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Understanding the role of low tide habitat, thermal predictability, and food availability in shaping the thermal performance of the California mussel

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ABSTRACT: Recent climate change models indicate that there will be an increase in thermal unpredictability and the frequency and intensity of hot days. How thermal unpredictability operates in intertidal low tide habitats with different habitat media, such as tidepool (submerged in water) or tidally exposed (circatidal exposure to air and water) environments is poorly understood, especially when coupled with other important determinants of physiological performance, such as food availability. We examined how acclimation to different levels of thermal predictability with either high or low food availability shapes performance during an acute thermal ramp in the California mussel *Mytilus californianus* in 2 low tide habitat treatments: tidepool and tidally exposed. Mussels were warmed at a rate of $6.5^{\circ}\text{C h}^{-1}$ for 6 h in their respective habitat medium (water or air). Cardiac performance, glycogen, and Hsp/Hsc70 were determined during the heat ramp. We found that low tide habitat was the largest driver for shaping thermal performance. Tidepool mussels exhibited higher maximum heart rates, but also multiple breakpoints and lower upper thermal tolerance in cardiac performance, coupled with an increase in Hsp/Hsc70 levels and glycogen usage in comparison to tidally exposed mussels. Mussels exposed to unpredictable thermal regimes exhibited elevated cardiac performance and thermal tolerance, supported by elevated initial glycogen stores. Food availability had a minimal effect on mussel thermal performance. Our results suggest that intertidal organisms that experience thermal stress in a tidepool habitat may be more susceptible to climate-induced increases in temperature, but thermal unpredictability may aid in increasing thermal tolerance.

KEY WORDS: Intertidal · Multiple stressors · Ecophysiology · Climate change · Environmental variability · Air exposure

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1. INTRODUCTION

Temperature has long been appreciated as a major determinant of organismal performance, and understanding the capacity of an organism to tolerate climate change-induced variations in temperature has become a central focus in ecophysiology (Kroeker et al. 2016, Burggren 2019, Sheldon 2019). The rocky intertidal zone is a model ecosystem for assessing the effects of climate warming due to dynamic, unpredictable fluctuations in temperature naturally experienced by intertidal organisms due to the ebb and the flow of

the tide. Though thermal fluctuation is an inherent characteristic of daily life in the intertidal, much of our understanding on thermal performance in intertidal organisms is based on acclimation to submerged, steady state conditions, where emphasis on increases in the mean or magnitude of acclimation temperature was thought to be the primary contributor for increasing thermal tolerance (Barry et al. 1995, Stillman 2002, Somero 2005). More recently, we are learning that there are many aspects of the thermal signal that can modulate thermal performance, including the temporal pattern of thermal stress (e.g. Helmuth et al. 2006,

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Zhang et al. 2014, Hui et al. 2020), prior thermal history (e.g. Tomanek & Somero 1999, Pasparakis et al. 2016, Schoepf et al. 2022), thermal variability (e.g. Oliver & Palumbi 2011, Han et al. 2020, Marshall et al. 2021), as well as interaction with other environmental variables (e.g. Todgham & Stillman 2013, Paganini et al. 2014, Kelly et al. 2016) and habitat medium (e.g. Stillman & Somero 1996, Bjelde & Todgham 2013, Tagliarolo & McQuaid 2015). Investigation into the complexity of the thermal signal has resulted in an increase in experiments incorporating thermal fluctuation within a tidal framework (e.g. Marshall et al. 2011, Paganini et al. 2014, Jimenez et al. 2016, Vajedsamiei et al. 2021, Nancollas & Todgham 2022), yet we still know very little about how thermal unpredictability shapes physiological performance.

In order to tolerate such a dynamic environment, intertidal organisms rely on a number of mechanisms to maintain functional integrity during acute thermal stress, including metabolic modulation (Dahlhoff et al. 2001, Marshall et al. 2011, Hui et al. 2020), the initiation of cellular stress response mechanisms, such as heat shock proteins (Hsps) (Hofmann & Somero 1995) and antioxidant enzymes (Dowd et al. 2013), and maintenance of sufficient energy stores (Sokolova et al. 2012). Cardiac function is important in establishing thermal tolerance limits (Dong et al. 2022), and the close linkage between cardiac performance and metabolism in ectotherms (Frederich & Pörtner 2000) makes heart rate an effective indicator of metabolic rate in molluscs. Expression of Hsps serves to maintain protein integrity during stress and plays an important role in increasing thermal tolerance, but requires a significant energetic investment, and thus maintaining sufficient energy stores and ATP turnover is crucial for intertidal organisms to survive stressful low tide periods (Somero 2002, Ivanina et al. 2008, Sokolova et al. 2012). The induction and magnitude of the inducible heat shock response can be altered by acclimation conditions (e.g. Hofmann & Somero 1995, Roberts et al. 1997, Tomanek & Somero 1999, Buckley et al. 2001, Dong et al. 2008, Wang et al. 2020), and some evidence suggests that intertidal organisms can use predictable environmental cues to preemptively upregulate Hsps for an anticipated period of stress, as a form of 'preparative defence' (Dong et al. 2008, Somero 2020) or cellular 'frontloading' (Barshis et al. 2013, Collins et al. 2020). As our understanding of how the thermal environment informs the cellular stress response develops, it is important to understand how exposure to representative thermal patterns may shape crucial determinants of the thermal stress response, such as Hsps,

energy stores, and cardiac function, in order to link mechanism to physiological performance.

The topography in the rocky intertidal can result in conspecifics experiencing differences in unpredictable thermal fluctuation in intertidal habitats with different physical attributes during low tide. Tidally exposed individuals experience circatidal exposure to both air and water and are typically exposed to complex environmental stimuli in conjunction with air exposure. In contrast, individuals that reside in shallow waters and tidepools that are isolated during low tide remain constantly submerged, but also experience stochastic fluctuations in temperature and other environmental variables (Helmuth & Hofmann 2001, Nakano & Iwama 2002). The habitat medium (air or water) in which an intertidal organism experiences thermal stress can dictate different metabolic profiles. Some organisms, such as gastropods and crabs, are able to maintain comparable metabolic profiles in air and water (Stillman & Somero 1996, Bjelde & Todgham 2013, Huang et al. 2015), whereas other species, like many bivalves, will metabolically depress in air in order to conserve energy and minimize the desiccation risk that can often co-occur with air exposure (e.g. McMahon et al. 1991, Sokolova & Pörtner 2001, Tagliarolo et al. 2012, Tagliarolo & McQuaid 2016, Nancollas & McGaw 2021a). When combined with air exposure, thermal unpredictability can have complex effects on thermal tolerance that may be correlated with metabolic reorganization and metabolic profile (Drake et al. 2017, Nancollas & Todgham 2022). However, we still know little about how it shapes performance in an aquatic setting.

Along with temperature, food availability has been shown to be among the most important determinants of survival, growth, and reproduction for a wide array of organisms (Menge et al. 2008, Schneider et al. 2010, Sokolova 2013), and available energy stores are crucial in mounting a stress response to demanding environments (Roberts et al. 1997, Sokolova 2013). Climate change-induced warming has been linked to reductions in food availability for filter-feeding marine organisms through alterations in phytoplankton abundance or community composition (Tortell et al. 2002, Hays et al. 2005, Rost et al. 2008, Boyce et al. 2010, Lesser et al. 2010, Henson et al. 2021) as well as reduced scope for growth and ability to assimilate energy from ingested food (Anestis et al. 2010). Recent evidence suggests that mussels in unpredictable thermal environments rely more heavily on elevated energy stores to tolerate stressful low tide conditions in comparison to mussels from predictable thermal environments (Nancollas & Todgham 2022), suggesting that declines in food availability could have

detrimental effects on physiological performance in intertidal mussels.

