The influence of stochastic temperature fluctuations in shaping the physiological performance of the California mussel, *Mytilus californianus*

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SUMMARY STATEMENT

Mussels acclimated to an unpredictable thermal regime produce different physiological performance in terms of energy stores, metabolic capacity, and thermal performance in comparison to mussels acclimated to a predictable thermal regime.

ABSTRACT

Climate change is forecasted to increase temperature variability and stochasticity. Most of our understanding of thermal physiology of intertidal organisms has come from laboratory experiments that acclimate organisms to submerged conditions and steady-state increases in temperatures. For organisms experiencing the ebb and flow of tides with unpredictable low tide aerial temperatures, the reliability of reported tolerances and thus predicted responses to climate change requires incorporation of environmental complexity into empirical studies. Using the mussel *Mytilus californianus,* our study examined how stochasticity of the thermal regime influences physiological performance. Mussels were acclimated to either submerged conditions or a tidal cycle that included either predictable, unpredictable or no thermal stress during daytime low tide. Physiological performance was measured through anaerobic metabolism, energy stores and cellular stress mechanisms just before low tide, and cardiac responses during a thermal ramp.

Both air exposure and stochasticity of temperature change were important in determining thermal performance. Glycogen content was highest in the mussels from the unpredictable treatment, but there was no difference in the expression of heat shock proteins between thermal treatments, suggesting that mussels prioritise energy reserves to deal with unpredictable low tide conditions. Mussels exposed to fluctuating thermal regimes had lower gill anaerobic metabolism, which could reflect increased metabolic capacity. Our results suggest that while thermal magnitude plays an important role in shaping physiological performance, other key elements of the intertidal environment complexity such as stochasticity, thermal variability, and thermal history are also important considerations for determining how species will respond to climate warming.

Keywords: Environmental predictability, Unpredictable, Climate change, Cardiac performance, Intertidal physiology, Bioenergetics.

INTRODUCTION

A central question of environmental biology is how organisms integrate environmental signals mechanistically via physiological systems and how this integration leads to variation in species performance (Doak and Morris, 2010; Helmuth et al., 2006; Somero, 2010). Due to the fundamental role of temperature on biological patterns and processes, understanding the capacity of an organism to tolerate climate change induced variations in temperature has been a central focus in ecophysiology (Burggren, 2019; Kroeker et al., 2016; Sheldon, 2019). Climate change is predicted to increase the annual mean global temperature, but will also increase the frequency and magnitude of short-term (weather) and long-term (climate) extreme events (Burggren, 2018; 2019; Stillman, 2019). A central prediction of recent climate change models is that there will be an increase in temperature variation and stochasticity, which will increase the probability of extreme warm temperatures (Angélil et al., 2017; Guo et al., 2018; IPCC, 2021; Stillman, 2019) and heat wave events (Angélil et al., 2017; IPCC, 2021). The majority of research to date investigating the link between climate change and organismal performance has focused on mean environmental conditions over time, yet temperature variability and extreme events can substantially impact organismal physiology and ultimately survival (Baldwin et al., 2019; Buckley and Kingsolver, 2012; Drake et al., 2017). This is particularly true with regard to studies based on the rocky intertidal ecosystem, where experiments have typically focused on steady

state or predictable increases in temperature when organisms are submerged in water, rather than stochastic variations in temperature during air exposure (low tide), which is more representative of the natural environment resulting from the ebb and flow of the tide.

The importance of environmental complexity in ecophysiological research has led to an increase in studies incorporating temperature change during a tidal cycle framework (Drake et al., 2017; Han et al., 2013; Jimenez et al., 2016; Mangan et al., 2019; Marshall and McQuaid, 1992; Marshall et al., 2011; McMahon et al., 1991; Paganini et al., 2014; Widdows and Shick, 1985). Recent work in particular has started to explore different aspects of thermal complexity, such as the medium in which thermal stress occurs (air or water). The results of such studies have shown that intertidal organisms have higher upper thermal tolerance limits when subjected to thermal stress in air in comparison to in water (Bjelde and Todgham., 2013; Bjelde et al., 2015; Drake et al., 2017) and that periodic air exposure can result in physiological adjustments that increase thermal tolerance (Drake et al., 2017; Roberts et al., 1997; Stillman and Somero, 2000). Moreover, changes in water level is perhaps the most predictable environmental fluctuation in the intertidal zone, and therefore it is thought that air exposure may act as the cue that primes intertidal organisms for anticipated periods of stress during low tide (Bjelde and Todgham, 2013; Drake et al., 2017). Similarly, it is becoming more apparent that thermal fluctuation, rather than constant temperatures, provides crucial complexity that is important in structuring stress tolerance. Exposure to predictable fluctuating thermal environments can increase thermal tolerance (da Silva et al., 2019; Drake et al., 2017; Feldmeth et al., 1974; Kern et al., 2015; Marshall et al., 2021; Oliver and Palumbi, 2011; Otto and Rice, 1974; Schaefer and Ryan, 2006; Threader and Houston, 1983; Vafeiadou et al., 2018), and intertidal species exposed to thermal fluctuations can be more tolerant to additional stressors than those that are exposed to constant temperatures (Collins et al., 2020; Drake et al., 2017; Giomi et al., 2016; Podrabsky and Somero, 2004; Stillman and Somero, 1996; Todgham et al., 2006; Tomanek and Sanford, 2003). While incorporating predictable temperature fluctuation into experimental design is more representative of what occurs naturally in the intertidal, temperature variation typically occurs stochastically (Burggren, 2019; Dillon et al., 2016) and thus, predictable cycles often do not accurately capture the thermal environment *in situ*, and can risk ‘overpredicting’ the degree of responses if the temperatures used are unrealistic. Therefore, studies with predictable temperature change may not capture the full repertoire of an organism’s natural physiological

responses associated with unpredictable temperature fluctuations and extreme weather events produced by climate change (Burggren, 2019). Our understanding of how thermal stochasticity influences the physiological performance of intertidal organisms is extremely limited.

Extensive investigation of the mechanisms underlying thermal tolerance have revealed the importance of cellular defence mechanisms and energy metabolism in enhancing performance and maintaining homeostasis during daytime low tide (Sokolova et al., 2012). Tolerance to thermal stress is enabled by employing cellular stress mechanisms such as heat shock proteins (Hsps), and the magnitude and induction temperature of the heat shock response has advanced our understanding of how the environmental signal is integrated to modulate thermal tolerance in intertidal organisms (Buckley et al., 2001; Dong et al., 2008; Han et al., 2013; Hofmann and Somero, 1995; Madiera et al., 2015; Rhee et al., 2009; Roberts et al., 1997; Sagarin and Somero, 2006; Tomanek and Somero, 1999; Tomanek and Sanford, 2003; Wang et al., 2020). Previous studies in limpets, gastropods and corals that have shown evidence of 'preparative defence' (Dong et al., 2008) or cellular 'frontloading' (Bashirs et al., 2013), where species prepare for a period of anticipated stress by upregulating Hsp70 or Hsc70 (Dong et al., 2008) in advance. Stress response mechanisms such as Hsps are energetically expensive, and require sufficient energy available to mount a comprehensive defence (Ivanina et al., 2008; Sokolova et al., 2012). For sessile organisms such as mussels, air exposure during low tide can result in valve closure to reduce desiccation during low tide (e.g. Bayne et al., 1976). Valve closure can often lead to hypoxic conditions resulting in a greater reliance on anaerobic metabolism (Demers and Guderley, 1994), which can be exacerbated by concurrent heat stress (Dowd and Somero, 2013). The comparable inefficiency of anaerobic pathways in producing energy in comparison to aerobic pathways results in mussels relying on strategies such as metabolic depression (Anestis et al., 2010), increased enzyme activity in anaerobic pathways (Collins et al., 2020) and sufficient energy stores to ensure enough energy is available to mount a sufficient response to low tide periods (Sokolova et al., 2012; Widdows and Shick, 1985). While we have a good broad understanding of the importance of energy metabolism and cellular defence mechanisms in tolerating thermal stress, we currently know very little about how the specific elements of the thermal signal influence these mechanisms to shape thermal performance of intertidal organisms.

The primary objective of this study was to investigate how varying levels of thermal complexity (air exposure, magnitude of fluctuation, and predictability) modulated preparatory mechanisms for energy metabolism and cellular stress defense mechanisms and how this shaped performance during thermal stress in the California mussel *Mytilus californianus.* The California mussel is predominantly distributed along the coast of California. In Northern California, Winter/Spring months can experience unpredictable warm days for mussels due to sun exposure creating high internal temperatures in comparison to the surrounding air temperature. We examined how acclimation to either submerged conditions or a tidal cycle that included either unpredictable, predictable, or no thermal stress during daytime low tide influenced growth rates, malate dehydrogenase (MDH) activity, glycogen content, succinate content and Hsp/Hsc70 levels just before low tide (i.e. just before being air exposed in tidal treatments), and cardiac performance during an acute thermal ramp during low tide. Performance was also compared to wild (termed "field" here) mussels to better understand how complexity modulated in the lab compared to mussels from the field to identify key underlying drivers influencing thermal performance. We hypothesized that mussels acclimated to an unpredictable thermal regime would need to be prepared for unexpected, but potentially high levels of thermal stress and thus would rely on greater energy expenditure and higher basal levels of cellular defense mechanisms to tolerate stressful low tide periods. We predicted that mussels acclimated to the unpredictable regime would exhibit elevated glycogen, succinate and Hsp/Hsc70 levels and higher MDH activity and lower growth rates compared to mussels acclimated to predictable or no thermal stress. The preparatory strategy utilized by mussels in the unpredictable regime was predicted to lead to elevated upper thermal tolerance during an acute stress event during daytime low tide. With the predicted increases in thermal unpredictability forecasted by climate change models, understanding how thermal predictability shapes performance during acute stress and assessing the underlying mechanisms controlling energy dynamics and stress tolerance will be paramount for predicting how climate change will affect intertidal organisms.

MATERIAL AND METHODS

Mussel collection

Mytilus californianus (Conrad, 1837) were collected during low tide from the mid-upper intertidal zone at Shell Beach, CA, USA (38°25'17" N, 123°06'47" W) in March 2019. Mussels (length range 47.5-52.5 mm) were then transported to the University of California Davis Bodega Marine Laboratory in Bodega Bay, CA, USA, cleaned of epibionts and placed in a flow through tank at 13°C, 33.5 ‰ salinity and 100% air saturation. Collection and transport lasted no longer than 2 hours.

