produced in goat, Sigma-Aldrich). The secondary antibody was diluted 1:1000 in TBST with 2.5% BSA, and the membrane was incubated for 45 min. The membranes were then washed four times for 5 min each in TBST.

The proteins were visualized by incubating membranes for 30 min in BCIP®/NBT Alkaline Phosphatase Substrate (B5655, SIGMAFAST™ BCIP®/NBT, Sigma-Aldrich). The intensities of the detected bands were analyzed with a FluorChem 8800 Imager (Alpha Innotech, San Leandro, CA, USA) using AlphaEase FC software (v. 3.1.2; Alpha Innotech). The density measurements of each protein sample band at 68, 72 and 76 kDa, were compared relative to the human HSP70 standard on each gel, allowing for comparison of relative density values across all western blots.

### Dot blots

Using the same protein samples as with the HSP70 assay, ubiquitin-conjugated protein expression was examined using immunohistochemical analysis. Nitrocellulose membranes (0.2 μm) were pretreated for 10 min in TBS then secured into the Bio-Dot Microfiltration apparatus (Bio-Rad). Wells were rehydrated once with TBS, then 0.5 μg of each protein sample and 0.05 μg of ubiquitin standard (AB218616, Recombinant Human Ubiquitin protein, His tag, Abcam) were blotted in triplicate and allowed to gravity filter. Wells were washed twice with 200 μL TBST and the membrane was removed from the apparatus and heat fixed at 55 °C for 20 min. Membranes were then developed and visualized using the same protocol as the HSP70 assay, except for a ubiquitin specific primary antibody (1:1000 dilution; UBCJ2, Mono- and polyubiquitinated conjugates monoclonal antibody, FroggaBio).

### Statistical analysis

Statistical analyses were performed using R (c. 3.5.1; R Development Core Team 2021). The R package “ggplot2” was used to generate Figs. 1, 2, 3, 4, 5, 6, 7 (Wickham 2016), the package “cowplot” was also used to generate Figs. 3, 4, 5, 7 (Wilke 2020). The R package “plyr” was also used to determine means and standard errors before plotting (Wickham 2011).

Logistic regression was used to test for significant differences in survival within different freeze–thaw cycle lengths. Then, a chi-squared test was used to test for significant differences in survival after freezing assessing shell length, total time frozen, and shore height as potential predictors of survival after freezing. Specific differences between survival groups were analyzed using an ANOVA and Tukey HSD post-hoc test (Tukey 1977). To determine how basal levels of HSP70 changed with shore height, HSP70 density values were standardized to the HSP70 human standard (so they could be accurately compared among separate blots) and then tested for normality using a Shapiro–Wilk test (Royston 1982). If data were normal, the means of the shore height were compared using a student’s t-test, and if they were not normal, a Mann–Whitney U (Mann and Whitney 1947) test was used. Finally, freeze time, recovery and shore position were analyzed in relation to HSP70 density. First, relative HSP70 density was calculated by taking the densitometry measurements
of the sample and dividing them against the densitometry value for the HSP70 human standard, and then by the means of their time matched controls. The resulting values were then log transformed (expressed as log₂ fold change) to better adjust for normality. A three-way ANOVA was used to determine if freeze time, recovery, or shore position had an effect on HSP70 density, as well as recording interaction terms between the factors. The same statistical tests were used to evaluate content of ubiquitin-conjugated protein in relation to shore height, freeze time, and recovery time. Values are reported as means ± standard error. Alpha was set to 0.05. All data is deposited on Github at https://github.com/laurentjgill/mussel-freeze-thaw.

Fig. 2 Western blot analysis of gill tissue HSP70 expression from mussels frozen at −6 °C. Labels indicate the treatment group or time-matched controls, and all treatment groups on this blot were allowed to recover for 20 h. The standard was 10 ng of human HSP70.
Fig. 4  
A Relative log fold change HSP70 expression of all bands measured in *M. trossulus*.  
B Relative log fold change HSP70 expression of bottom bands (68 + 70 kDa).  
C Relative log fold change HSP70 expression of top bands (76 kDa).  
Measurements are relative to 10 ng human HSP70 standard, and then expressed as log2 fold change relative to time matched control. Mussels were collected from both the high and low intertidal zones (50/50). Letters ‘a’ and ‘b’ represent statistical significance. Dashed line represents no change in relative HSP70 expression relative to time-matched control. Error bars represent standard error of the mean. *n* = 8