In this study, we examined how acclimation to various combinations of low tide habitat, thermal predictability, and food availability shaped cardiac performance, the cellular stress response (heat shock protein 70 and heat shock cognate 70 [Hsp/Hsc70]), and energy reserves (glycogen content) of the California mussel *Mytilus californianus* during an acute thermal ramp. We predicted that: (1) mussels experiencing constant submersion in tidepool treatments would have lower thermal tolerance than mussels acclimated to circatidal exposure of air and water in tidally exposed treatments; (2) 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 levels of cellular defence mechanisms to tolerate stressful low tide periods; and (3) mussels subjected to low food availability would have reduced resources to dedicate towards the stress response and thus would have a reduced thermal tolerance. With the predicted increases in thermal unpredictability, as well as the magnitude and frequency in extreme temperatures forecasted by climate change models, understanding how thermal predictability interacts with food availability in different low tide habitats to shape performance during acute stress will be paramount for predicting how climate change will affect intertidal communities.

2. MATERIALS AND METHODS

2.1. Mussel collection

A total of 432 *Mytilus californianus* (Conrad, 1837) were collected during low tide from the mid-upper intertidal zone at Shell Beach, California, USA (38° 25' 17" N, 123° 06' 47" W) in September 2020. Mussels (length range: 47.5–52.5 mm) were then transported to the University of California Davis Bodega Marine Laboratory in Bodega Bay, California, 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 h.

2.2. Acclimation conditions

Mussels were weighed, measured, labelled, and randomly divided equally between 1 of 12 different acclimation treatments and were held under these

conditions for 3 wk. The 12 acclimation treatments consisted of 3 different temperature regimes (unpredictable, U; predictable, P; no heat, N) and 2 levels of food (high, H; low, L) within 2 different low tide habitats: tidepool (always submerged, S) and tidally exposed (circatidal exposure to air and water, A) (Fig. 1A). Experimental tanks and treatment design were modeled off the methods outlined in Nancollas & Todgham (2022), with several modifications. For the 6 tidally exposed treatments, tanks were built to simulate natural intertidal conditions by replicating semi-diurnal circatidal changes in water height, periodically exposing mussels to air during 'low tides' and submerging mussels in seawater during 'high tides'. Water height was manipulated using Arduino micro-controllers (Arduino YUN, Adafruit; Miller & Long 2015, Drake et al. 2017) and oscillated based on a semidiurnal tidal pattern. Tanks were flowthrough, where incoming seawater was first passed through a 30µm sand filter and then a 5µm filter to remove biological matter, including natural food sources. For the 6 tidepool treatments, mussels were held in identical conditions as tidally exposed treatments, but experienced no air exposure and were always held in submerged conditions.

Within each low tide habitat, 3 temperature regimes occurred, which differed in degree of thermal stress and predictability: unpredictable, predictable, no heat (Fig. 1B). In the intertidal, both tidepool and tidally exposed mussels experience thermal stress during daytime low tide due to solar radiation either through direct exposure (tidally exposed), or indirectly, by warming the surrounding water (tidepool). As such, temperature manipulation for all treatments occurred during each daytime low tide and was performed in the appropriate corresponding medium based on low tide habitat—tidepool mussels were warmed in water and tidally exposed treatments were warmed in air. Temperature experienced during daytime low tide was dependent on thermal regime. Temperature profiles for treatments were based off daytime low tide data from 2 'robomussel' temperature loggers (Maxim Integrated Products): 1 embedded on the rock next to tidally exposed *M. californianus*, and 1 in a tidepool (length: 1m, width: 0.6m, depth: 0.25m) surrounded by *M. californianus* in Bodega Marine Reserve. This size tidepool was chosen because it represents the typical size of tidepools containing *M. californianus* in the mid-intertidal on this and nearby shores. Each robomussel continuously monitored temperature every 10 min from May 2020 to October 2020. Assessment of the logger data revealed that the tidally exposed robomussel and the tidepool robo-

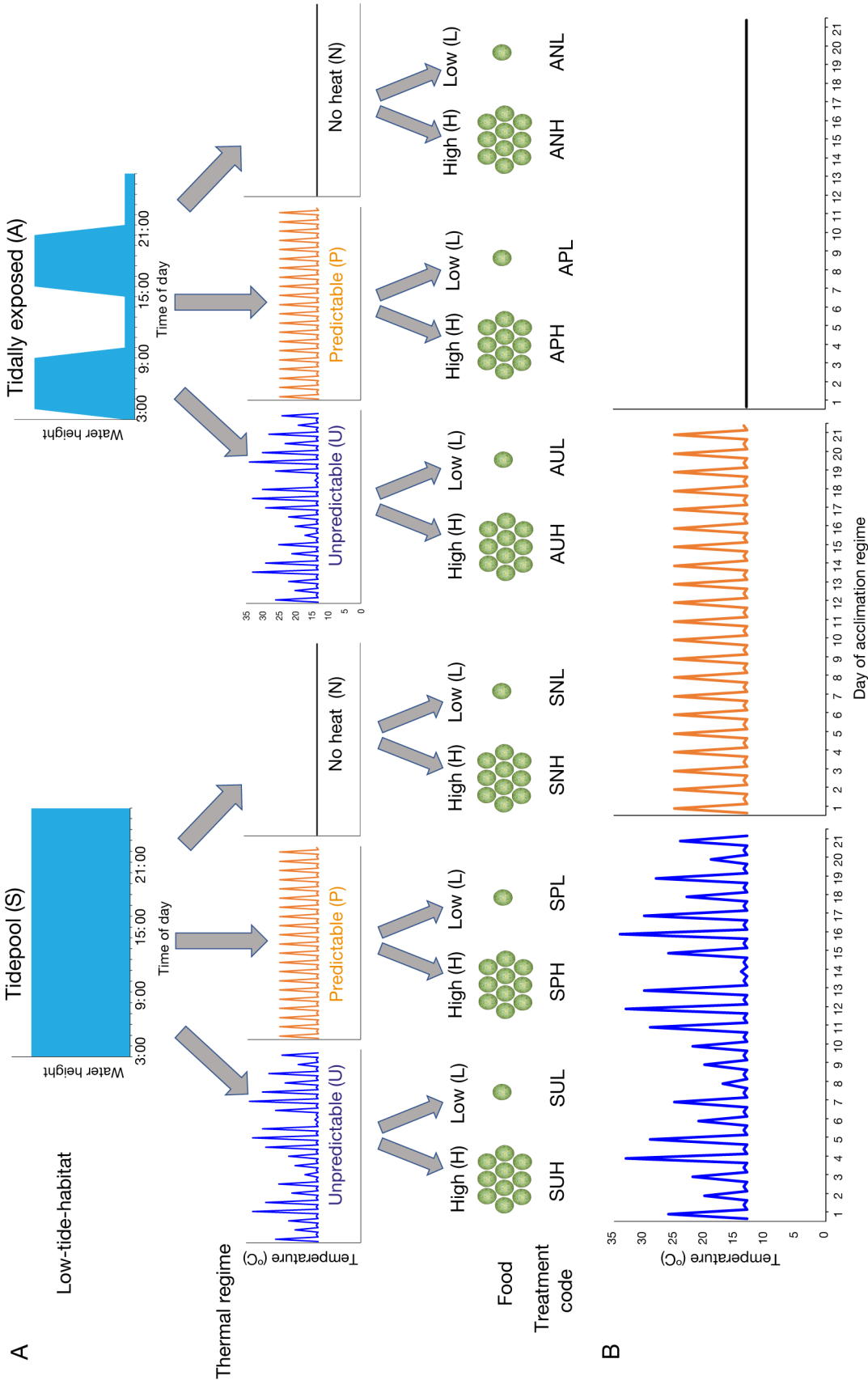


Fig. 1. (A) *Mytilus californianus* were acclimated for 3 wk to either tidepool (S) or tidally exposed (A) treatments combined with either unpredictable (U), predictable (P) or no (N) thermal stress and high (H) or low (L) food availability for a total of 12 separate acclimation treatments. Tidepool treatments were permanently submerged and experienced temperature change in water, whereas tidally exposed treatments were subjected to a semi-diurnal cycle of immersion and emersion and experienced temperature change during air exposure. Warming only occurred during daytime low tide, and mussels were fed during daytime high tide which followed the warming period. (B) Closer examination of the 3 thermal regimes incorporated during acclimation. Mussels subjected to unpredictable thermal stress (blue) were warmed to a different maximum temperature during daytime low tide, and this profile mimicked a 3 wk period of maximum temperatures from the 'master' logger profile. Mussels under predictable (orange) thermal stress were warmed to the same maximum temperature every daytime low tide (25°C) which was the average of the maximum temperatures in the unpredictable regime. Mussels from no heat treatments (black) were not warmed during daytime low tide and experienced ambient temperatures (13°C)

mussel experienced comparable levels of thermal unpredictability, with peaks in temperature occurring on the same days, but the tidally exposed robomussel experienced slightly higher maximum temperatures and larger thermal variability. Daily maximum temperatures for the tidally exposed robomussel ranged from 13 to 36°C, whereas tidepool temperatures ranged from 13 to 32°C. In order for direct comparisons to be made between the tidepool and tidally exposed treatments, the temperature profiles of the tidally exposed and tidepool robomussels were combined to create a single 'master' logger thermal profile, which was used to inform the temperature profiles for both the tidepool and tidally exposed acclimation treatments (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m747p061_supp.pdf).