Acclimation conditions

Experimental tanks and treatment design were modelled off the methods outlined in Drake et al. (2017), with some modifications. Tanks were built to simulate natural intertidal conditions by replicating circatidal changes in water height and temperature. Tanks were flow through and continuously flushed with fresh seawater during high and low tides. Temperature and water height were manipulated using Arduino microcontrollers (Arduino YUN, Adafruit, New York, NY, USA; Miller and Long, 2015, Drake et al., 2017). For mussels, the dominant driver controlling body temperature is solar radiation (Helmuth, 1998; Helmuth et al., 2016), therefore, heat lamps with 150 W ceramic bulbs were used to modulate mussel body temperature during daytime low tide periods. The Arduino microcontroller manipulated mussel body temperature through a feedback system between a temperature sensor encased in a mussel shell with silicone (similar design as "Robomussels" [Fitzhenry et al., 2004]) and the heat lamp. Temperature of the heat lamp (and mussel body temperature) was regulated and ramped at specific rates depending on the acclimation treatment. As orientation to the sun can also have a large impact of the warming rate and ultimate body temperature (Harley, 2008; Miller and Dowd, 2017), mussels were individually housed in small mesh baskets to allow similar orientation to the heat lamp and promote a uniform heating rate among individuals in each tank. Mesh baskets were attached to a plastic grate platform (height = 5.5 inches), which enabled mussels to either be immersed or emersed depending on changes in water height.

Mussels were weighed, measured, labelled, and randomly divided between one of four different acclimation treatments (unpredictable, predictable, air, submerged) that incorporated varying levels of natural predictability and were held under these conditions for 2 weeks.

Temperature profiles for treatments were based off daytime low tide data from 'Robomussel' temperature loggers (Maxim Integrated Products, Dallas, TX, USA) embedded on the rock next to *M. californianus* in Bodega Marine Reserve that continuously monitored temperature every 10 minutes from 11 January 2019 to 1 March 2019. California experiences mixed semidiurnal tides where successive daytime low tides can often be progressively later in the morning, resulting in subsequent days experiencing potentially higher low tide temperatures during a period of warm weather; and unpredictable warm days can occur more frequently in this season due to the difference in sun exposure and air temperature.

The unpredictable treatment was a circatidal regime (6 h emersed, 6 h immersed) with ambient seawater conditions (~13°C) and varying aerial temperatures within the range of (13-28°C; Fig. 1) during daytime low tide. This temperature profile mirrored a 2-week period of natural cycles in environmental temperature within the logger data, which was equal to the average temperature of daytime low tide during the 7-week logger period (20°C) and included the maximum temperature recorded (28°C). The predictable treatment was a circatidal regime with ambient seawater conditions (~13°C) with a consistent maximum aerial temperature of 20°C every daytime low tide. The predictable treatment was designed to subject mussels to the same degree of heating throughout the 2 weeks as the unpredictable treatment, but in a predictable manner. To understand the role of periodic air exposure on physiological performance, the air treatment was a circatidal regime with ambient seawater conditions (~13°C) and no heating during daytime low tide (13°C). The submerged treatment acted as a control and had no tidal regime, mussels were submerged in ambient seawater (~13°C) and experienced no aerial exposure. For all treatments that experienced a tidal cycle (unpredictable, predictable, air), no heating occurred during night-time low tide (i.e. constant 13°C aerial exposure).

In order to understand which components of the acclimation treatments were key drivers for *in situ* performance, physiological responses of mussels from the four acclimation treatments were also compared to wild mussels (hereafter termed "field treatment"). To avoid the effects of collection and handling stress on the performance of field mussels, mussels of the same size range were collected from the same sampling location (Shell Beach) during daytime low tide, cleaned of epibionts and placed in tanks under a circatidal regime that coincided with the tidal

cycle of Shell Beach. Mussels were held under these conditions for 18 hours before being sampled for the physiological parameters described below.

Each of treatments had two replicate tanks (10 tanks in total) and were conducted simultaneously. During acclimation trials, temperature, salinity, and dissolved oxygen were measured every day, and nitrate, nitrite and ammonia were checked twice a week to ensure acceptable conditions for mussels. Temperature, salinity, and dissolved oxygen were measured using a YSI Model 85 m (YSI Incorporated, Yellow Springs, OH, USA) while API saltwater test kit (API, Chalfont, PA, USA). Mussels were fed live *Nannochrolopsis* sp. every daytime high tide at an average density of 150,000 cells ml^{-1} $tank^{-1}$, which correlated to approximately 7500 cells ml^{-1} $mussel^{-1}$. Mortality was low in all tanks ($\leq$ 5%) and if occurred, food was adjusted to maintain consistent per mussel density across tanks.

Heart rate

Difference in upper thermal tolerance limits were estimated by examining upper critical thermal limits of cardiac performance. Heart rate was monitored for each individual using a non-invasive technique introduced by Depledge et al. (1996) and modified by Burnett et al. (2013). A sensor consisting of an infrared emitter and phototransistor, was permanently glued next to the mid-dorsal posterior hinge area that corresponds to the position of the heart. Similarly to Tagliarolo and McQuaid (2015), preliminary tests showed that the heart rate signals stabilised 10 to 15 minutes after handling. Therefore, mussels were left undisturbed for 15 minutes after attachment of the sensor to recover before the start of recording. The signal from the sensor was amplified using AMP-03 (Newshift LDA, Leiria, Portugal), digitised using a data acquisition system (PowerLab 16/35, ADInstruments, Colorado Springs, CO, USA) and recorded with the associated software (LabChart 8.0, ADInstruments). A temperature probe (Type T thermocouple) inserted into a 'robomussel' was also attached to the data acquisition system via a thermocouple meter (TC-2000, Sable Systems International, Las Vegas, NV, USA) to give a live temperature feed during the thermal ramp that was also recorded through the LabChart software. Fifteen mussels from each of the five treatments were exposed to ramped increases in temperature in air starting at 13°C (ambient ocean temperature) at rate of 6.5°C h^{-1}. The heat ramp was timed to so that it would occur at the start of the scheduled daytime low tide period

during acclimation. Temperature was ramped using the Arduino microcontroller - heat lamp system described previously.

Cardiac performance analysis

Cardiac performance was analysed following methods previously described for limpets (Bjelde and Todgham, 2013; Drake et al., 2017). Several measures of performance were used to determine overall cardiac performance. Firstly, final break point temperature (BPT) was measured, which is defined as the highest temperature reached before a steep decline in heart rate and is considered to be the upper critical thermal limit of intertidal organisms (Stillman and Somero, 1996), including mussels (Tagliarolo and McQuaid, 2015). Final BPT was calculated as described elsewhere (Bjelde and Todgham, 2013, Drake et al., 2017). Briefly, individual mussel heart rate (beats min^{-1}) were plotted against temperature and the intersection point (BPT) was determined by two best-fit regression lines for the ascending and descending portion of the heart rate. Secondly, flat line temperature (FLT) was determined by manually observing the temperature of the last heartbeat on LabChart recordings. The difference (FLT-BPT) was also calculated to determine the temperature range of suboptimal performance (Drake et al., 2017). Maximum heart rate for each individual mussel was determined as a measure of cardiac capacity and defined as the highest heart rate recorded during the heat ramp. Lastly, temperature sensitivity of heart rate was examined using thermal performance curves.

Shell growth

Increases in shell length was used as a proxy for growth. Shell length (measurement of umbo to shell edge) of each individual was measured just before mussels were placed in the acclimation tanks and after the 2-week acclimation period. Mussels were labelled to ensure correct tracking of each individual and measurements were made using a caliper (precision 0.1 mm). As mussels from the field treatment were only held overnight to recover from collection, changes in shell length were not measured for this treatment.

Mussel tissue sampling

We predicted that mussels in the unpredictable treatment would have preparatory mechanisms in place to tolerate unpredictable, but potentially high levels of thermal stress. To test this prediction, tissue samples (gill and mantle) were dissected from mussels (n = 9) from each treatment immediately prior to cardiac performance trials (therefore immediately prior to day-time low tide). Physiological condition was measured through biochemical analyses by assessing cellular stress mechanisms using gill tissue (Hsp/Hsc70 protein), energy stores using mantle tissue (glycogen content) and anaerobic capacity using both gill and mantle tissue (malate dehydrogenase (MDH) activity and succinate content). Mussels were removed from treatment tanks and gill and mantle tissue were dissected quickly (i.e. under 45s). Tissue samples were immediately frozen on dry ice and stored at -80°C until analysis.

Sample preparation for Hsp/Hsc70 and total protein

Frozen gill samples (~100 mg) were used for total protein and Hsp/Hsc70 quantification. Tissue preparation, subsequent sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were completed according to the methods of Todgham et al., (2005) with some modifications. Samples were homogenized on ice in a 1:2 solution of homogenisation buffer (100 mM Tris-HCl, pH 7.5; 0.1% SDS [w/v], 0.5 M EDTA) containing a combination of protease inhibitors: 0.7 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 1 µg/ml aprotinin, 20 µg/ml phenylmethylsulfonyl fluoride (Sigma, St Louis, MO, USA). Homogenates were then centrifuged at 13,000 ***g*** for 10 minutes. Supernatants were transferred to a new microcentrifuge tube with an equal volume of 2 x Laemmli's sample buffer (0.5 M Tris-HCl, pH 6.8; 20% glycerol [v/v], 4% SDS [w/v], 10% ß-mercaptoethanol [v/v], 0.25% bromophenol blue) for SDS-PAGE. Samples were then heated for 3 minutes at 100°C and stored at -20°C before electrophoresis. The remaining supernatant was transferred to a new tube and stored at -20°C until analysed for total protein concentration using the bicinchoninic method (Smith et al., 1985) (Thermo Fisher Scientific, Waltham, MA, USA).