Fig. 5  
A Relative log fold change HSP70 expression of all bands measured in *M. trossulus*.  
B Relative log fold change HSP70 expression of bottom bands (68 + 70 kDa).  
C Relative log fold change HSP70 expression of top bands (76 kDa).  
Measurements are relative to 10 ng human HSP70 standard, and then expressed as log2 fold change relative to time matched control. Letters ‘a’ and ‘b’ represent statistical significance. Dashed line represents no change in HSP70 expression relative to time-matched control. Error bars represent standard error. *n* = 8
Results

Effects of repeated freeze thaw on mussel survival

To test the effects of repeated freeze thaw cycles on mussels, 168 mussels were frozen either for 1 × 8 h, 2 × 4 h or 4 × 2 h. On average, mussels froze at −3.3 ± 0.15 °C (n = 60), and every mussel in this experiment froze, as evidenced by the characteristic exotherm in the temperature trace graph that follows the supercooling point. There was no significant effect of shore position within the intertidal zone on survival (df = 1, 68, z = 1.01, p = 0.15), and shell length was also not a significant predictor of survival (df = 1, 67, F = 3.5, p = 0.06). However, mussels that were repeatedly frozen for 4 × 2 h (proportion = 0.88) or 2 × 4 h (proportion = 0.61) had higher survival than mussels that had a single prolonged freeze exposure (proportion = 0.21; df = 1,70, p < 0.0001; Fig. 1). Freezing twice for 4 h each time caused similar mortality as freezing four times for 2 h each (p = 0.106; Fig. 1). Cumulative survival in *M. trossulus* repeatedly frozen for two hours stayed constant for the first two freezes (proportion = 1), but then decreased slightly at the third (proportion = 0.96) and fourth (proportion = 0.88) freeze–thaw cycles, but this trend was not statistically significant (Z = −1.76, p = 0.08).

Relative HSP70 expression analysis via western blot

A new subset of 88 mussels was collected on Dec 13th, 2020 and frozen for either 4 × 2 h or 1 × 8 h at −6 °C, and all but 1 survived until their dissection time. Mussels froze at −4.4 ± 0.8 °C (n = 136), and every mussel in this experiment froze.
HSP70 expression was determined through semi-quantitative densitometry analysis using Western blots. The monoclonal antibody used in the immunoblot analysis (Fisher-Scientific, MA3-007) recognized three different isoforms of HSP70 (at 68, 70 and 76 kDa) in the gill tissue of *M. trossulus*. In some specimens, the bottom two bands were not well resolved, therefore comparisons of HSP70 expression were performed using either all bands, the top band (76 kDa) or the bottom two bands (68+70 kDa). This aligns with a previous study, which found up to four different isoforms of HSP70 (ranging between 68 and 76 kDa) in *M. trossulus*, with not all isoforms being detectable in all samples (Hofmann ands Somero 1995). A representative western blot is shown in Fig. 2.

The concentration of the 76 kDa isoform was significantly higher in gills of mussels from the high intertidal zone compared to the low intertidal zone before any freezing event occurred (t = 4.0, p < 0.001; Fig. 3C). Despite the significant difference in the 76 kDa isoform, this difference was not observed in the bottom two bands (p = 0.74; Fig. 3B) or when all bands were combined (p = 0.32; Fig. 3A).

**HSP70 expression in response to repeated freeze–thaw**

Each frozen mussel was directly paired with a control mussel that remained in seawater that was dissected at the same time to reduce any circadian effects. Therefore, densitometries have been displayed as HSP70 expression relative to the average of the time-matched controls (expressed as log₂ fold change).

Neither freeze treatment nor recovery time had a significant effect on relative HSP70 density for the sum of the 68+70 kDa isoforms (Freeze treatment: \( F_{(1, 24)} = 0.13, p = 0.72 \); recovery: \( F_{(1, 24)} = 0.96, p = 0.34 \)) or the 76 kDa isoform (freeze treatment: \( F_{(1, 24)} = 0.02, p = 0.88 \); recovery: \( F_{(1, 24)} = 1.0, p = 0.32 \)). When all HSP70 isoforms were combined, recovery time had a significant effect on HSP70 density, but not freeze treatment (freeze treatment: \( F_{(1, 32)} = 2.8, p = 0.1 \); recovery time: \( F_{(2, 32)} = 4.3, p = 0.045 \)). In mussels exposed to repeated freeze thaw cycles, average density of total HSP70 protein within gill tissue was greater following a 20-h recovery (freeze treatment × recovery: \( F_{(1, 24)} = 5.2, p = 0.03 \); Fig. 4A). Expression of HSP70 in the bottom two bands (\( F_{(1, 24)} = 1.6, p = 0.21 \); Fig. 4B) and the top band isoform (\( F_{(1, 24)} = 1.9, p = 0.18 \); Fig. 4C) did not have a significant interaction term between recovery times and freeze treatment.