For the unpredictable treatments, mussels experienced varying temperatures within the range of 13–34°C during daytime low tide (Fig. 1B). This temperature profile mirrored a 3 wk period of natural cycles in environmental temperature within the 'master' logger data, which was equal to the average temperature of daytime low tide during the 4 mo logger period (25°C) and included the maximum temperature (34°C). Mussels in the predictable treatments were consistently warmed to the average temperature (25°C) every daytime low tide. The predictable treatment was designed to subject mussels to the same degree of heating (average of daily maxima) throughout the 3 wk as the unpredictable treatment, but in a predictable manner. As a control, a final thermal regime with no heat during daytime low tide (13°C) was included. For all treatments that experienced a tidal cycle, no heating occurred during night-time low tide (i.e. constant 13°C aerial exposure), and submerged/high tide conditions were maintained at 13°C.

For tidally exposed 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 to 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. Tanks were insulated to ensure uniform heating throughout the tank, which was confirmed with preliminary testing of the system. As orientation to the sun can also have a large impact on the warming rate and ultimate body temperature (Har-

ley 2008, Miller & 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 = 14 cm), which enabled mussels to either be immersed or emersed depending on changes in water height. As mussel body temperature in tidepools are not modulated by direct exposure to the sun, but rather the indirect process of the sun warming the surrounding water, mussels in the 6 tidepool treatments were held in identical conditions as described above, but temperature was manipulated with a 150 W aquarium heat bar attached to the Arduino microcontroller system, rather than a heat lamp.

To manipulate food levels, mussels were fed live *Nannochloropsis* sp. and *Isochrysis* sp. mix (1:1) every daytime high tide, after the low tide period. Prior to feeding, tanks were first flushed with fresh seawater for 1 h to ensure temperature in all tanks had returned to ambient (13°C) conditions and to remove any waste product build up that had occurred during daytime low tide. High food treatments were fed 100 000 cells ml⁻¹ (~10 µg chl l⁻¹), whereas low food treatments were fed 10 000 cells ml⁻¹ (~1 µg chl l⁻¹), which equated to 50 cells g⁻¹ mussel wet weight and 5 cells g⁻¹ mussel wet weight, respectively. These food levels were chosen to represent the average high and low levels of chlorophyll observed near Bodega Bay (data access: <https://boon.ucdavis.edu/>; https://coastwatch.pfeg.noaa.gov/infog/MW_chla_las.html).

Each of the treatments had 2 replicate tanks (24 tanks in total) and acclimation for all treatments was conducted simultaneously. During acclimation, temperature, salinity, and dissolved oxygen were measured multiple times a day, and nitrate, nitrite, and ammonia were checked twice a week to ensure zero levels were maintained. Temperature, salinity, and dissolved oxygen were measured using a YSI Model 85 while ammonia, nitrate and nitrite levels were monitored with an API saltwater test kit. Mortality was low in all tanks (≤ 1%), and if it occurred, food was adjusted to maintain consistent feed densities per mussel wet weight across tanks.

2.3. Heart rate

To understand how low tide habitat, thermal predictability, and food availability influenced the cardiac performance of *Mytilus californianus* during thermal stress, heart rate was measured in 6 mussels from each of the 12 treatments during an acute ther-

mal ramp starting at 13°C (ambient ocean temperature) and increasing at rate of 6.5°C h⁻¹ for 6 h. 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/bar system described previously. Similar to acclimation, mussels were warmed in their respective medium: mussels from the 6 tidepool treatments were warmed in water and mussels from the 6 tidally exposed treatments were warmed in air. Heart rate was monitored for each individual as described by Nancollas & Todgham (2022). Briefly, 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. The signal from the sensor was amplified using AMP-03 (Newshift), digitized using a data acquisition system (PowerLab 16/35, ADInstruments), 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) to give a live temperature feed during the thermal ramp that was also recorded through the LabChart software. Similar to Tagliarolo & McQuaid (2015) and Nancollas & Todgham (2022), preliminary tests showed that the heart rate signals stabilised 10 to 15 min after handling. Therefore, mussels were left undisturbed for 15 min after attachment of the sensor to recover before the start of recording.

2.4. Cardiac performance analysis

Cardiac performance was analysed following methods previously described (Bjelde & Todgham 2013, Nancollas & Todgham 2022). Several measures of performance were used to determine overall cardiac performance. Overall temperature sensitivity of heart rate was examined using thermal performance curves. Final breakpoint temperatures (BPTs) were measured, which are defined as the highest temperature at which the heart rate rapidly declines and is considered to be the upper critical thermal limit of intertidal organisms (Stillman & Somero 1996), including mussels (Tagliarolo & McQuaid 2015). Final BPT was calculated as described elsewhere (Bjelde & Todgham 2013, Drake et al. 2017, Nancollas & Todgham 2022). Briefly, individual mussel heart rates (beats min⁻¹) were plotted against temperature, and the intersection point (BPT) was determined using the segmented package in R. Average number of

breaks in heart rate by individuals was also reported. Flat line temperature (FLT) was determined by manually observing the temperature of the last heartbeat on LabChart recordings, and 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.

2.5. Mussel tissue sampling

To understand how low tide habitat, thermal predictability, and food availability influence the preparedness for an anticipated period of thermal stress, baseline tissue samples (gill and mantle) were dissected from mussels ($n = 6$) from each treatment immediately prior to the acute thermal ramp (therefore immediately prior to daytime low tide, ambient temperature ~13°C). Tissues samples were also taken at 20, 30, and 40°C (see Fig. S2 in the Supplement for diagrammatic representation of sampling) during the acute thermal ramp to understand how low tide habitat, thermal predictability, and food availability influence the physiological condition of mussels during acute thermal stress. Due to tidepool treatments being close to their flat line temperature at 40°C, a reduced sample size ($n = 3$ per treatment) was taken for the 6 tidepool treatments at the 40°C sampling point. Cellular stress mechanisms (Hsp/Hsc70 protein) were assessed in gill tissue due to the importance of this tissue during stress and for respiration. In *M. californianus*, glycogen is primarily stored in the mantle, so this tissue was used to assess energy stores (glycogen content). Mussels were removed from treatment tanks, and gill and mantle tissue were dissected quickly (i.e. under 20 s). Tissue samples were immediately frozen on dry ice and stored at -80°C until analysis.

2.6. SDS-PAGE and Western blot analysis for Hsp/Hsc70

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 Nancollas & Todgham (2022). The primary antibody (Mouse IgG Hsp70; MA3-007; Thermo Fisher Scientific) was used at a 1:1000 dilution with 2% BSA in TTBS. The horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1 706 516,

Bio-Rad) was diluted to 1:5000 in 2% BSA in TTBS. Membranes were then developed with chemiluminescent SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific), and imaging was performed immediately on wet membranes placed directly on the image screen of a ChemiDoc XRS imager (Bio-Rad). Analysis for determination of relative Hsp/Hsc70 protein quantity was quantified using Image Lab software (version 6.1, Bio-Rad). Hsp/Hsc70 protein levels are presented relative to the band density of the internal standard.

2.7. Glycogen content

Glycogen content was measured as described by Nancollas & Todgham (2022), modified from Fanguie et al. (2008). Samples for glycogen determination were enzymatically digested following previous methods (Hassid & Abraham 1957), and all samples were analysed for glucose following a method (Bergmeyer 1983) modified for a microplate spectrophotometer (Synergy HT, Biotek). Glycogen content was then corrected for starting free glucose.