SDS-PAGE and Western blot analysis for Hsp/Hsc70

Levels of Hsp/Hsc70 were measured using the discontinuous SDS-PAGE method of Laemmli (1970). Equal amounts of total protein (10 µg) were resolved with a 4% stacking and 10% resolving gel on a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA). One lane in each gel was loaded with a prestained molecular marker (PageRuler, Thermo Fisher Scientific) and a second lane was loaded with an internal standard to calibrate protein expression within and among gels. The internal standard was a gill tissue sample from the submerged group that had shown positive expression of Hsp/Hsc70. Proteins were separated by SDS-PAGE at 75 V for 15 minutes followed by 150 V for 1 hour. After separation of proteins by SDS-PAGE, gels were trimmed at the 35kDa protein band of the ladder lane and transferred onto nitrocellulose (Bio-Rad, 0.2 µm pore size) via semi-dry transfer apparatus (Bio-Rad Trans-Blot) at 15 V for 20 minutes with transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS [w/v], 20% methanol [v/v], pH 9.3). Transfer membranes were then blocked in 2% bovine serum albumin (BSA) in Tween-20 Tris-buffered saline (TTBS; 20 mM Tris-HCl, 0.14 M NaCl, 0.1% Tween-20 [v/v]) for 1 hour. Membranes were then rinsed once and soaked in TTBS for 5 minutes. Membranes were then soaked in primary antibody (Mouse IgG Hsp70; MA3-007; Thermo Fisher Scientific) at a 1:1000 dilution with 2% BSA in TTBS. Membranes were then washed 3 times for 5 minutes each in TTBS and then soaked in horseradish peroxidase-conjugated goat antimouse IgG secondary antibody (1706516, Thermo Fisher Scientific) diluted 1:5000 in 2% BSA in TTBS for one hour. Membranes were then washed 3 times in TTBS for 5 minutes each, followed by one wash in Tris-buffered saline to remove Tween-20. Membranes were then developed with chemiluminescent SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) for 5 minutes. Imaging was performed immediately on wet membranes placed directly on the image screen of a ChemiDoc XRS imager (Bio-Rad) and analysis for determination of relative Hsp/Hsc70 protein quantity was quantified using Image J software (Rasband, 2013). Hsp/Hsc70 protein levels are presented relative to the band intensity of the internal standard.

Glycogen content

Glycogen content was measured as described by Bjelde and Todgham (2013), modified from Fangue et al. (2008). Tissue was ground into a fine powder using liquid nitrogen and an insulated mortar and pestle. Glycogen was extracted by adding 1 ml of ice cold 8% $HClO_4$ to ~20 mg of powdered tissue, which was then homogenized on ice for ~20 seconds with a Pro200 Bio-Gen Series homogenizer (PROScientific, Oxford, CT, USA). A sample of homogenate (200 µl) was placed in a microcentrifuge tube and frozen at -80°C for later glycogen quantification. The remaining homogenate was centrifuged at 10,000 ***g*** for 10 minutes at 4°C and the supernatant was extracted and neutralized with 3 mol l^{-1} K_2CO_3. The neutralized solution was centrifuged at 10,000 g for 10 min at 4°C and frozen at -80°C for later free glucose assays. Samples for glycogen determination were enzymatically digested following previous methods (Hassid and Abraham, 1957), and all samples were analysed for glucose following a method (Bergmeyer, 1983) modified for a microplate spectrophotometer (Synergy HT, Biotek, Winooski, VT, USA). Glycogen content was then corrected for starting free glucose.

Malate dehydrogenase (MDH) activity

Malate dehydrogenase is an important enzyme involved in several different functions within the citric acid cycle and plays a key role in the aerobic pathway of ATP generation in bivalves (Hochachka and Somero, 2002; Logan et al., 2012). MDH activity was measured in gill and mantle tissue as described by Logan et al. (2012) with some modifications. Tissues were homogenized in ice cold 50 mM potassium phosphate buffer (pH 6.8 at 20°C) at a ratio of 1:10 for ~20 seconds with a Pro200 Bio-Gen Series homogenizer (PROScientific). Tissue homogenates were then centrifuged at 17,500 ***g*** for 30 minutes at 4°C. The supernatant was transferred into fresh tubes and spun for an additional 5 minutes. Aliquots of the resulting supernatant were made, with 100 µl stored at -20°C for total protein analysis and the remaining supernatant stored at -80°C for MDH activity. The activity of MDH was determined by monitoring the conversion of NADH to NAD+ spectrophotometrically (Synergy HT, Biotek) at 340 nm at 25°C. MDH was assayed in 200 mM imidazole buffer (pH 7.0 at 20°C), 200 µM oxaloacetate and 150 µM NADH in triplicate. Total protein was quantified as described previously and MDH activity is expressed per mg protein.

Succinate content

Succinate has been found to be the most significant end-product of anaerobiosis in mussels (Connor and Gracey, 2012; Tagliarolo and McQuaid, 2015). Tissue was weighed and deproteinized with ice-cold 8% $HClO_4$ at a ratio of 1:4, and was then and homogenized for 1 minute using a Pro200 Bio-Gen Series homogenizer (PROScientific). The homogenate was then centrifuged at 13,000 ***g*** for 10 minutes at 4°C. The supernatant was extracted and neutralized with 1 M KOH and kept on ice for 20 minutes and then centrifuged again at 13,000 ***g*** for 10 minutes at 4°C. The extract was stored at -80°C before analysis. Succinic acid content was assayed using a commercial kit according to the manufacturer's instructions (Megazyme, International Ireland). The succinic acid assay procedure applies the method of succinyl-CoA synthetase, pyruvate kinase and lactate dehydrogenase, according to Beutler (1989).

Statistical analysis

All data sets were analysed using R (R Development Core Team, 2008) and assessed for homogeneity and normality using residual and q-q plots, and tested with Shapiro Wilks and Levene's test. BPT, FLT, MDH activity, glycogen and succinic acid content met assumptions and were analysed with a one-way ANOVA with treatment as the main effect. Differences between acclimation treatments were differentiated using a Tukey's HSD. Shell length data did not meet the normality assumption and were analysed with a non-parametric Kruskal-Wallis test, followed by a Dunn's Multiple Comparison test with Bonferroni adjustment to differentiate differences between acclimation groups. Hsp/Hsc70 data did not meet normality or homogeneity of variance assumptions due to no detectable expression of Hsp70/Hsc70 in the predictable and unpredictable treatments (expressed as 0). As such, Hsp/Hsc70 data was analysed with a Tobit model with left censoring using a log normal distribution in the survival package (Therneau and Lumley, 2015) within R. Pairwise comparisons with multiple-comparison correction (Tukey method) were then used to differentiate any differences between acclimation groups using the emmeans package (Length et al., 2019). To assess differences in thermal sensitivity of heart rates between acclimation treatments, a generalized additive mixed model (GAMM) was used based off Zurr et al., 2009 and Angilletta et al., (2013) incorporating modifications from Drake et al., (2017). To account for repeated measures, the identity of each mussel was included as a random

factor. Analyses were performed with the mgcv (Wood, 2004) and nlme (Pinheiro et al., 2013) libraries in R.

RESULTS

Cardiac performance during thermal ramp

Acclimation treatment affected upper critical temperature of heart function, measured at final break point temperature (BPT) during ramping increases in temperature (one-way ANOVA, $F_{4,53}$= 4.849, P= 0.002; Fig. 2). Mussels in the submerged treatment had the lowest final BPT of 34.70 ± 0.61°C, which was statistically similar to the air and predictable treatments, but lower than the unpredictable and field treatments. The highest final BPT was observed in mussels in the unpredictable treatment (40.47 ± 1.08°C). There was no significant difference between air, predictable, unpredictable and field treatments.

Similarly, acclimation treatment had a significant effect on the thermal limits of heart function, measured as flat line temperature, FLT (one-way ANOVA, $F_{4,53}$= 4.059, P= 0.006; Fig. 3). Mussels in the submerged treatment had the lowest FLT at 41.04 ± 0.66°C, which was statistically lower than all other treatments. Mussels acclimated to unpredictable treatment had the highest FLT 46.91 ± 0.99°C, but this was statistically similar to air, predictable and field treatments.

There was no difference in the temperature range between final BPT and FLT (FLT-BPT), suggesting similar suboptimal performance between treatments following final BPT (one-way ANOVA, $F_{4,53}$=0.586, P=0.674; Table 1). Maximum heart rate was different across acclimation treatments (one-way ANOVA, $F_{4,53}$=4.872, P=0.002; Table 1). Mussels from the unpredictable treatment had the highest heart rate (24.06 ± 1.79 bpm) and this was statistically similar to mussels from field and air treatments. Submerged mussels had the lowest maximum heart rate (16.87 ± 1.51 bpm), which was statistically similar to predictable and air treatments.

The relationship between temperature and heart rate displayed slightly different patterns of temperature sensitivity of heart rate, depending on treatment. All acclimation treatments exhibited a non-linear response to warming, initially appearing relatively temperature insensitive and then exhibiting a steady increase in heart rate with increasing temperature until the ultimate decline in function (Fig. 4). In contrast, mussels from the field treatment appeared to display a more temperature dependent increases in heart rate until the ultimate decline. GAMM analysis of

the thermal performance curves show that treatment had a significant effect on temperature sensitivity of mussel heart rates when compared with the submerged treatment (Table S1).

Shell length

Acclimation treatment had a significant effect on the increase in shell length (Kruskal-Wallis, c^2 = 14.93, d.f = 3, P = 0.002, Fig. 5). Mussels acclimated to submerged conditions exhibited the greatest increase in shell length (0.46 ± 0.06 mm) and this was significantly higher than air, predictable and unpredictable treatments. Mussels from unpredictable treatments experienced the smallest increase in shell length (0.128 ± 0.035 mm) but this was statistically similar to air and predictable treatments.

Hsp/Hsc70 protein levels

When samples were taken just before the daytime low tide period, we found that acclimation treatment had a significant effect on Hsp/Hsc70 levels in gill tissue (Tobit model, x^2 = 44.41, d.f = 4, P = <0.0001; Fig. 6). Mussels from the submerged treatment displayed the highest relative protein levels of Hsc/Hsp70, but also the highest variation (118.4 ± 35.5% of internal standard) and levels in mussels from the submerged group were significantly elevated above air, predictable and unpredictable treatments, but statistically similar to the field treatment. Mussels from the predictable and unpredictable treatments displayed no Hsp/Hsc70 protein expression, and thus had the lowest relative levels (0%).