There was a significant interaction between shore position and freeze treatment such that only mussels from the low intertidal exhibited increased relative 76 kDa isoform expression following repeated freeze thaw cycles (Position: \( F_{(1, 28)} = 8.4, p < 0.01 \); freeze time × position: \( F_{(1, 28)} = 6.1, p = 0.02 \); Fig. 5C). When exposed to repeated freeze–thaw cycles, low intertidal mussels had a higher expression of the 76 kDa isoform (mean = 0.53 ± 0.19) than their high intertidal counterparts (mean = −0.82 ± 0.36; Fig. 5C). Low intertidal mussels exposed to freeze thaw cycles induced higher expression of HSP70 compared to low intertidal mussels that were exposed to a single, prolonged freeze, in all bands (mean = 0.58 ± 0.31), bottom bands (mean = 0.47 ± 0.25) and top band (mean = 0.53 ± 0.19; Fig. 5).

There was no effect of shore position on HSP70 expression in the mussels that experienced a sustained freezing event (1 × 8 h) in any of the isoforms (\( p > 0.09 \) in all cases; Fig. 5).

To determine if mussels were able to upregulate HSP70 in response to a singular freeze event of any length, *M. trossulus* were frozen for a single freeze of either 2, 4, 6, or 8 h. No significant upregulation in HSP70 expression was observed among any of the freeze times (\( p > 0.3 \); Figure S2).

**Relative ubiquitin-conjugated protein analysis via dot blot**

Using the same protein samples as in the HSP70 analysis, the amount of ubiquitin-conjugated protein was analyzed using dot blotting. Basal content of ubiquitin-conjugated proteins did not differ between *M. trossulus* from the high intertidal and mussels from the low intertidal (\( p > 0.35 \); Figure S3). When exposed to freeze–thaw cycles, mussels that recovered for 20 h had a significantly higher levels of ubiquitin-conjugated proteins (mean = 0.06 ± 0.05) than those that recovered for 2 h after freeze exposure (mean = −0.16 ± 0.03; \( p < 0.005 \); Fig. 6A). There was no significant effect of shore position on the amount ubiquitin-conjugated protein (\( p = 0.45 \); Fig. 6B).

**HSP70 and ubiquitin-conjugated protein correlation**

The relative content of ubiquitin-conjugated proteins and the relative levels of HSP70 are positively correlated when looking at treatment group averages (\( R = 0.85; p < 0.01 \)) and when looking at each sample individually (\( R = 0.46; p < 0.05 \); Fig. 7).

**Discussion**

Here, we show for the first time that HSP70 and ubiquitin-conjugated proteins are upregulated in response to repeated freezing, but not single freezes in *M. trossulus*. HSP70 expression and ubiquitin-conjugated proteins in repeatedly frozen *M. trossulus* were investigated to better understand changes to the cellular protein pool during freezing. Overall,
we found: (1) survival of repeatedly frozen *M. trossulus* was significantly higher than mussels that were frozen only once, (2) basal levels of HSP70 were initially lower but then significantly upregulated after freezing in low intertidal mussels, as compared to high intertidal mussels (3) both HSP70 expression and the content of ubiquitin-conjugated proteins were upregulated 20 h after, but not 2 h after repeated freezing. Taken together, this may indicate that repair processes during immersion following freezing are important for mussels to survive sub-zero air temperatures.

**Survival**

*Mytilus trossulus* living on polar and temperate shores can experience many freeze–thaw cycles throughout the winter, and therefore must respond to cellular damage from freezing to survive. Generally, to minimize the effects of freeze-induced damage, animals can either upregulate protective mechanisms before and during freezing, or they can repair their cellular damage after the freeze exposure. Given that animals can respond biochemically to a freezing event, this suggests that the timing and repetition of freezing events might be important for predicting survival. Sustained freezing in *M. trossulus* induced more mortality than repeated freezing (Fig. 1), even though the cumulative amount of time spent frozen was held constant. This suggests that during RFT, either cellular repair occurs during each recovery period to mitigate damage, and/or that mussels can upregulate defense mechanisms between each freeze.