2.8. Statistical analysis

All data sets were analysed using R (version 4.2.1) and assessed for homogeneity and normality visually by plotting using residual, density and q–q plots of the models to ensure parametric analysis of assumptions were met. All assumptions were met unless stated otherwise. For all models, an ANOVA table was generated to obtain statistical differences, and relevant post hoc tests were performed using the Tukey HSD method with the package emmeans (Lenth et al. 2019). Significance level (α) for all analyses was set at 0.05.

2.8.1. Cardiac performance

To assess for differences in thermal sensitivity of heart rates between acclimation treatments during the thermal ramp, we used generalized additive mixed modeling (GAMM) due to the nonlinear responses exhibited following Zuur et al. (2009) and Angilletta et al. (2013). Low tide habitat, thermal regime and food availability were fitted as fixed predictor factors, and temperature as a function of treatment was fitted as a smooth term. To account for repeated measures, the identity of each mussel was included as a random fac-

tor. The GAMM was performed using the restricted maximum likelihood method (REML) in order to account for small sample sizes and to avoid overfitting. Analyses were performed with the mgcv (Wood 2004) and nlme (Pinheiro et al. 2017) packages in R.

Separate linear mixed effect models (LMEs) using the REML method were fit using the nlme package with final BPT, FLT, and maximum heart rate as the response variables with low tide habitat, thermal regime, and food availability as fixed effects, and acclimation tank included as a random effect. As tidepool treatments exhibited 2 distinct breakpoints, an additional LME was run on only tidepool treatments for the first BPT, where thermal regime and food availability were considered as the fixed effects. A stepwise approach was taken for model creation, where separate models were created that included various combinations of the fixed factors and their interactions to analyze which statistical model was the best fit for the data. Model selection was achieved by comparing the AICc (to account for small sizes) of the models using the package MuMIn (Bartoń 2015). The models with the lowest AICc were deemed the most appropriate and used in analysis. Maximum heart rate data was transformed using the package bestnormalize (Peterson 2021), where an orderNorm transformation was implemented.

2.8.2. Hsp/Hsc70 and glycogen

To investigate mussel preparedness for anticipated thermal stress, linear mixed effect models were created with baseline (just before daytime low tide, 13°C) levels of Hsp/Hsc70 or glycogen as the response variable and low tide habitat, thermal regime, and food availability as the fixed factors, and acclimation tank as the random effect. To understand how Hsp/Hsc70 and glycogen change over the course of the thermal ramp, a separate set of LMEs were created that included the fixed factors low tide habitat, thermal regime, and food availability as well as sampling temperature (4 levels: baseline [13°C], 20°C, 30°C, 40°C; Fig. S2), with acclimation tank as a random effect. Similar to the cardiac performance models, a stepwise approach was taken for statistical model creation, where separate models were created that included various combinations of fixed factors and their interactions to analyze which model was the best fit for the data, and the AICc of each model was compared and the lowest was chosen for analysis. Hsp/Hsc70 data was transformed using the package bestnormalize, where a box-cox transformation was implemented.

3. RESULTS

3.1. Cardiac performance

3.1.1. Thermal performance curve

The relationship between temperature and heart rate, as temperature was increased, displayed different patterns of temperature sensitivity depending on treatment. The heart rates of mussels from all acclimation treatments were significantly influenced by temperature (Fig. 2, Table 1). Temperature sensitivity of heart rate during the acute thermal ramp was significantly affected by low tide habitat and thermal regime, but there was no effect of food availability (Table 1). Mussels from tidepool and tidally exposed treatments displayed very different cardiac responses during the acute thermal ramp. Tidepool mussels were much more thermally sensitive, exhibiting a rapid increase in heart rate with increasing temperatures, resulting in higher maximum heart rates but also 2 distinct breakpoints in heart rate. In contrast, heart rates of tidally exposed mussels were characterized by a much more gradual increase as temperature increased and generally experienced a single breakpoint close to their FLT. Thermal regime also played a significant role on thermal sensitivity (Table 1), with mussels from unpredictable treatments exhibiting higher performance curves in comparison to mussels acclimated to no heat treatments (Tukey HSD, $p = 0.006$).

3.1.2. Breakpoint temperatures

Tidepool mussels exhibited 2 distinct breakpoints, the first occurring between (mean $\pm$ SE) $26.51 \pm 1.35^\circ\text{C}$ and $28.72 \pm 0.35^\circ\text{C}$, which also coincided with the maximum heart rate, and the second (and final) BPT occurring between $36.89 \pm 0.6^\circ\text{C}$ and $40.34 \pm 0.25^\circ\text{C}$. In contrast, the majority of tidally exposed mussels exhibited a single BPT between $40.2 \pm 1.11^\circ\text{C}$ and $45.19 \pm 0.45^\circ\text{C}$. For the first breakpoint exhibited by tidepool treatments, the best model had only thermal regime as a fixed effect, as food availability did not contribute significantly to the trend. There was a significant effect of thermal regime ($F_{2,28} = 5.198$, $p = 0.012$), where predictable and unpredictable treatments had significantly higher BPTs than mussels acclimated to no heat treatments (Tukey HSD, $p = 0.049$, $p = 0.014$, respectively).

For the final BPT, which corresponds to the second breakpoint for tidepool treatments and the only breakpoint for tidally exposed treatments, the statistical model with the lowest AICc incorporated all fixed factors plus their interactions. We found that there was a significant effect of low tide habitat ($F_{1,60} = 67.011$, $p < 0.0001$; Fig. 3), with tidally exposed treatments having higher final BPT in comparison to tidepool treatments. There was also a significant effect of thermal regime ($F_{2,60} = 9.485$, $p < 0.001$; Fig. 3), where predictable and unpredictable treatments had significantly higher BPT than mus-

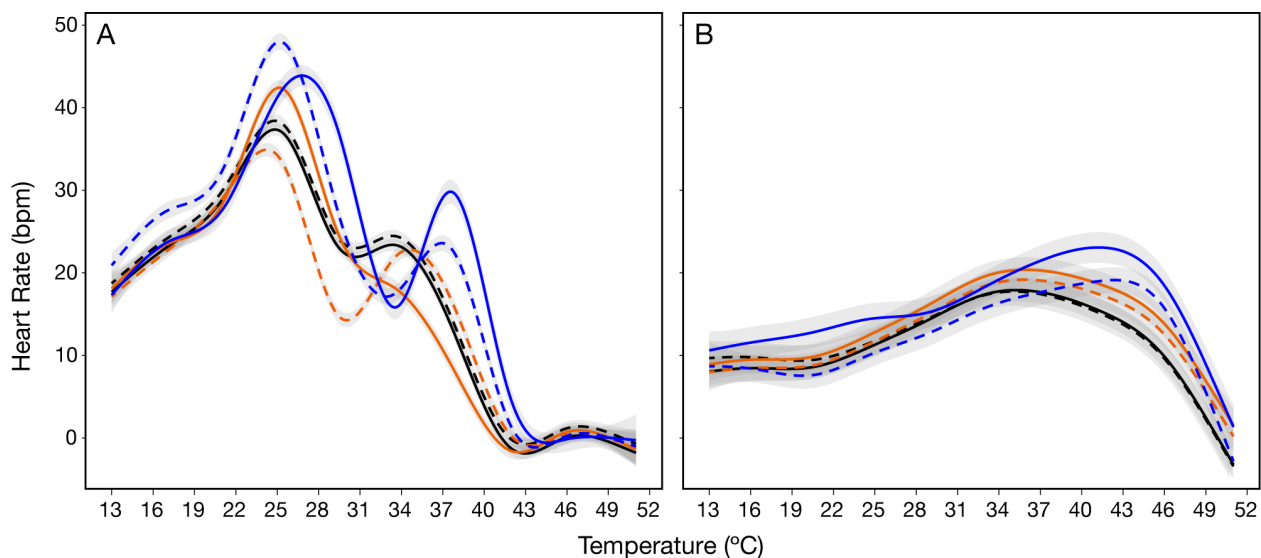


Fig. 2. Thermal performance curves produced by generalized additive mixed modeling (GAMM) for heart rates throughout the acute thermal ramp for mussels from the (A) tidepool and (B) tidally exposed treatments. Unpredictable treatments are in blue, predictable in orange and no heat treatments in black. Solid lines represent high food treatments and dashed lines represent low food. All treatments ($n = 6$ treatment $^{-1}$) were subjected to an acute thermal ramp from 13 to 52°C at a rate of 6.5°C h^{-1} ; mussels from tidepool treatments were warmed in water, whereas mussels from tidally exposed treatments were warmed in air