Glycogen content

When samples were taken just before the daytime low tide period, we found that acclimation treatment had a significant effect on mantle glycogen content (one-way ANOVA, $F_{4,37}$= 6.324, P = 0.001; Fig. 7). Mussels acclimated to the unpredictable treatment exhibited the highest glycogen content (48.86 ± 7.7 μmol glucosyl units g^{-1} tissue) and this was significantly elevated above submerged and predictable treatments, but similar to air and field treatments. Mussels acclimated to the predictable regime had the lowest glycogen content (20.62 ± 3.89 μmol glucosyl units g^{-1} tissue).

MDH activity

When samples were taken just before the daytime low tide period, we found that acclimation treatment had a significant effect on gill malate dehydrogenase (MDH) activity (one-way ANOVA, $F_{4,37}$= 5.495, P = 0.00142; Fig. 8). Mussels acclimated to the submerged treatment exhibited the highest MDH activity (2.16 ± 0.15 μmol mg protein^{-1} min^{-1}) and this was significantly elevated above unpredictable and field treatments, but similar to air and predictable treatments. Mussels acclimated to the field regime had the lowest gill MDH activity (1.05 ± 0.05 μmol mg protein^{-1} min^{-1}). Comparatively, mantle MDH activity showed no difference across acclimation treatments (one-way ANOVA, $F_{4,37}$= 2.559, P = 0.0546; Fig. 8). Mussels from the field treatment had the highest mantle MDH activity (2.07 ± 0.31 μmol mg protein^{-1} min^{-1}) whereas mussels acclimated to the air treatment had the lowest mantle MDH activity (1.25 ± 0.07 μmol mg protein^{-1} min^{-1}).

Succinate content

When samples were taken just before the daytime low tide period, we found that acclimation treatment had a significant effect on gill succinate content (one-way ANOVA, $F_{4,38}$= 9.105, P = 2.93 x 10^{-5}; Fig. 9). Mussels acclimated to the air treatment exhibited the highest gill succinate content (1.14 ± 0.19 μmol g^{-1} tissue) and this was significantly elevated above predictable, unpredictable and field treatments, but similar to the submerged treatment. Mussels from the field treatment had the lowest gill succinate content (0.25 ± 0.1 μmol g^{-1} tissue). Comparatively, mantle succinate content showed no difference across acclimation treatment (one-way ANOVA, $F_{4,37}$= 1.685, P = 0.174; Fig. 9). Mussels from the predictable treatment had the highest mantle succinate content (1.83 ± 0.39 μmol g tissue^{-1}) whereas mussels acclimated to the field treatment had the lowest mantle MDH activity (0.87 ± 0.23 μmol g tissue^{-1}).

DISCUSSION

Intertidal organisms must be able to integrate a large number of environmental signals in order to inhabit a widely fluctuating environment. A significant amount of attention has been focused on the thermal physiology of intertidal animals; however, there remains a large gap in understanding of how the complexities of the thermal signal (e.g. aerial exposure, magnitude, predictability)

integrate to modulate stress tolerance. In our study we were interested to see how different levels of environmental complexity shaped the biochemical and physiological responses of *Mytilus californianus*, particularly in relation to energy allocation, metabolic capacity, and thermal tolerance. We predicted that mussels acclimated to an unpredictable regime would tailor their physiology to be able to tolerate a large temperature range by maintaining high levels of energy stores, elevated anaerobic capacity, and anticipatory cellular stress response leading to an elevated thermal tolerance. Our results suggest that acclimation to an unpredictable thermal regime does shape upper thermal tolerance, but acclimation to cyclic air exposure was the predominant factor in increasing upper thermal tolerance in mussels. Furthermore, while mussels in the unpredictable treatment did have elevated glycogen content, there was no priming of cellular stress or anaerobic mechanisms in anticipation of an upcoming low tide period. This suggests a reliance on energy stores to tolerate unpredictable emersion conditions under the temperature range used.

One of the few predictable elements of the intertidal habitat is the movement of the tide, resulting in the shift between aquatic and aerial environments. Thermal stress typically occurs during air exposure, yet we know surprisingly little about how the predictable environmental element of air exposure may shape thermal performance. Our results suggest that acclimation to cyclic air exposure is the predominant environmental signal for shaping thermal performance and plays a crucial role in shaping upper thermal tolerance. This parallels similar research that has been performed in limpets (Drake et al., 2017) and supports previous studies that have shown that thermal tolerance is elevated in air in intertidal organisms compared to thermal tolerance when submerged (Bjelde and Todgham, 2013; Bjelde et al., 2015; Drake et al., 2017; Fusi et al., 2016; Huang et al., 2015; Pasparakis et al., 2016).

Mussels acclimated to cyclic air exposure (i.e. air, predictable, unpredictable and field treatments) had elevated upper thermal limits of cardiac performance (i.e. flat line temperature (FLT)) compared to submerged mussels. This is perhaps not surprising as the thermal tolerance trial was performed in air. Acclimation to cyclic air exposure could have increased FLT in mussels through physiological adjustments in metabolism that facilitated greater energy efficiency. During air exposure, to avoid desiccation, many *Mytilus* species often do not gape, which results in a shift to anaerobic metabolism (Collins et al., 2020; Connor and Gracey et al., 2011; Demurs and Gurdley et al., 1994; Tagliarolo et al., 2012; Tagliarolo and McQuaid, 2015;

Widdows and Shick, 1985). The shift to anaerobic metabolism is often accompanied by a depression of heart rate activity (Collins et al., 2020; Connor and Gracey, 2011; Tagliarolo and Mcquaid, 2015) as well as the reduction of activities such as digestion or excretion to enhance or maintain energy balance (Connor and Gracey, 2011; Widdows and Shick, 1985) during air exposure. Studies have shown that when acclimated to cyclic air exposure, *Mytilus* sp. may decrease resting heart rate (Bakhmet et al., 2005; Collins et al., 2020; Pickens, 1965) and metabolic rate (Demers and Guderley, 1994; Widdows and Shick, 1985) via methods such as enhanced metabolic depression (Demers and Guderley; 1994) or increased metabolic capacity (Andrade et al., 2019), which improved energy efficiency to sustain physiological performance during stress. Together, this suggest that mussels acclimated to a tidal cycle in this study could have made metabolic adjustments that enhanced energy efficiency during air exposure, which promoted increased tolerance to thermal stress.

Mussels acclimated to an unpredictable regime can prolong optimal cardiac performance to higher temperatures as evidenced by having higher final break point temperature (BPT) in comparison to submerged mussels. Acclimation to elevated or fluctuating temperatures has also been shown to increase upper thermal tolerance of intertidal organisms (Cheng et al., 2018; Giomi et al., 2016; Kern et al., 2015; Oliver and Palumbi, 2011; Schaefer and Ryan, 2006; Schoepf et al., 2015). It is noteworthy that mussels in the predictable regime did not show an increase in BPT in comparison to submerged mussels. Instead, elements of the stochastic temperature regime rather than thermal fluctuation alone influenced BPT. Higher BPTs in mussels from the unpredictable thermal regime could be due to number of reasons that centre around differences in predictability, magnitudes of temperature variability and magnitude, and immediate thermal history between the two thermal acclimation treatments. While mussels acclimated to the predictable and unpredictable regime experienced the same total heating over the acclimation period, mussels from the unpredictable treatments experienced a wider variety of temperatures (13°C-28°C) and higher maximum temperature (28°C) compared to mussels in the predictable treatment (13°C-20°C). Organisms that experience high thermal variability in comparison to medium/low thermal variability have higher thermal tolerance (Kern et al., 2015; Otto and Rice, 1974, Shaefer and Ryan, 2006; Schoepf et al., 2015) and both the increased thermal variability and exposure to a higher maximum temperature could have contributed to the difference in BPT between mussels acclimated to the predictable and unpredictable treatment.

Current literature investigating the influence of unpredictable thermal environments in comparison to predictable has primarily focused on evolutionary differences through offspring or life history traits (Maneti et al., 2014; 2015; 2017; Shama, 2017; Sørenson et al., 2020). While there are limited studies that have investigated the effects thermal stochasticity in a short-term capacity, Drake and colleagues (2017) investigated the role of different unpredictable thermal regimes that had the same set of daily maximum temperatures but in different orders in the intertidal limpet *Lottia digitalis* and concluded that it was likely immediate thermal history (i.e. low tide temperatures the last few days before) in addition to unpredictability that influenced upper thermal tolerance. In our unpredictable treatment, two of the three daytime low tide periods prior to the thermal ramp were relatively warm (28°C, 18°C and 25°C), compared to the consistent 20°C low tide conditions of the predictable treatment, and this immediate history could have influenced cardiac responses to the subsequent upper thermal tolerance trial. In *L. digitalis*, prior exposure to aerial temperatures between 25°C-35°C in the summer and 20°C in winter the day before a critical thermal ramp increased thermal tolerance (Pasparakis et al., 2016). This 'heat-hardening' effect has been shown in other mollusc species (Dong et al., 2008; Dunphy et al., 2018; Zhang et al., 2021), including *M. californianus* (Moyen et al., 2020), and could contribute towards the elevated thermal tolerance exhibited by mussels acclimated to the unpredictable treatment in this study. From our study we cannot discern if it is the stochastic nature of temperature exposure, the degree of thermal variability or immediate thermal history (or likely, a combination of all) of the unpredictable regime that are the important modulators for upper critical thermal limits of cardiac performance. Our results do provide support that complexities in environmental temperature beyond simple shifts in mean temperature are important considerations in understanding thermal tolerance of intertidal organisms.

Our results for the temperature at BPT and FLT are higher than has been previously reported for this species (see Moyen et al., 2019). A possible reason for this is the difference between actual mussel temperature and our 'robomussel' temperature. While robomussels have been shown to accurately reflect live mussel temperatures within 2.5°C (Fitzhenry et al., 2004; Helmuth et al., 2001; Helmuth et al., 2016; Jost et al., 2007), as we did not directly measure each live mussel temperature during the thermal ramp it may be possible that our robomussels over estimated mussel body temperature at BPT and FLT.