While this is the first study to examine the physiological effects of freeze–thaw cycles in intertidal species, there have been previous studies examining the effects of RFT in insects, which have yielded contradictory results about the impact of RFT on survival (Marshall and Sinclair 2012). RFT resulted in increased mortality for insect species like the goldenrod gall fly *Eurosta solidaginis* (Doebling et al. 2014), woolly bear caterpillar *Pyrrharctia isabella* (Marshall and Sinclair 2011), and hoverfly *Syrrphus ribesii* (Brown et al. 2004). However, *Belgica antarctica* midge larvae had increased survival after RFT compared to a single, sustained freeze (Teets et al. 2011). The findings from our study reveal that *M. trossulus* exposed to RFT have significantly increased survival compared to those that experienced prolonged freeze exposures. During recovery, *M. trossulus* may recover, repair damage, and upregulate defensive mechanisms in preparation for the next freezing event. Perhaps since intertidal animals experience frequent thawing periods in their natural environment in accordance with daily immersion during high tide, they are better able to utilize these “recovery periods” to repair damage after freezing, as compared to insects which are not exposed to frequent, predictable thawing periods and are therefore more accustomed to sustained bouts of freezing.

**Intertidal shore height effects**

Mussels living higher on the shore are exposed to aerial conditions for longer, and must contend with greater thermal stress compared to their low intertidal counterparts (Halpin et al. 2002). This suggests that mussels from the high edges of the mussel bed may be better adapted to frequent freeze exposures. However, our results indicated no difference in overall survival between high and low intertidal mussels when exposed to freezing events of any duration. In contrast, Kennedy et al. (2020) showed that *M. trossulus* from the high intertidal zone survived significantly better than ones from the low intertidal zone during single three hour freezes. The differences found by Kennedy et al. (2020) were relatively small, however, and since the authors of that study used multiple test temperatures, we may not have been able to detect these small differences as only one test temperature was used in our study.

Aside from survival, intertidal organisms from high and low shore positions may also have differences in constitutively expressed cryoprotectants or proteins other than HSP70. The difference in *M. trossulus* survival due to shore height is likely not driven by low molecular weight cryoprotectants (Kennedy et al. 2020), which leaves high molecular weight molecules such as ice nucleating agents, heat shock proteins, or antifreeze proteins to explain these differences. Despite finding no difference in survival proportion, our results showed that *M. trossulus* from the high intertidal zone had a greater basal expression of the 76 kDa heat shock protein compared to mussels from the low intertidal zone. The 76 kDa isoform of HSP70 in *M. trossulus* has been previously identified to be less heat-inducible (Hofmann and Somero 1995), and combined with our findings that its endogenous expression varies based on shore position, suggests that the 76 kDa isoform is constitutively expressed in *M. trossulus*. Past research has similarly found that HSP expression can vary with shore height, as *M. trossulus* from a more stressful intertidal habitat had higher basal HSP70 expression compared to a subtidal habitat (Hofmann and Somero 1995). The mussel *M. californianus* and a limpet species of the genus *Lottia* from high intertidal sites also had higher HSP70 expression, compared to their low intertidal counterparts (Dong et al. 2008; Halpin et al. 2002). In summary, animals inhabiting the high intertidal zone may have higher expression of constitutive HSPs as a preparative and/or protective function for more frequent and extreme thermal stress.

Aside from constitutively expressed molecules, intertidal organisms from high and low shore positions may differ in the expression of protective molecules during and after freezing. Our results showed that low intertidal mussels...
exposed to freeze thaw cycles induced greater expression of HSP70 compared to high intertidal mussels. This may suggest that freeze thaw cycles are more stressful for low intertidal mussels, and because of their lower levels of constitutive protective molecules, they must induce higher levels of HSP70 during and/or after freezing to mitigate protein damage. High intertidal mussels, on the other hand, may express greater basal levels of high molecular weight molecules, like HSP70, to pre-emptively combat the more frequent and extreme thermal stress they experience in situ.

**HSP70 expression and levels of ubiquitin conjugates**

HSP70 was upregulated in *M. trossulus* in response to freeze thaw cycles only later in recovery. This response was only observed when mussels had been repeatedly frozen, not after a single 2 h or 8 h sustained freeze, and only 20 h post freezing. *M. trossulus* also upregulated ubiquitin-conjugated proteins after being repeatedly frozen. Similarly, ubiquitin-conjugated proteins were only upregulated 20 h after the last repeated freezing event. This may come as a result of two separate scenarios: (1) repair of cellular damage after freezing or (2) upregulation of protective mechanisms after freezing to prevent further damage from subsequent freezing events.