Table 1. ANOVA table results of the generalized additive mixed modeling (GAMM) analysis, displaying the effect of low tide habitat, thermal regime, food availability (fixed effect predictors) and their interactions, as well as the influence of temperature (smooth factor) on mussel heart rate from all 12 treatments ($n = 6$ for each treatment) during the acute thermal ramp ($6.5^{\circ}\text{C h}^{-1}$). Treatment names combine low tide habitat (S: tidepool; A: tidally exposed) with thermal regime (N: no heat; P: predictable; U: unpredictable) and food availability (L: low; H: high). F and p -values are displayed for all factors, with degrees of freedom (df) for predictor factors and effective degrees of freedom (edf) for temperature

Predictor factor	df	F	p
Low tide habitat	1	14.073	0.002
Thermal regime	2	4.098	0.017
Food	1	0.054	0.817
Low tide habitat $\times$ thermal regime	2	1.269	0.281
Low tide habitat $\times$ food	1	0.095	0.758
Food $\times$ thermal regime	2	1.887	0.152
Low tide habitat $\times$ thermal regime $\times$ food	2	0.861	0.423
s(Temperature)	edf		
SNL	8.315	129.896	<0.0001
SNH	8.321	139.045	<0.0001
SPL	8.655	113.309	<0.0001
SPH	8.530	171.461	<0.0001
SUL	8.775	118.729	<0.0001
SUH	8.887	160.115	<0.0001
ANL	4.818	18.906	<0.0001
ANH	5.556	33.844	<0.0001
APL	3.89	36.329	<0.0001
APH	4.638	25.894	<0.0001
AUL	5.236	14.409	<0.0001
AUH	4.853	8.937	<0.0001

sels acclimated to no heat treatments (Tukey HSD, $p = 0.049$, $p = 0.002$, respectively). There was no effect of food availability on final BPT ($F_{1,60} = 0.378$, $p = 0.541$), but mussels from the low food treatments, especially in the tidally exposed habitat, exhibited a higher degree of individual variability than mussels from the high food groups for final BPT (Fig. 3).

3.1.3. Flatline temperature

The best statistical model included all fixed factors without their interactions. We found that there was a significant effect of low tide habitat ($F_{1,67} = 109.52$, $p < 0.0001$; Fig. 4) with tidally exposed treatments having higher FLT than tidepool treatments. The FLT for tidepool treatments ranged from $40 \pm 0.12^{\circ}\text{C}$ to $42.28 \pm 0.22^{\circ}\text{C}$, whereas the FLT of tidally exposed treatments were much more variable, ranging from $43.28 \pm 0.38^{\circ}\text{C}$ to $47.7 \pm 0.36^{\circ}\text{C}$. There was also a significant effect of thermal regime ($F_{2,67} = 10.55$, $p < 0.001$;

Fig. 4), where the unpredictable treatments had significantly higher FLT than mussels acclimated to no heat and predictable treatments (Tukey HSD, $p = 0.001$, $p = 0.029$, respectively). There was no effect of food availability on FLT ($F_{1,67} = 1.99$, $p = 0.163$).

3.1.4. Maximum heart rate

The best statistical model included all fixed factors with their interactions. Maximum heart rate was significantly different between low tide habitat ($F_{1,60} = 103.794$, $p < 0.0001$; Table 2), where tidepool mussels had much higher (almost double) maximum heart rates than tidally exposed mussels. In tidepool mussels, maximum heart rates ranged from (mean $\pm$ SE) 44.15 ± 1.49 bpm to 49.56 ± 1.12 bpm, whereas tidally exposed treatments ranged from 23.55 ± 1.54 bpm to 26.52 ± 1.38 bpm. There was no difference in maximum heart rate due to thermal regime ($F_{2,60} = 0.520$, $p = 0.597$) or food availability ($F_{1,60} = 0.055$, $p = 0.816$).

3.2. Cellular stress mechanisms and energy stores

3.2.1. Hsp/Hsc70

For analysis of baseline (13°C , taken just before low tide) levels to understand preparedness for an anticipated period of stress, the best statistical model included all fixed factors and their interactions. Comparisons of baseline Hsp/Hsc70 levels revealed that thermal regime ($F_{2,60} = 4.385$, $p = 0.017$) significantly impacted baseline Hsp/Hsc70 levels, but there was also a significant interaction between low tide habitat and thermal regime ($F_{2,60} = 3.243$, $p = 0.046$). Analysis of post-hoc tests revealed that in the tidepool habitat, mussels from both the predictable and unpredictable treatments had higher baseline Hsp/Hsc70 levels than mussels acclimated to the no heat treatment (Tukey HSD, $p = 0.041$, $p = 0.033$, respectively). In contrast, while tidally exposed mussels exhibited a similar trend, there was no difference in baseline Hsp/Hsc70 levels between the different thermal regimes. Food availability also affected the baseline

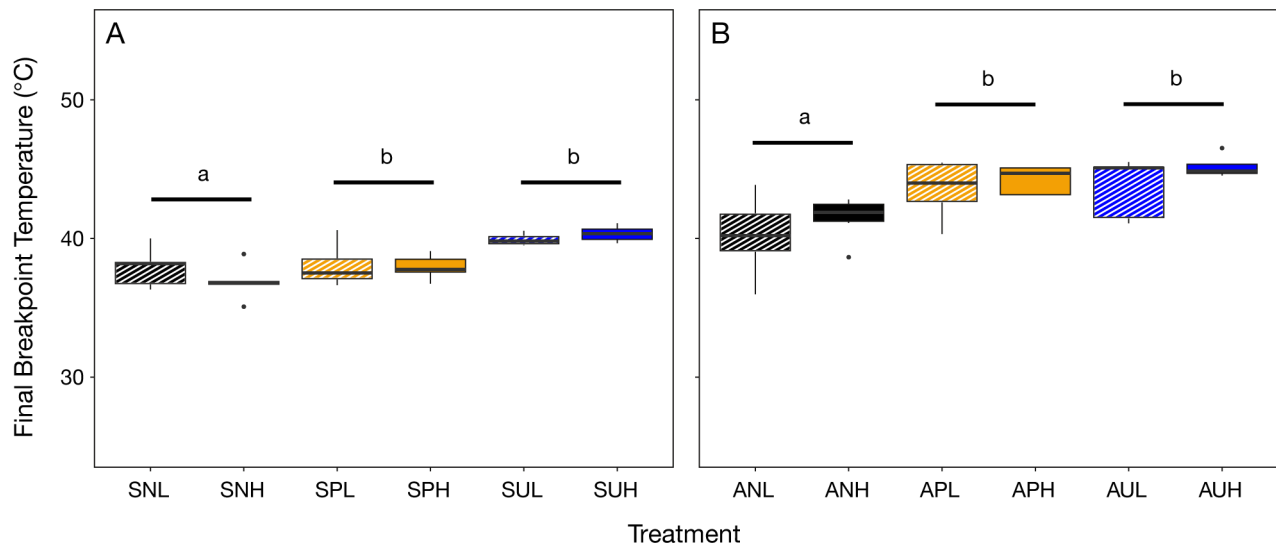


Fig. 3. Final breakpoint temperatures for *Mytilus californianus* from (A) tidepool and (B) tidally exposed treatments. Unpredictable (U) treatments are in blue, predictable (P) in orange and no heat (N) treatments are in black. Solid plots represent high food (H) treatments and plots with diagonal lines represent low food (L) treatments. All treatments ($n = 6$ treatment⁻¹) were subjected to an acute thermal ramp from 13 to 52°C at a rate of 6.5°C h⁻¹; mussels from tidepool treatments were warmed in water, whereas mussels from tidally exposed treatments were warmed in air. The lines on the boxplots represent the median, the box represents the inter-quartile range (IQR), the whiskers extend 1.5× IQR. Points beyond the whiskers are outliers. Different letters represent a significant difference between thermal regimes (Tukey HSD, $p < 0.05$) within a given low tide habitat type. Tidally exposed mussels had a significantly higher final breakpoint temperature than tidepool mussels (linear mixed effect model [LME], $p < 0.0001$). There was no significant difference between low and high food treatments (LME, $p > 0.05$).