The mechanisms underpinning the enhanced cardiac performance (exhibited by the thermal performance curves) displayed by mussels acclimated to the unpredictable treatment is likely due to an increase in cardiac capacity (represented here by maximum heart rate) and elevated initial energy stores allowing for prolonged maintenance of optimal performance with increasing temperatures. Performance during thermal stress relies on sufficient energy reserves to meet the rising energy demand (Kleptsatel et al., 2016; Sokolova et al., 2012) and elevated initial glycogen stores can result in increased thermal tolerance (Cheng et al., 2018; Dunphy et al., 2006; Willis et al., 2021). In line with our predictions, acclimation to an unpredictable regime increased mantle glycogen content in mussels, suggesting that having sufficient energy stores is an important component for tolerating unpredictable thermal stress during low tide periods. Interestingly, acclimation to predictable or unpredictable thermal regimes had markedly different effects on glycogen content in *M. californianus*. While the unpredictable treatment had the highest glycogen content, mussels from the predictable treatment exhibited the lowest levels of glycogen content. This suggests that mussels could exhibit different strategies for energy allocation when presented with predictable or unpredictable thermal stress. Environmental predictability has been shown to be a crucial determinant of energy allocation in empirical studies (Fischer et al., 2009; 2011). Under stochastic conditions, energy stores are expected to play a central role in buffering unpredictable fluctuations, whereas in predictable environments, they play a smaller role and more emphasis is placed on maximising reproductive capacity (Fischer et al., 2011). This has been seen in the limpet *Siphonaria japonica*, where individuals from hotter, but predictable environments have a higher reproductive output than those from more benign, but unpredictable thermal environments (Wang et al., 2020). Moreover, gametogenesis in bivalves is often at the expense of glycogen reserves, resulting in low levels of glycogen but elevated reproductive output (Berthelin et al., 2000). As there was no difference in growth of mussels between predictable and unpredictable treatments in this study, and metabolic capacity was similar (see discussion below), the difference in glycogen content between mussels acclimated to the predictable and unpredictable treatment could be due to a difference in reproductive investment.

Similarity of MDH activity and succinate content between predictable, unpredictable (and field) mussels suggests comparable metabolic demands. We predicted that acclimation to unpredictable regime would result in elevated MDH activity in preparation for utilizing

anaerobic metabolism during unpredictable thermal stress during daytime low tide. We predicted this preparation for anaerobic metabolism would be reflected in higher succinate content as well. Surprisingly, we found that in gill tissue of mussels acclimated to unpredictable and field (and to a lesser degree, predictable) treatments had lower levels of MDH activity and succinate content in comparison to mussels in the submerged treatment. Both MDH and succinate play an important role in the TCA cycle as well as anaerobic pathways in bivalves (Logan et al., 2012; Dunphy et al., 2018). Therefore, MDH activity is thought to be a good biochemical proxy for metabolic rate (Dahlhoff & Menge, 1996, Dahlhoff et al., 2002, Dahlhoff, 2004; Logan et al., 2012), especially as anaerobic metabolism can contribute ~ 20% of total metabolic rate during normoxia in *Mytilus spp.* (Hammon, 1980; Wang and Widdows, 1993). The lower levels of MDH activity and succinate content exhibited by mussels in the tidal treatments accompanied by temperature fluctuations could indicate evidence of increased metabolic efficiency, as lower MDH activities are associated with lower maintenance costs in *Mytilus spp.* (Lockwood and Somero, 2011) and thus lower metabolic rates (Dahlhoff et al., 2002). Studies have shown that exposure to fluctuating thermal regimes can also be more energetically costly (Kern et al., 2015; Ruel and Ayres, 1999; Schafer and Ryan, 2006; Williams et al., 2012) and result in lower routine metabolic rate (Dame and Verberg 1978; Dong and Dong, 2006; Marshall et al., 2021; Tian et al., 2004; Verheyen and Stoks, 2019; Widdows, 1976) than constant regimes at the same mean temperature. Together, it could be possible that in our study, acclimation to fluctuating thermal stress during daytime low tide resulted in increased metabolic efficiency and a decreased reliance on anaerobic metabolism, which is reflected with lower levels of MDH activity and succinate content. Seasonal comparisons of MDH activity in oysters display a similar pattern, where summer-acclimated *Crassostrea gigas* exhibit lower MDH activity in the gills than winter-acclimated individuals under the same temperature (Greenway and Storey,1999), indicating some degree of seasonal thermal acclimatization (Greenway and Storey, 1999). Little is known about how thermal acclimation affects succinate levels in the gills of bivalves, but due to its role in similar pathways as MDH, it is reasonable to suggest that the lower levels here are also a product of increased metabolic efficiency.

We measured MDH activity and succinate content in both gill and mantle because we were interested to see how thermal predictability influenced metabolic changes in an energy dependent tissue (gill) and an energy storage tissue (mantle) to better understand metabolic

dynamics in relation to tissue function. Our results suggest tissue-specific differences in MDH activity and succinate content that are likely reflective of differences in energy demand between the two tissues. The primary function of the gill is for gas exchange and filter feeding, which are energetically demanding processes that constitute a significant portion of whole organism metabolic rate in *M. edulis* (Riisgård and Larsen, 2015; Stapp et al., 2017). Whole organism measures of these traits (such as oxygen consumption and filtration rate) as well as gill enzyme activity have been shown to be highly influenced with thermal acclimation (Koehn and Immerman, 1981; Widdows, 1971). In comparison, the mantle is the primary site for reproductive activity and energy storage in *Mytilus spp.* and is overall a less metabolically active tissue than gill tissue (Stapp et al., 2017) and mantle metabolism has previously been shown to be relatively insensitive to thermal acclimation (Gabbot and Bayne, 1973; Koehn and Immermann, 1981; Widdows, 1978). A seasonal comparison of MDH activity between winter and summer in gill and mantle tissues of *M. edulis* has shown that MDH activity of gill tissue was lower in the summer than the winter, but MDH activity of mantle tissue remained the same (Greenway and Storey, 1999), suggesting that differences in physiological function could underlie the tissue-specific effects in the current study.

In terms of energy allocation to growth (indicated here by increase in shell length), we found that acclimation to a tidal cycle was the predominant factor affecting growth rates in *M. californianus*. We predicted that the combination of cyclic air exposure from the tidal cycle coupled with unpredictable thermal stress during daytime low tide would present the most energetically costly scenario, and therefore expected mussels in the unpredictable treatment to have the lowest growth rates. We found that restrictions in shell growth was predominantly controlled by acclimation to a tidal cycle in *M. californianus*, with mussels in tidal treatments having lower growth rates than those in the submerged treatment. Intertidal species often exhibit reduced growth in comparison to their subtidal conspecifics (Harger, 1970; Menge et al., 1994), predominantly due to decreased feeding and/or calcification time coupled with increased stress during air exposure. Tidal treatments (air, predictable, unpredictable) had varying levels of stress during air exposure, but similar growth rates, suggesting that the decreased growth rate in tidal treatments was not strongly influenced by stress during air exposure. In the current study food levels were controlled, and therefore the difference in growth was likely due to the differences in available calcification time between the submerged and tidal treatments. Daily growth rates

typically increase with submergence time in *Mytilus spp*. (Pannella 1976, Buschbaum & Saier 2001) as calcification cannot occur in air (Tagliarolo et al., 2012). It is also possible that by only using increases in shell length as a proxy for growth, we may have missed changes in other attributes associated with growth in tidal mussels. For example, previous studies have reported that mussels permanently submerged grow longer thinner shells, whereas intertidal individuals possess thicker shells and larger adductor muscles to increase protection from waves, desiccation, and temperature variations (Alyakrinskaya, 2005; Tagliarolo and McQuaid, 2012; Vermeij 1973). Assessment in other aspects of shell morphometrics and/or other measurements for growth over a longer time frame would be needed to confirm if there is a difference in growth between the treatments.

Acclimation to predictable or unpredictable temperatures during daytime low tide did not produce a preparatory response for Hsp/Hsc70 in *M. californianus.* The antibody used in the present study can detect both the inducible Hsp70 and its constitutively expressed form, heat shock cognate 70 (Hsc70) (but it cannot distinguish between the two) and thus we were surprised that acclimation to predictable or unpredictable temperatures did not result in an increase in inducible Hsp70 or constitutive Hsc70. This is in contrast with previous studies in limpets, gastropods and corals that have shown evidence of 'preparative defence' (Dong et al., 2008) or cellular 'frontloading' (Bashirs et al., 2013), where species prepare for a period of anticipated stress by upregulating Hsp70 or Hsc70 (Dong et al., 2008) in advance. In the current study we expected mussels that were anticipating thermal stress to prime their cellular stress response to proactively defend against cellular damage, particularly in the predictable treatment, where heat stress was anticipated and upregulate Hsc/Hsp70 prior to daytime low tide periods. With regards to the inducible form, it is possible that Hsp70 was induced during thermal stress in the predictable and unpredictable treatments, but returned to basal levels before the next day time low tide period (18 hours later), which matches the temporal pattern seen in the intertidal snails *Tegula* sp (Tomanek and Somero, 2000). It is also possible the temperature during daytime low tide in the predictable treatment (20°C: 7°C increase from water temperature) was not stressful enough to result in protein denaturation, an important signal to initiate anticipatory Hsc/Hsp70 mechanisms. Studies on the induction of Hsp70 production in *M. californianus* in response to thermal stress has shown that although heat shock transcription factor 1 (HSF1) is activated with relatively small increases in temperature, Hsp70 synthesis in the gill does not occur on average

until 23°C in mussels acclimated to similar temperature as this study (13°C) (Buckley et al., 2001). Similarly, Roberts et al. (1997) found synthesis of Hsp70 to be between in 20-25°C in field-acclimatized and lab-acclimated *M. californianus*, as did Halpin et al. (2004). Nevertheless, as we did not measure Hsp70 protein expression during the daytime low tide period, it is not possible to know if that was the case.