In this case, *M. trossulus* most likely upregulated HSP70 and ubiquitin-conjugated proteins after freeze thaw cycles to repair damage. This is because HSP70 was uniquely upregulated after RFT, which means that RFT either uniquely induces damage, or that 8 h of freezing induces such severe damage that *M. trossulus*’ ability to repair freezing-induced damage is extremely hindered. Evidence points to the latter, because mussels that were frozen for 8 h had significantly higher mortality. Another key finding was that HSP70 peaks in its expression at 20 h post freezing but is not significantly different at the 2 h mark. The closely related congener, *M. galloprovincialis*, similarly exhibits peak HSP70 expression 15 h following a single heat shock, indicating that it takes some time post-thermal stress for significant upregulation of HSP70 to occur in mussels (Cellura et al. 2006). Depending on the type of tide cycle, 20 h may be enough time for mussels to repair freezing damage before the next low tide exposure that may bring another freezing event. Overall, the findings of this study suggest that *M. trossulus* elevates HSP70 after repeated freezing, likely to repair protein denaturation due to freezing.

Alternatively, this observed effect could be due to HSP70 being upregulated as a way to prevent further freeze induced damage, in preparation for potential subsequent freezing exposures. Pasparakis et al. (2016) found that the intertidal limpet, *L. digitalis*, has a plastic upper thermal tolerance that is likely modulated by low tide aerial exposures experienced the day prior. This may suggest that low tide emersion periods are an important mechanism to prime stress tolerance for the next aerial exposure, perhaps by providing a cue for repair or maintenance of the cellular protein pool. The freezing events in this experiment were separated by 22 h in seawater, modelling the immersion/emersion times of a low intertidal mussel experiencing a mixed semi-diurnal tidal cycle with one aerial exposure per day. For this reason, it is a possibility that the elevated expression of HSP70 in *M. trossulus* shortly before the next freezing event serves a preparative or protective function in animals exposed to repeated freezing.

The upregulation of ubiquitin-conjugated proteins 20 h after RFT also gives support to the repair scenario, through a few possible mechanisms. The number of ubiquitin-conjugated proteins may directly reflect the number of denatured proteins within the cell (Hofmann and Somero 1995). If this is the case, we can infer that RFT induces more protein damage compared to sustained freezes, or that 8 h sustained freezes are so damaging that *M. trossulus* was not able to tag denatured proteins with ubiquitin. This response may also be a result of disrupted proteostasis within cells, causing a more complex chain of reactions due to freezing. For example, proteins that degrade ubiquitin tagged molecules, called proteases, could exhibit reduced activity due to freezing stress (Todgham et al. 2007). This would result in a build-up of ubiquitin conjugates within the cell over the thawing period, as we observed in *M. trossulus* that had been repeatedly frozen. In either of these cases, ubiquitin molecules seem to play a role in repairing mussel cells’ disrupted protein environment after repeated freeze thaw.

**Concluding remarks**

In summary, freeze–thaw cycles result in higher survival in *M. trossulus* compared to sustained freezes of the same total duration. Additionally, after repeated freeze thaw cycles, but not after sustained freezing, mussels upregulate HSP70 and ubiquitin conjugates. This likely indicates that freeze–thaw cycles, which happen naturally in the intertidal, provide mussels with a period to repair damage and are therefore crucial for *M. trossulus* survival in sub-zero temperatures. We have seen an increasing frequency of extreme weather events linked to climate change, such as the 2018/2019 extreme cold temperatures in North America caused by a weakening of the jet stream air flow (MacQuarrie et al. 2019; Overland and Wang 2016). Because winters are becoming more variable in northern regions, studying the limits and mechanisms behind freeze tolerance of intertidal invertebrates will help paint a better picture of how these animals will respond and adapt to these changes. Future studies should examine the time course of HSP70 and ubiquitinated protein.
levels throughout and after freezing, to see when elevated expression starts, peaks, and ends. Comparing these results to mussels that have been exposed to low (but non-freezing) temperatures would further differentiate between the impact of cooling versus ice formation on proteostasis. Additionally, examining other biochemical indicators such as oxidative stress proteins or cell cycle regulators under RFT may help form a more comprehensive idea of the mechanistic basis of freeze tolerance, particularly to repeated freeze–thaw. Further investigations into other freeze tolerant intertidal species would also be beneficial to understand if other intertidal species modify HSP70 and/or ubiquitinated protein levels in response to freezing in the same way as *M. trossulus*.