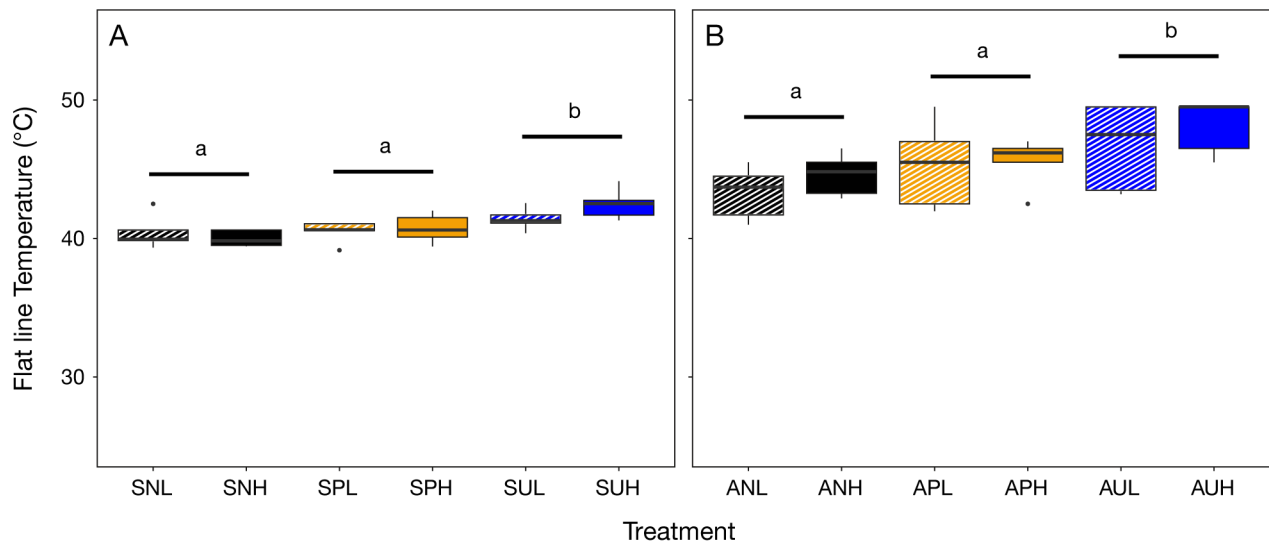


Fig. 4. Flat line temperatures for *Mytilus californianus* from (A) tidepool and (B) tidally exposed treatments. Unpredictable (U) treatments are in blue, predictable (P) in orange and no heat (N) treatments are in black. Solid plots represent high food (H) treatments and plots with diagonal lines represent low food (L) treatments. All treatments ($n = 6$ treatment⁻¹) were subjected to an acute thermal ramp from 13 to 52°C at a rate of 6.5°C h⁻¹; mussels from tidepool treatments were warmed in water, whereas mussels from tidally exposed treatments were warmed in air. The lines on the boxplots represent the median, the box represents the inter-quartile range (IQR), the whiskers extend 1.5× IQR. Points beyond the whiskers are outliers. Different letters represent a significant difference between thermal regimes (Tukey HSD, $p < 0.05$) within a given low tide habitat type. Tidally exposed mussels had significantly higher flat line temperature than tidepool mussels (LME, $p < 0.0001$). There was no significant difference between low and high food treatments (LME, $p > 0.05$).

Table 2. Heart rate metrics for mussels exposed to an acute thermal ramp ($6.5^{\circ}\text{C h}^{-1}$) that had been acclimated to tidepool (S) or tidally exposed (A) habitats with either no heat (N), predictable (P) or unpredictable (U) thermal stress during daytime low tides with low (L) or high (H) food availability. Metrics are: initial heart rate (IHR) taken at the start of the acute thermal ramp at 13°C and maximum heart rate (MHR) achieved during the acute thermal ramp; number of breaks in cardiac function exhibited by an individual. Values are presented as mean $\pm$ SE for 6 individuals

Acclimation treatment	IHR	MHR	No. of breaks
SNL	19.48 ± 1.57	48.12 ± 1.50	2.17 ± 0.17
SNH	21.01 ± 0.45	49.09 ± 3.09	2 ± 0
SPL	14.51 ± 0.93	44.15 ± 1.49	2.5 ± 0.22
SPH	18.92 ± 1.05	48.52 ± 1.42	2.17 ± 0.17
SUL	21.29 ± 2.31	49.13 ± 2.25	2.5 ± 0.22
SUH	17.27 ± 2.17	49.85 ± 3.27	2.17 ± 0.17
ANL	9.18 ± 1.48	23.88 ± 2.58	1 ± 0
ANH	8.42 ± 0.92	24.69 ± 0.82	1 ± 0
APL	8.88 ± 1.04	24.55 ± 1.72	1 ± 0
APH	8.10 ± 0.63	23.55 ± 1.54	1.17 ± 0.17
AUL	11.03 ± 0.35	24.99 ± 0.25	1 ± 0
AUH	7.27 ± 0.92	26.51 ± 1.38	1.17 ± 0.17

Hsp/Hsc70 levels ($F_{1,60} = 10.16$, $p = 0.002$), where low food groups had lower baseline levels of Hsp/Hsc70 than high food groups.

When comparing Hsp/Hsc70 levels during the thermal ramp, mussels exhibited different responses of Hsp/Hsc70 levels, depending on acclimation treatment. The best statistical model included low tide habitat, food availability, and sampling temperature with their interactions, as thermal regime did not contribute significantly to the trend. Both low tide habitat ($F_{1,235} = 8.527$, $p = 0.004$; Fig. 5) and sampling temperature ($F_{3,235} = 13.134$, $p < 0.0001$) had a significant effect on Hsp/Hsc70 levels during the acute ramp, but there was also a significant interaction effect between low tide habitat, food availability, and sampling temperature ($F_{3,235} = 3.794$, $p = 0.011$). With post hoc analysis, it was revealed that this interactive effect was due to the Hsp/Hsc70 levels of the high food tidepool treatments increasing over the course of the acute thermal ramp, where Hsp/Hsc70 levels at 30 and 40°C were significantly elevated over baseline (13°C) and 20°C levels (Tukey HSD, $p < 0.05$ for all). In comparison, low food tidepool treatments saw an increase between baseline (13°C) levels and 20°C (Tukey HSD, $p = 0.013$), largely driven by the tidepool, predictable, low treatment, but then returned to baseline (13°C) levels by 30°C . High food tidally exposed treatments saw no

change in Hsp/Hsc70 during the thermal ramp, but low food treatments did show a small, but progressive, increase over the course of the ramps, whereby levels at 40°C were elevated over baseline (13°C) and 20°C levels (Tukey HSD, $p = 0.003$, $p = 0.014$, respectively)

3.2.2. Glycogen

For analysis of baseline (13°C , taken just before low tide) levels of glycogen, to understand preparedness for an anticipated period of stress, the best statistical model included all fixed factors and their interactions. Baseline (13°C) glycogen levels were affected by thermal regime ($F_{2,60} = 3.597$, $p = 0.034$), with mussels from unpredictable treatments having elevated glycogen levels over mussels acclimated to no heat treatments (Tukey HSD, $p = 0.025$), but there was no effect of low tide habitat ($F_{1,60} = 0.143$, $p = 0.706$) or food availability ($F_{1,60} = 0.725$, $p = 0.398$) on baseline (13°C) levels.