The lack of Hsc70 protein expression suggests that neither the predictable nor unpredictable temperatures triggered a thermally sensitive increase in protein synthesis rates, which has been associated with a rise in Hsc70 (Buckley et al., 2001). Our results also reflect that of Buckley et al., 2001, who saw no increase in Hsc70 in *M. trossulus* that were acclimatized to warm summer temperatures. Therefore, unlike the preparatory response that has been reported in limpets when acclimated to predictable thermal stress (Dong et al., 2008), *M. californianus* does not appear to increase Hsc70 levels as a preparatory response to thermal stress within the temperature range of this study. For mussels acclimated to the unpredictable thermal regime, we predicted that the mussels would prime higher levels of Hsp/Hsc70 to be able to tolerate unexpected, but potentially high levels of thermal stress. While mussels acclimated to the unpredictable treatment did experience temperatures above the threshold for Hsp70 synthesis, mussels experienced these temperatures in an unpredictable fashion. Synthesis of Hsp70 is energetically expensive, and while a preparatory defence makes this energy investment worthwhile for species/individuals that regularly experience high temperatures (Dong et al., 2008), our results indicate that when these temperatures are experienced stochastically, it is perhaps more beneficial to maintain high energy stores to tolerate high temperatures when they occur, rather than maintain a constitutive elevated level of molecular chaperones to tolerate unpredictable warm days. This is especially true considering our experiment was conducted in March, where temperatures are generally cooler (but where sun exposure produces unpredictably warm days). An avenue for future work would be to see if the predictable and unpredictable treatments are still similar when the average temperature (i.e predictable treatment) reflects Hsp70 induction temperatures (i.e 25°C+). Interestingly, the highest levels of Hsp/Hsc70 were found in mussels acclimated to the submerged treatment. This could be related to the episodes of spontaneous bradycardia and metabolic shifts produced by *M. californianus* acclimated to permanent submersion (Gracey and Connor, 2016) and indicates that acclimation to permanent

submersion in intertidal organisms may result in cellular stress and perhaps are not optimum conditions for an intertidal individual.

In conclusion, the results of the current study highlighted two main findings. Firstly, cyclic air exposure is a major factor in shaping thermal performance that has been highlighted in earlier studies (Bjelde and Todgham, 2013; Collins et al., 2020; Drake et al., 2017; Roberts et al., 1997). Secondly, the predictability of the thermal regime affects cardiac performance and mechanisms of energy storage in the form of glycogen. Mussels from the unpredictable treatment had elevated cardiac capacity, which lead to elevated BPT over submerged mussels that was not reflected in mussels from the predictable treatment. Mussels acclimated to unpredictable thermal regimes also had elevated glycogen levels in comparison to mussels from the predictable treatment, and combined with the elevated cardiac capacity could have aided in increasing cardiac performance during thermal stress. Moving forward, physiological studies performed in the lab to predict the physiological performance of wild mussels should look towards incorporating thermal complexity within a tidal framework to more accurately simulate the intertidal environment and assess sensitivity and tolerance to environmental change. Differences in preparatory strategy between unpredictable and predictable mussels warrants further investigation, and assessment of the mechanistic underpinnings of these responses during thermal stress will help to understand strategic differences in physiological performance. Similarly, from our results and others (e.g Drake et al., 2017; Moyen et al., 2019; Pasparakis et al., 2016), it is becoming increasingly clear that the magnitude of thermal stress is not the only aspect of the thermal signal that plays a role in shaping thermal performance. We focused on incorporating some key aspects of the *in-situ* thermal signal (air exposure, thermal fluctuation, predictability) to understand how these elements shaped physiological performance in *M. californianus*. Our work identified important physiological differences in the unpredictable treatment, but our design did not allow for us to tease apart what specific underlying aspects of the unpredictable regime led to differences in physiological performance. Further work is needed to identify how different elements of the unpredictable regime (unpredictability, thermal history, thermal magnitude, thermal variability) specifically shapes physiological performance, especially under hotter (summer) conditions. For example, comparing predictable and unpredictable thermal variation that experience the same maximum temperatures would aid in understanding specifically the role of unpredictability in shaping physiological performance. Furthermore, as

the intertidal has varying levels of predictability on a temporal scale, assessing how stochasticity integrates across a longer time scale (month, season or year) will highlight the importance of predictability (and unpredictability) in shaping physiological performance. Lastly, identifying how specific components of the thermal signal, such as the medium of thermal stress (air or water) interacts with other variables that are often intertwined with changes in temperature (such as food availability) will be crucial to accurately predict how intertidal organisms will respond to climate change. Climate change has been linked with changes in abundance and community composition of phytoplankton (Boyce et al., 2010; Tortell et al., 2002) and alterations in upwelling gradients (Wang et al., 2015). How these variations will interact with thermal stochasticity could be crucial in informing performance.

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COMPETING INTERESTS

The authors declare no competing or financial interests.

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Figures and Table

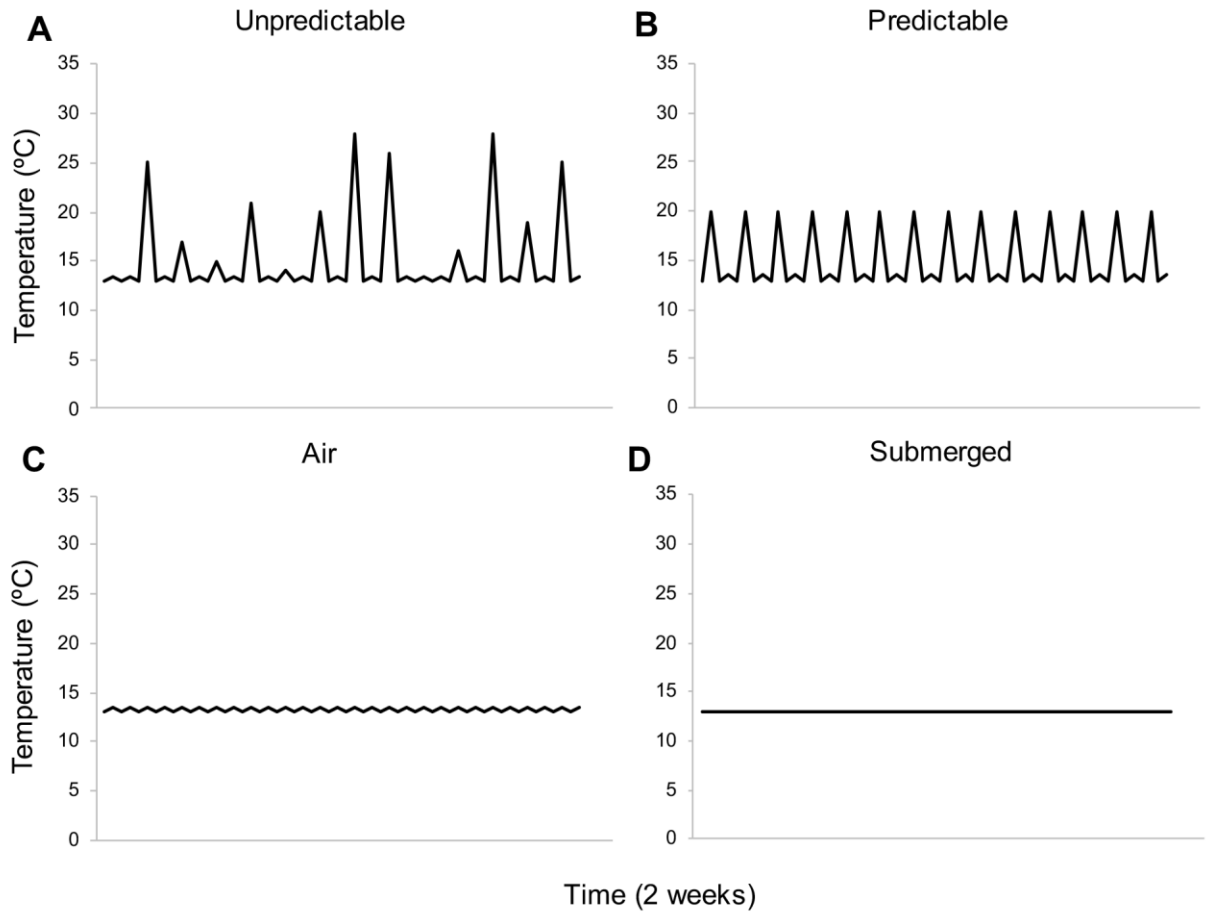

Figure 1. Temperature profiles for each acclimation treatment over the 2-week acclimation period. (A) Unpredictable treatment: circatidal regime and varying aerial temperatures during each daytime low tide. (B) Predictable treatment: circatidal regime with consistent, predictable warming to 20°C each daytime low tide. (C) Air treatment: circatidal regime with no warming during daytime low tide. (D) Submerged treatment: control where mussels were permanently submerged (no tidal regime, no air exposure).

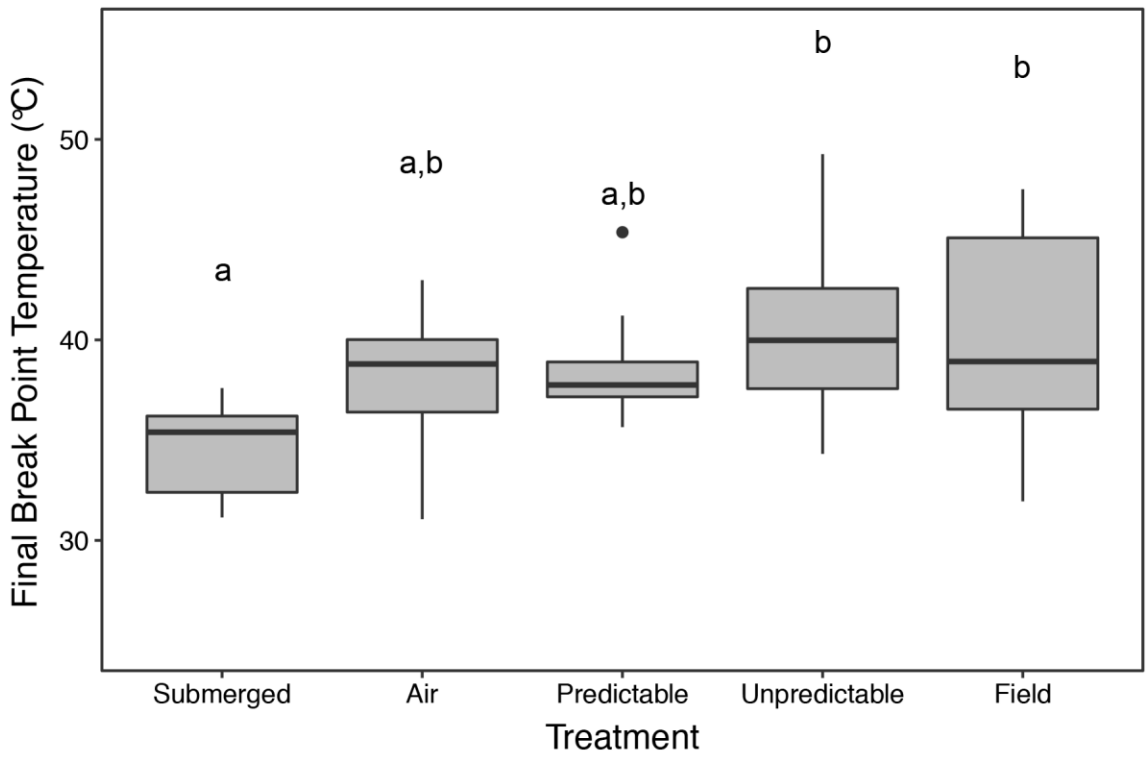

Figure 2. Final break point temperature in heart rate for mussels from submerged (n=11), air (n=11), predictable (n=11), unpredictable (n=12) and field (n=12) acclimation treatments measured during a thermal ramp in air. The line on the boxplots represents the median, the box represents the inter-quartile range (IQR), the whiskers extend 1.5 times IQR. Points beyond the whiskers are outliers. Differences in letters represents significant differences between acclimation treatments (one-way ANOVA, Tukey's HSD, $p < 0.05$).