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**Data availability** All data is deposited on Github at https://github.com/laurentigilli/mussel-freeze-thaw.

**References**

Aarset AV (1982) Freezing tolerance in intertidal invertebrates (a review). Comp Biochem Physiol Part A Physiol 73(4):571–580. https://doi.org/10.1016/0300-9629(82)90264-X

Al-Fageeh MB, Smales CM (2006) Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. Biochem J 397(2):247–259. https://doi.org/10.1042/BJ20060166

Aleng NA, Sung YY, MacRae TH, Wahid MEA (2015) Non-lethal heat shock of the Asian Green Mussel, *Perna viridis*, promotes Hsp70 synthesis, induces thermotolerance and protects against vibrio infection. PLoS ONE 10(8):1–16. https://doi.org/10.1371/journal.pone.0135603

Baust JG (1973) Mechanisms of cryoprotection in freezing tolerant animal systems. Cryobiology 10(3):197–205. https://doi.org/10.1016/0011-2240(73)90031-X

Boroda AV, Kipryushina YO, Odintsova NA (2020) The effects of cold stress on *Mytilus* species in the natural environment. Cell Stress Chaperones. https://doi.org/10.1007/s12192-020-01109-w

Brown CL, Bale JS, Walters KFA (2004) Freezing induces a loss of freeze tolerance in an overwintering insect. Proc R Soc B Biol Sci 271(1547):1507–1511. https://doi.org/10.1098/rspb.2004.2760

Carrington E, Moeser GM, Dimond J, Mello JJ, Boller ML (2009) Seasonal disturbance to mussel beds: field test of a mechanistic model predicting wave dislodgement. Limnol Oceanogr 54(3):978–986. https://doi.org/10.4319/lo.2009.54.3.0978

Cellura C, Toubiana M, Parrinello N, Roch P (2006). HSP70 gene expression in *Mytilus galloprovincialis* hemocytes is triggered by moderate heat shock and *Vibrio anguillarum*, but not by *V. splendidus* or *Micococcus lysodeikticus*. Developal and Comparative Immunology 30(11):984–997. https://doi.org/10.1016/j.dci.2005.12.009

Clark MS, Peck LS (2009) Triggers of the HSP70 stress response: Environmental responses and laboratory manipulation in an Antarctic marine invertebrate (*Nacella concinna*). Cell Stress Chaperones 14(6):649–660. https://doi.org/10.1007/s12192-009-0117-x

Davenport J, Davenport JL (2005) Effects of shore height, wave exposure and geographical distance on thermal niche width of intertidal fauna. Mar Ecol Prog Ser 292:41–50. https://doi.org/10.3354/meps292041

Doelling ARW, Griffis N, Williams JB (2014) Repeated freezing induces oxidative stress and reduces survival in the freeze-tolerant goldenen gall fly, *Eurosta solidaginis*. J Insect Physiol 67:20–27. https://doi.org/10.1016/j.jinsphys.2014.05.024

Dong Y, Liao M, Han G, Somero GN (2021) An integrated, multi-level analysis of thermal effects on intertidal molluscs for understanding species distribution patterns. Biol Rev. https://doi.org/10.1111/brv.12811

Dong Y, Miller LP, Sanders JG, Somero GN (2008) Heat-shock protein 70 (Hsp70) expression in four limpets of the genus *Lottia*: interspecific variation in constitutive and inducible synthesis correlates with in situ exposure to heat stress. Biol Bull 215(2):173–181. https://doi.org/10.2307/25470698

Duman JG (2001) Antifreeze and ice nucleator proteins in terrestrial arthropods. Annu Rev Physiol 63:327. https://doi.org/10.1146/annurev.physiol.63.1.327

Finke GR, Navarrete SA, Bozinovic F (2007) Tidal regimes of temperate coasts and their influences on aerial exposure for intertidal organisms. Mar Ecol Prog Ser 343:57–62. https://doi.org/10.3354/meps06918

Halpin PM, Sorte Cj, Hofmann GE, Menge BA (2002) Patterns of variation in levels of Hsp70 in natural rocky shore populations from microscale to mesoscales. Integr Comp Biol 42(4):815–824. https://doi.org/10.1111/jcb.12815

Helmut B, Hofmann GE (2001) Microhabitats, thermal heterogeneity, and patterns of physiological stress in the rocky intertidal zone. Biol Bull 201(3):374–384. https://doi.org/10.2307/1543615