For assessment of glycogen stores during the acute thermal ramp, the best statistical model included low tide habitat, thermal regime, and sampling temperature with their interactions, as food availability did not contribute significantly to the trend. Low tide habitat ($F_{1,235} = 15.997$, $p < 0.0001$; Fig. 6) and sampling temperature ($F_{3,235} = 5.435$, $p = 0.001$; Fig. 6) had significant effect on glycogen levels during the thermal ramp, but there was also a significant interaction between low tide habitat and sampling temperature ($F_{3,235} = 6.985$, $p < 0.001$). Post hoc analysis revealed that this interaction was due to tidepool treatments experiencing a decline in glycogen levels over the course of the thermal ramp, where glycogen was significantly lower than baseline (13°C) and 20°C levels at 30 and 40°C (Tukey HSD, $p < 0.05$ for all). The tidepool, unpredictable, high treatment had the largest decline in glycogen (72%) during the acute ramp, starting with the highest baseline (13°C) glycogen levels of the tidepool treatments (mean $\pm$ SE, 159.78 ± 15.01 $\mu\text{mol glucosyl units g}^{-1}$) and resulting in the lowest glycogen levels at 40°C (46.35 ± 6.28 $\mu\text{mol glucosyl units g}^{-1}$). In contrast, tidally exposed treatments maintained similar levels of glycogen throughout the thermal ramp. Thermal regime also significantly affected glycogen levels ($F_{2,235} = 3.930$, $p = 0.021$), with mussels from unpredictable treatments having significantly different glycogen levels in comparison to mussels acclimated to no heat treatments (Tukey HSD, $p = 0.048$).

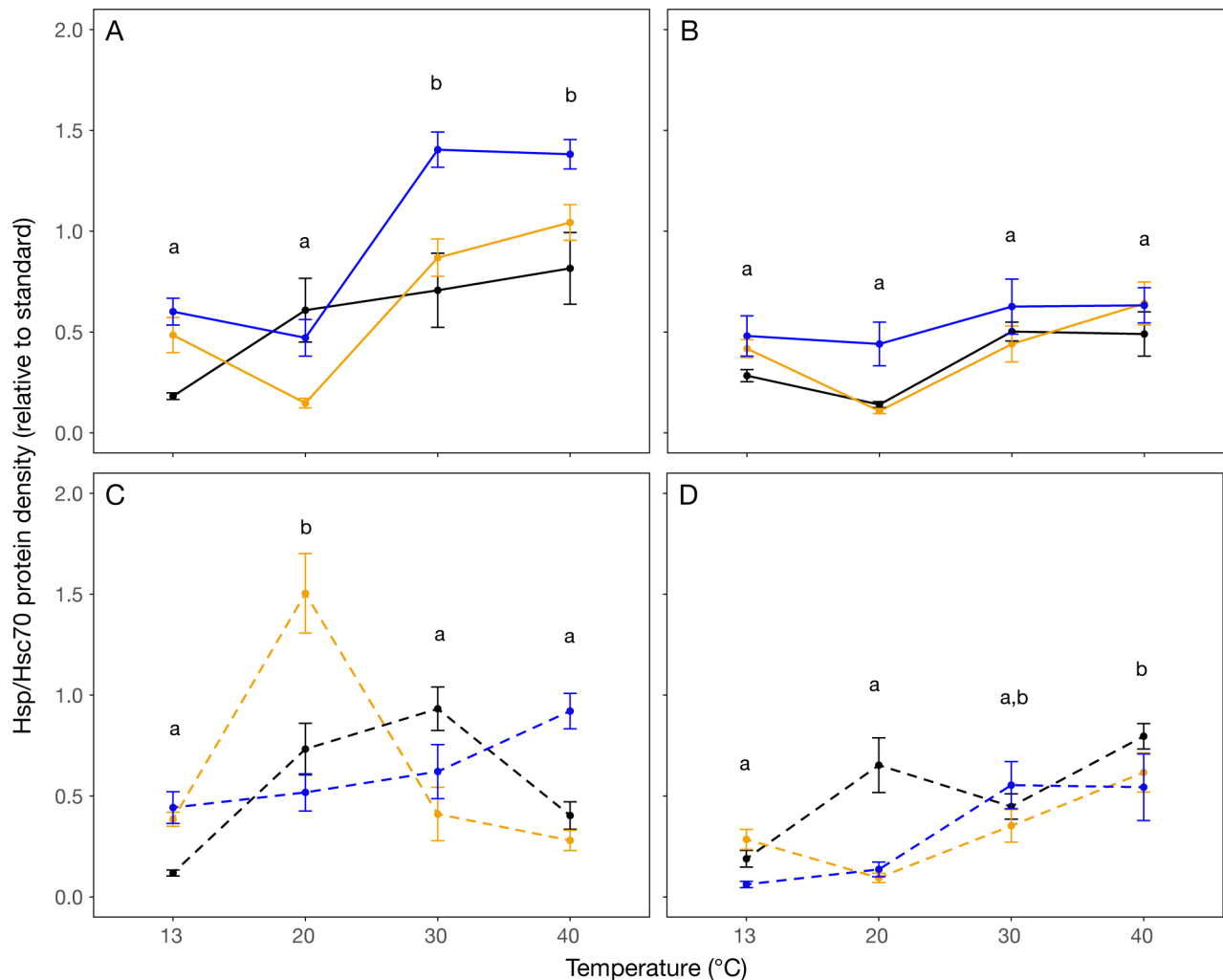


Fig. 5. Hsp/Hsc70 of *Mytilus californianus* gill tissue taken just before the daytime low tide (baseline:13°C) and at 20, 30, and 40°C during an acute thermal ramp. Due to a statistically significant interactive effect between low tide habitat, food availability, and sampling temperature, plots have been separated out into (A) tidetpool high food; (B) tidally exposed high food; (C) tidetpool low food; (D) tidally exposed low food treatments. (A–D) Unpredictable treatments are in blue, predictable in orange and no heat treatments are in black. For continuity with other plots, solid lines represent high food treatments and dashed lines represent low food treatments. All treatments ($n = 6$ treatment⁻¹) were subjected to an acute thermal ramp from 13 to 52°C at a rate of 6.5°C h⁻¹; mussels from tidetpool treatments were warmed in water, whereas mussels from tidally exposed treatments were warmed in air. Values are mean $\pm$ SE. Different letters represent a significant difference in levels at different sampling temperatures (Tukey HSD, $p < 0.05$), within a given low tide habitat $\times$ food availability plot. There was no significant difference in thermal regime (LME, $p > 0.05$)

4. DISCUSSION

A significant amount of attention has been focused on the thermal physiology of intertidal animals; however, there remains a large gap in our understanding of how the complexities of the thermal signal (e.g. low tide habitat, predictability, interaction with other stressors) integrate to modulate thermal tolerance. Our results suggest that low tide habitat was the largest driver for modulating physiological responses during an acute thermal ramp. Tidetpool mussels (i.e.

submerged during low tide vs. exposed to air) exhibited higher heart rates with multiple breaks, but lower upper thermal tolerance (both final BPT and FLT). This was combined with greater glycogen usage and elevated cellular stress response, suggesting that thermal stress in an aquatic medium is more stressful than in air. Mussels in unpredictable treatments also had increased FLT and baseline glycogen content, indicating that prior exposure to unpredictable thermal fluctuations serves to increase thermal tolerance, possibly through boosting carbohydrate stores.