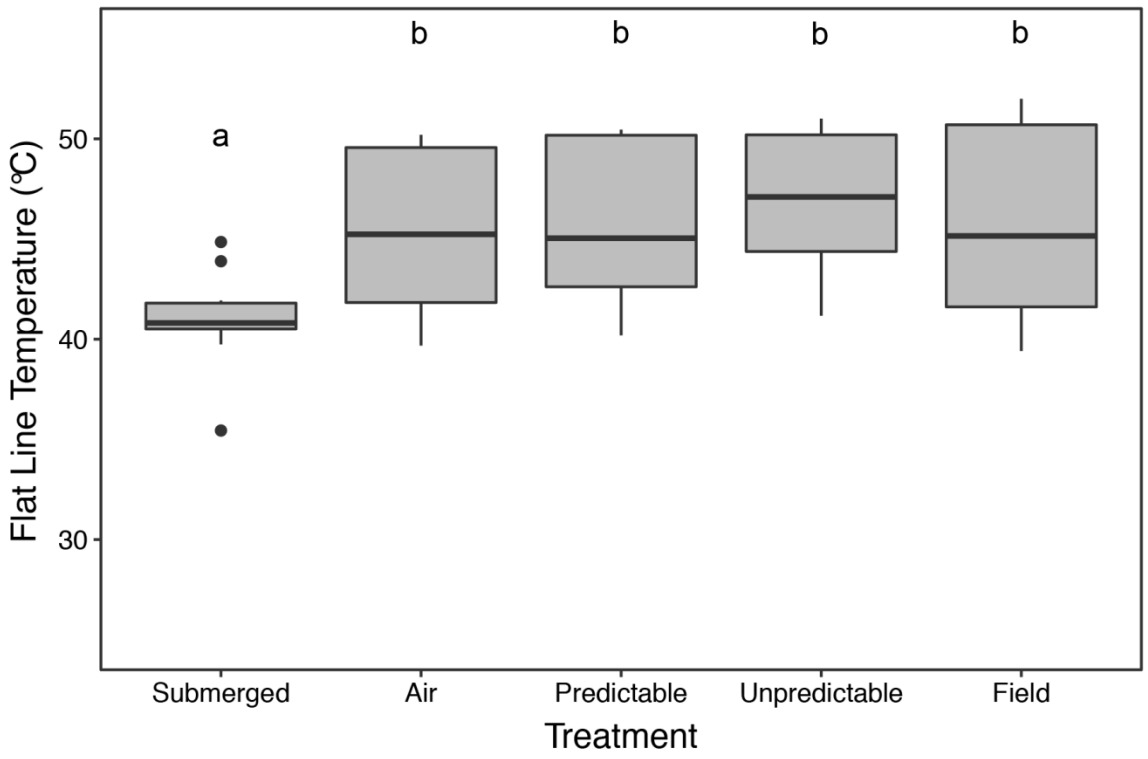

Figure 3. Flat line temperature in heart rate for mussels from submerged (n=11), air (n=11), predictable (n=11), unpredictable (n=12) and field (n=12) acclimation treatments measured during a thermal ramp in air**.**. The line on the boxplots represents the median, the box represents the inter-quartile range (IQR), the whiskers extend 1.5 times IQR. Points beyond the whiskers are outliers. Differences in letters represents significant differences between acclimation treatments (one-way ANOVA, Tukey's HSD, $p < 0.05$).

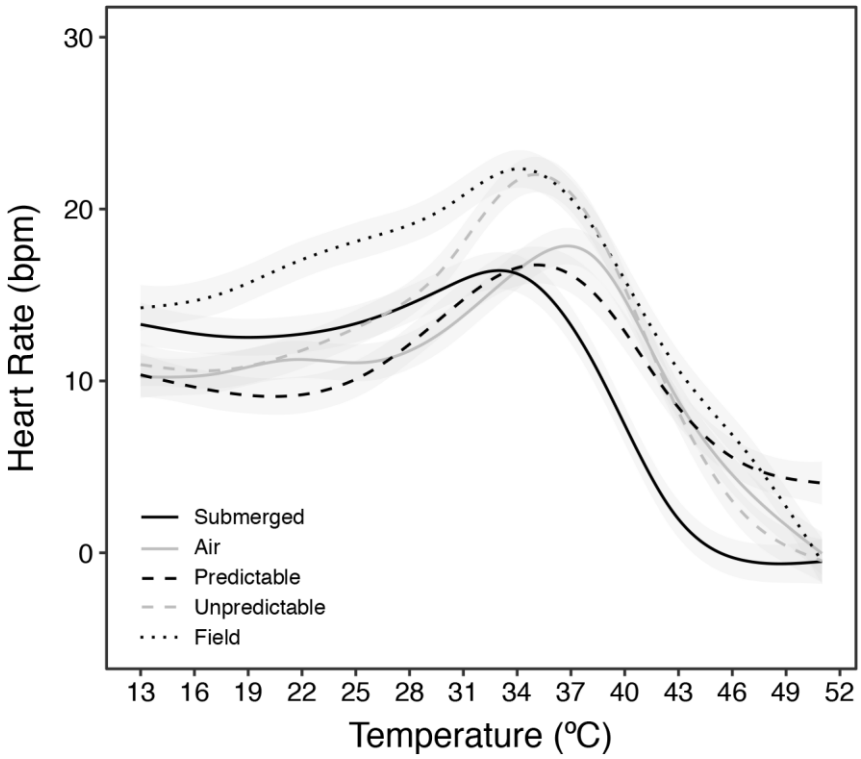

Figure 4. Generalized additive mixed modelling (GAMM) for heart rates throughout the heat ramp (performed in air) for mussels from the submerged (n=11), air (n=11), predictable (n=11), unpredictable (n=12) and field (n =12) acclimation treatments. Statistical differences are reported in Table S1.

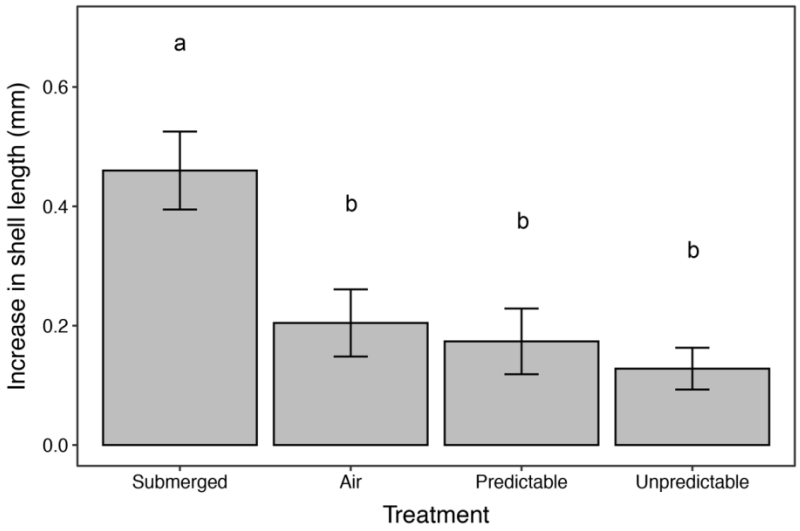

Figure 5. Increases in shell length (mm) of *Mytilus californianus* during the 2-week acclimation period in air (n=19), predictable (n=18), submerged (n=17) and unpredictable (n=20) acclimation treatments. Each bar represents the mean (± S.E.M). Differences in letters represents significant differences between acclimation treatments (Kruskal Wallis test, Dunn's Multiple Comparison Test, $p < 0.05$).

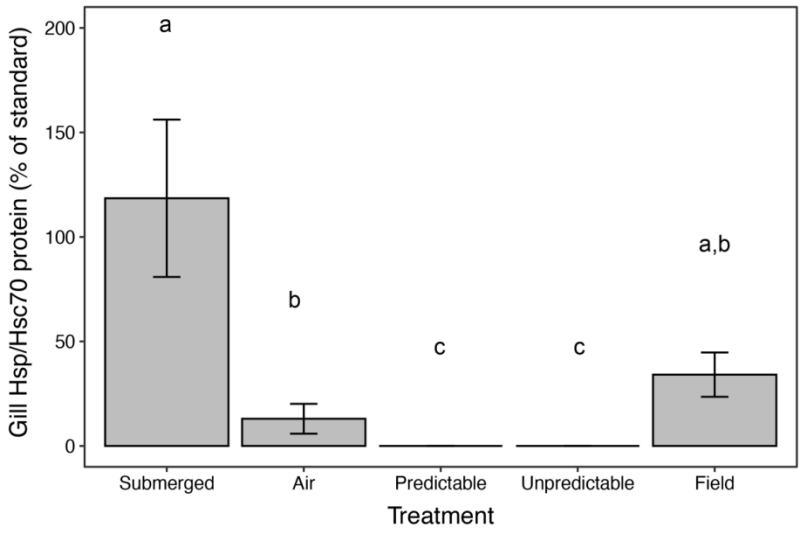

Figure 6. Relative levels of Hsp/Hsc70 protein in the gill tissue of *Mytilus californianus* taken just before daytime low tide acclimated to either submerged (n=8), air (n=9), predictable (n=7), unpredictable (n=9) or field (n=9) treatments. Protein levels are shown as relative values based on band intensities standardised with the level of Hsc/Hsp70 in a gill tissue sample from the submerged group that had shown positive expression of Hsc/Hsp70 (mean ± S.E.M). Differences in letters represents significant differences between acclimation treatments (Tobit model, pairwise comparisons with multiple-comparison correction (Tukey method), $p < 0.05$).