Hochstrasser M (1995) Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr Opin Cell Biol 7(2):215–223. https://doi.org/10.1016/0955-0674(95)90031-X

Hofmann S (1995) Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel *Mytilus trossulus*. J Exp Biol 198(Pt 7):1509–1518

Jensen MM, Denny MW (2016) Life in an extreme environment: characterizing wave-imposed forces in the rocky intertidal zone using high temporal resolution hydrodynamic measurements. Limnol Oceanogr 61(5):1750–1761. https://doi.org/10.1002/lno.10327

Kanwisher JW (1955) Freezing in intertidal animals. Biol Bull 109(1):56–63. https://doi.org/10.2307/1538658

Kelley AL, de Rivera CE, Buckley BA (2013) Cold tolerance of the invasive Carcinus maenas in the east Pacific: molecular mechanisms and implications for range expansion in a changing climate. Biol Invasions 15(10):2299–2309. https://doi.org/10.1007/s10530-013-0454-7

Kennedy J, Harley CDG, Marshall KE (2020) Drivers of plasticity in freeze tolerance in the intertidal mussel *Mytilus trossulus*. J Exp Biol. https://doi.org/10.1242/jeb.233478

Lee RE (2010) A primer on insect cold-tolerance. In: DL Denlinger, RE Lee (eds) Low temperature biology of insects. Cambridge University Press, Cambridge, pp 3–34. https://doi.org/10.1017/CBO9780511675997.002
Loomis SH, Carpenter JF, Crowe JH (1988) Identification of strombine and taurine as cryoprotectants in the intertidal bivalve *Mytilus edulis*. BBA Biomembranes 943(2):113–118. https://doi.org/10.1016/0005-2736(88)90542-1

Mahmood T, Yang P-C (2012) Western blot: technique, theory, and trouble shooting. N Am J Med Sci 4(9):429–434. https://doi.org/10.4103/1947-2714.100998

Mann HB, Whitney DR (1947) On a test of whether one of two random variables is stochastically larger than the other. Ann Math Stat 18:50–60

MacQuarrie CJK, Cooke BJ, Saint-Amant R (2019) The predicted effect of the polar vortex of 2019 on winter survival of emerald ash borer and mountain pine beetle. Can J for Res 49(9):1165–1172. https://doi.org/10.1139/cjfr-2019-0115

Marshall KE, Anderson KM, Brown NEM, Dytnerski JK, Flynn KL, Marshall KE, Sinclair BJ (2018) Repeated freezing induces a trade-off between cryoprotection and egg production in the goldenrod gall fly, *Eurosta solidaginis*. J Exp Biol. https://doi.org/10.1242/ejb.177956

Mazur P (2010) A biologist’s view of the relevance of thermodynamics and physical chemistry to cryobiology. Cryobiology 60(1):1–4. https://doi.org/10.1016/j.cryobiol.2009.12.001

Miller LP, Harley CDG, Denny MW (2009) The role of temperature and desiccation stress in limiting the local-scale distribution of the owl limpet, *Lottia gigantea*. Funct Ecol 23(4):756–767

Overland JE, Wang M (2016) Recent extreme arctic temperatures are due to a split polar vortex. J Clim 29(15):5600–5616. https://doi.org/10.1175/JCLI-D-16-0320.1

Pasparakis C, Davis BE, Todgham AE (2016) Role of sequential low-temperature periods on the thermal physiology of summer and winter laboratory-acclimated fingered limpets. *Lottia Digitalis*. Mar Biol 163:23. https://doi.org/10.1007/s00227-015-2779-5

Privalov PL (1990) Cold denaturation of protein. Crit Rev Biochem Mol Biol 25(4):281–306. https://doi.org/10.3109/1049239009090612

Pulgar J, Waldispeger M, Galbán-Malagón C, Maturana D, Pulgar VM, Aldana M (2017) UV radiation impacts body weight, oxygen consumption, and shelter selection in the intertidal vertebrate *Girella laevisirius*. Sci Total Environ 578:317–322. https://doi.org/10.1016/j.scitotenv.2016.10.157

R Development Core Team (2011) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Ramløv H (2000) Aspects of natural cold tolerance in ectothermic animals. Hum Reprod 15(SUPPL. 5):26–46. https://doi.org/10.1093/humrep/15.suppl_5.26

Richardson C, Seed R, Naylor E (1990) Use of internal growth bands for measuring individual and population growth rates in *Mytilus edulis* from offshore production platforms. Mar Ecol Prog Ser 66:259–265. https://doi.org/10.3354/meps060259

Royston P (1982) An extension of Shapiro and Wilk’s W test for normality to large samples. Appl Stat 31:115–124. https://doi.org/10.2307/2347973

Sagarin RD, Somero GN (2006) Complex patterns of expression of heat-shock protein 70 across the southern biogeographical ranges of the intertidal mussel *Mytilus californianus* and snail *Nucella ostrina*. J Biogeogr 33(4):622–630. https://doi.org/10.1111/j.1365-2699.2005.01403.x

Sinclair BJ, Renault D (2010) Intracellular ice formation in insects: unresolved after 50 years? Comp Biochem Physiol Part A Mol Integr Physiol 155(1):14–18. https://doi.org/10.1016/j.cbpa.2009.10.026

Štětina T, Koštál V, Korbelová J (2015) The role of inducible Hsp70, and other heat shock proteins, in adaptive complex of cold tolerance of the fruit fly (*Drosophila melanogaster*). PLoS ONE. https://doi.org/10.1371/journal.pone.0128976

Storey KB, Storey JM (1988) Freeze tolerance in animals. Physiol Rev 68(1):27–84

Storey KB, Storey JM (1996) Natural freezing survival in animals. Ann Rev Ecol Syst 27:365–386

Storey KB, Storey JM (2013) Molecular biology of freezing tolerance. Compr Physiol 3:1283–1308. https://doi.org/10.1002/cphy.c130007

Teets NM, Kawarasaki Y, Lee RE, Denlinger DL (2011) Erratum: survival and energetic costs of repeated cold exposure in the Antarctic midge, *Beliogga antarctica*: a comparison between frozen and supercooled larvae. J Exp Biol 214:806–814. https://doi.org/10.1242/jeb.060624

Todgham AE, Hoaglund EA, Hofmann GE (2007) Is cold the new hot? Elevated ubiquitin-conjugated protein levels in tissues of Antarctic fish as evidence for cold-denaturation of proteins in vivo. J Comp Physiol [b] 177(8):857–866. https://doi.org/10.1007/s00360-007-0183-2

Tomaneck L, Sanford E (2003) Heat-shock protein 70 (Hsp70) as a biochemical stress indicator: an experimental field test in two congeneric intertidal gastropods (genus: *Tegula*). Biol Bull 205(3):276–284. https://doi.org/10.2307/1543291

Toxopeus J, Koštál V, Sinclair BJ (2019) Evidence for non-colligative function of small cryoprotectants in a freeze-tolerant insect. Proc R Soc B 286(1899):20190050. https://doi.org/10.1098/rspb.2019.0050

Toxopeus J, Sinclair BJ (2018) Mechanisms underlying insect freeze tolerance. Biol Rev 93(4):1891–1914. https://doi.org/10.1111/brv.12425

Toyohara H, Hosoi M, Hayashi I, Kubota S, Hashimoto H, Yokoyama Y (2005) Expression of HSP70 in response to heat-shock and its DNA cloning from Mediterranean blue mussel. Fish Sci 71:327–332

Tukey JW (1977) Exploratory data analysis, vol 2, pp 131–160

Venetianer A, Pirity M, Hever-Szabo A (1994) The function of heat-shock proteins in stress tolerance. Cell Biol Int 18(6):605–616. https://doi.org/10.1007/bf00680870

Wickham H (2016) ggplot2: elegant graphics for data analysis. Springer, New York. ISBN 978–3–319–24277–4. https://ggplot2.tidyverse.org

Wickham H (2011) The split-apply-combine strategy for data analysis. J Stat Softw 40(1):1–29. http://www.jstatsoft.org/v40/i01/

Wilke C (2020) cowplot: Streamlined plot theme and plot annotations for ‘ggplot2’. R package version 1.1.1. https://CRAN.R-project.org/package=cowplot

Williams EE, Somero GN (1996) Seasonal-, tidal-cycle- and microhabitat-related variation in membrane order of phospholipid vesicles from gills of the intertidal mussel *Mytilus californianus*. J Exp Biol 199(7):1587–1596

Zhang G, Storey JM, Storey KB (2018) Elevated chaperone proteins are a feature of winter freeze avoidance by larvae of the goldenrod
gall moth, *Epiblema scudderiana*. J Insect Physiol 106:106–113. 
https://doi.org/10.1016/j.jinsphys.2017.04.007

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