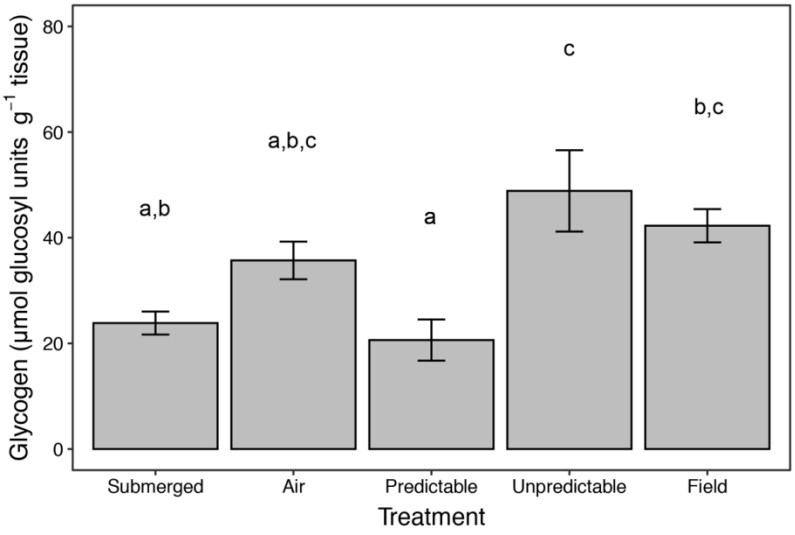

Figure 7. Glycogen content of mantle tissue in *Mytilus californianus* taken just before daytime low tide acclimated to either submerged (n=8), air (n=9), predictable (n=7), unpredictable (n=9) or field (n=9) treatments. Each bar represents the mean (± S.E.M). Differences in letters represents significant differences between acclimation treatments (one-way ANOVA, Tukey's HSD, $p < 0.05$).

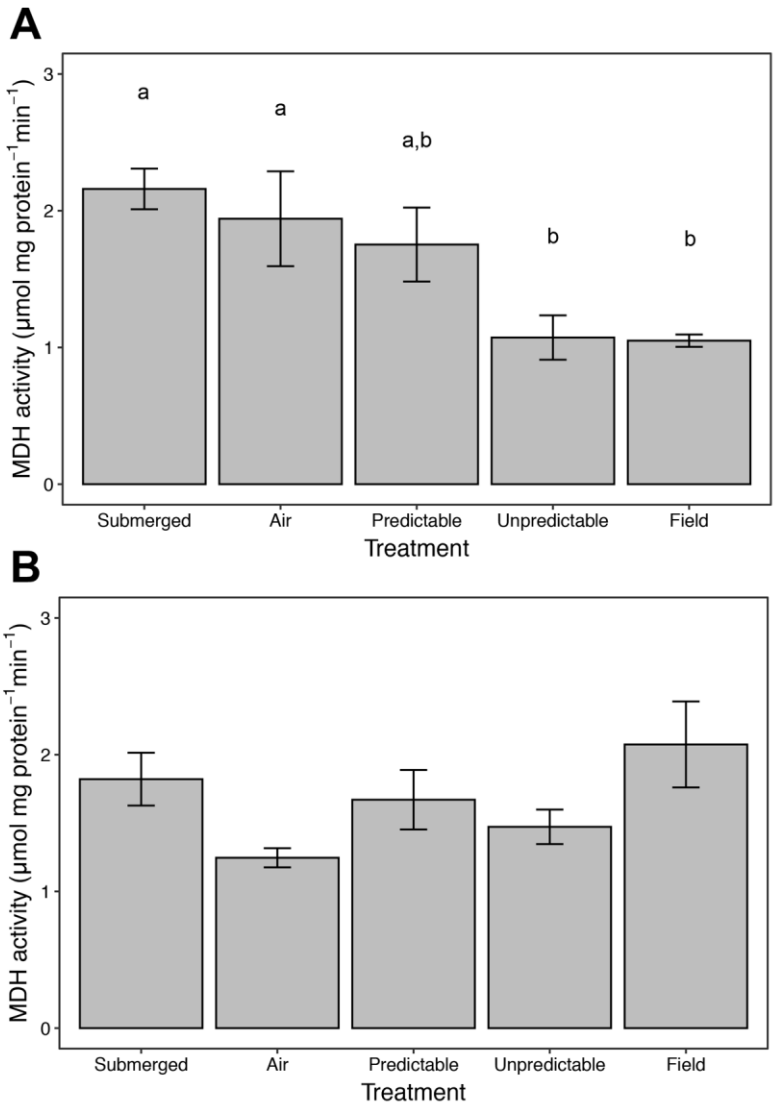

Figure 8. MDH activity of gill (A) and mantle (B) tissue in *Mytilus californianus* taken just before daytime low tide acclimated to either submerged (n=8), air (n=9), predictable (n=7), unpredictable (n=9) or field (n=9) treatments. Each bar represents the mean (± S.E.M). Differences in letters represents significant differences between acclimation treatments (one-way ANOVA, Tukey's HSD, $p < 0.05$).

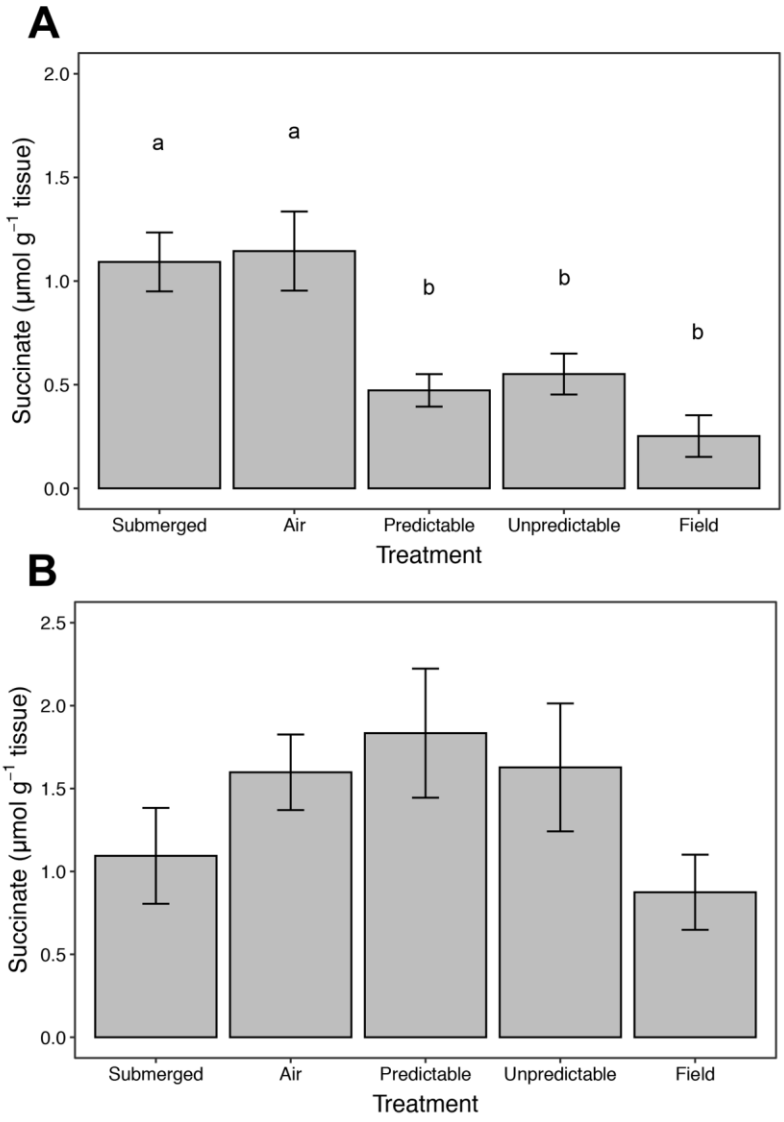

Figure 9. Succinate content of gill (A) and mantle (B) tissue in *Mytilus californianus* taken just before daytime low tide acclimated to either submerged (n=9), air (n=9), predictable (n=7), unpredictable (n=9) or field (n=9) treatments. Each bar represents the mean (± S.E.M). Differences in letters represents significant differences between acclimation treatments (one-way ANOVA, Tukey's HSD, $p < 0.05$).

Table 1. Differences between flat line temperature (FLT) and final break point temperature (BPT), and maximum heart rate (Max HR) of mussels from the five acclimation treatments.

Acclimation treatment	FLT-BPT	Max HR	N
Submerged	6.65 ± 0.96	16.87 ± 1.51	11
Air	8.01 ± 0.85	20.91 ± 1.52	11
Predictable	7.02 ± 1.34	18.61 ± 1.26	11
Unpredictable	7.17 ± 0.95	24.06 ± 1.79	12
Field	6.01 ± 0.83	21.64 ± 1.79	12

Data are means ± S.E.M.

Table S1. Comparisons of generalized additive mixed models (GAMM) of heart rate as a function of temperature, *f*(*T*). Tidal treatments were referenced to the curve of Submerged treatment and the degree of deviation was measured.

Acclimation treatment	e.d.f	F-value	P-value
f(*T*) for Submerged	8.307	165.60	<0.0001
Deviation from *f*(*T*) for Air	8.103	28.91	<0.0001
Deviation from *f*(*T*) for Predictable	8.410	31.86	<0.0001
Deviation from *f*(*T*) for Unpredictable	8.604	38.41	<0.0001
Deviation from *f*(*T*) for Field	7.816	26.34	<0.0001

e.d.f., effective degrees of freedom