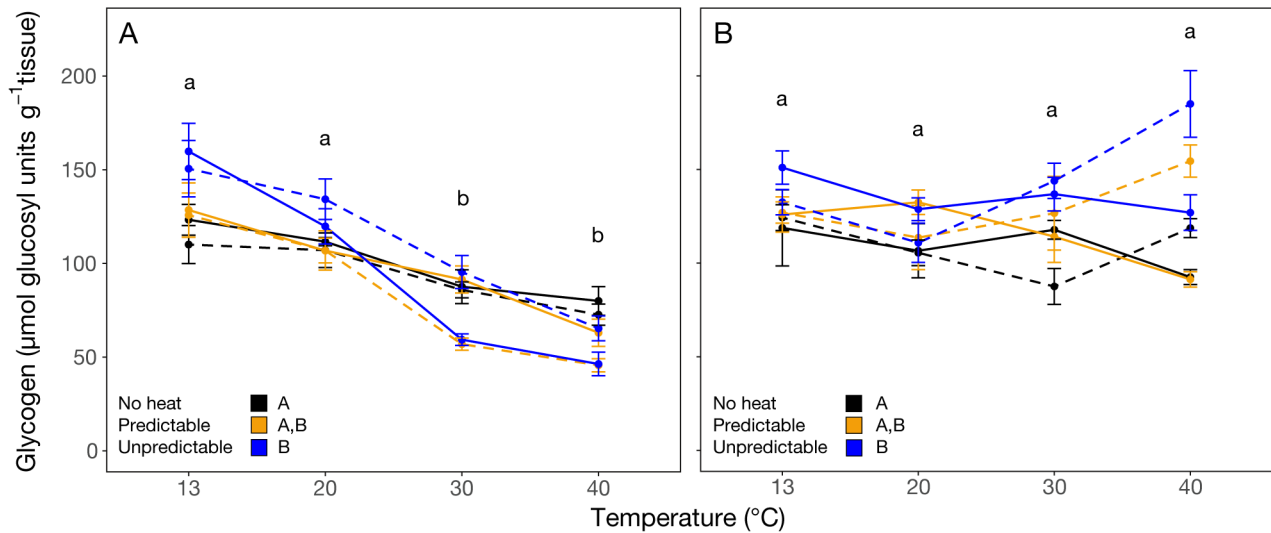


Fig. 6. Glycogen content of *Mytilus californianus* mantle tissue taken just before the daytime low tide (baseline: 13°C) and at 20, 30, and 40°C during an acute thermal ramp from (A) tidepool; (B) tidally exposed treatments. Unpredictable treatments are in blue, predictable in orange and no heat treatments are in black. Solid lines represent high food treatments and dashed lines represent low food treatments. All treatments ($n = 6$ treatment⁻¹) were subjected to an acute thermal ramp from 13 to 52°C at a rate of 6.5°C h⁻¹; mussels from tidepool treatments were warmed in water, whereas mussels from tidally exposed treatments were warmed in air. Values are mean $\pm$ SE. Different lowercase letters represent a significant difference in levels at different sampling temperatures (Tukey HSD, $p < 0.05$) within a given low tide habitat. Different uppercase letters represent a significant difference between thermal regimes. There was no effect of food availability

Surprisingly, food availability had little impact on cardiac performance or energy stores, but did affect baseline and subsequent induction of Hsp/Hsc70, which had low tide habitat-specific effects. Taken together, our results indicate that the thermal physiology of *Mytilus californianus* is regulated by several different environmental signals, and the effects of climate change on performance of mussels will depend upon specific changes to the physical and temporal characteristics of their thermal environment.

4.1. Low tide habitat

The cardiac performance and thermal sensitivity of mussels acclimated to tidepool and tidally exposed habitats were markedly different, suggesting that low tide habitat plays an important role in modulating physiological performance during thermal stress. Tidepool mussels exposed to an acute thermal ramp in water exhibited much higher maximum heart rates, multiple breakpoints in heart rate, but a lower upper thermal tolerance (i.e. final BPT and FLT) in comparison to mussels from tidally exposed treatments that experienced thermal stress during air exposure. Our results align with previous studies that show thermal tolerance of rocky intertidal organisms is higher during emersion in comparison to immersion (e.g. Jones

et al. 2009, Bjelde & Todgham 2013, Huang et al. 2015, Tagliarolo & McQuaid 2016). These differences suggest that the interaction of air exposure and increasing temperatures may confer enhanced stress tolerance in adult mussels. The mechanism behind this enhanced tolerance is most likely related to differences in valve gaping behaviour coupled with contrasts in metabolic efficiency and metabolic strategy to tolerate thermal stress between tidepool and tidally exposed mussels.

When immersed, *M. californianus* gapes and actively engages in ventilation to promote gas exchange and aerobic respiration, resulting in higher initial and overall heart rates in comparison to tidally exposed mussels due to increased energy demands associated with filtration and ventilation (Bayne et al. 1976). In the current study, tidepool mussels have 2 distinct breakpoints during warming, the first between 26 and 29°C and a second between 36 and 40°C. Maximum heart rate is reached at the first breakpoint, but not the second, suggesting that the first breakpoint could indicate capacity limitations coupled with shifts in behavioural and metabolic responses to increasing temperatures. This initial breakpoint is comparable with the critical temperature found in other *Mytilus* spp. experiencing aquatic warming at a similar rate (Braby & Somero 2006, Tagliarolo & McQuaid 2015). Valve closure has been shown to occur at the critical

temperature in *Mytilus* spp. during aquatic warming (Braby 2004), which could explain the sharp drop in heart rate exhibited after the first breakpoint (Braby & Somero 2006). It is unclear however, whether heart rate depression or valve closure occurs first (Braby & Somero 2006). Valve closure and/or heart rate depression at this critical temperature results in a shift from aerobic to anaerobic metabolism (Braby & Somero 2006), as restrictions to gas exchange result in the mantle cavity environment becoming hypercapnic and hypoxic (Burnett 1988). In intertidal molluscs, the critical temperature is often considered the onset of sublethal stress and the initiation of the heat shock response (Dong et al. 2022). This consequently results in a decline in energy stores due to the relative inefficiency of anaerobiosis in generating ATP coupled with the high energetic costs of the heat shock response (Dowd & Somero 2013). In tidepool mussels, glycogen content started to significantly decline and Hsp/Hsc70 levels started to rise at 30°C, just after the first breakpoint, supporting the likelihood of a shift to anaerobic metabolism and status of sublethal stress. Typically, continued performance in this sublethal range is time limited and can quickly progress into lethal temperatures (FLT) unless further behavioural and/or metabolic modifications occur (Sokolova et al. 2012, Dong et al. 2022). In the case of tidepool mussels, individuals may be able to extend this sublethal range by maintaining valve closure and depressing heart rate for a short period of time in an effort to reduce metabolic demands and conserve energy (Braby & Somero 2006, Hui et al. 2020, Liao et al. 2021), as valve closure during warming has been shown to elicit metabolic depression (Ortmann & Grieshaber 2003, Anestis et al. 2007). This could allow for heart rate to fluctuate between the zones of temperature sensitivity and insensitivity, which may act as a reprieve in order to maintain cardiac function at higher temperatures, and has been observed by a number of intertidal mollusc species during a thermal ramp (Braby & Somero 2006, Bjelde & Todgham 2013, Zhang et al. 2014, Hui et al. 2020). More investigation into valve gaping behaviour as well as metabolic and energy dynamics that occurs between multiple breakpoints may help elucidate the physiological function of multiple breaks in heart rate in intertidal molluscs. While tidepool mussels may be able to employ cardiac modulation to extend cardiac performance at increasing temperatures, the early capacity limitations coupled with the prolonged period of time spent in a sublethal performance range in comparison to tidally exposed mussels ultimately resulted in a lower upper thermal tolerance than tidally exposed mussels.

The ability of tidally exposed mussels to circumvent the early limitations on capacity and thus result in elevated final BPT and FLT is likely due to a combined effect of the behaviour and metabolic strategy utilized to tolerate thermal stress coupled with increased metabolic efficiency. When emersed, *M. californianus* does not typically exhibit gaping behaviour (Widdows & Shick 1985, Gracey & Connor 2016), and valve closure is accompanied by hypercapnia, hypoxia, and lowered pH of residual water within the mantle cavity due to restrictions on gas exchange (Burnett 1988), resulting in a reliance on anaerobic metabolism (Bayne et al. 1976). In order to tolerate these conditions, many mollusc species metabolically depress in order to reduce energy demands and reliance on anaerobic metabolism (Connor & Gracey 2012, Lesser 2016, Connor & Gracey 2020). Consequently, intertidal organisms acclimated to cyclical regime of periodic air exposure exhibit a number of physiological modifications that promote metabolic efficiency to reduce metabolic demand during emersion (De Zwaan 1983, Widdows & Shick 1985, Demers & Guderley 1994, Nancollas & McGaw 2021a,b) such as reduced ammonia excretion (Widdows & Shick 1985), and metabolism of alternative substrates during air exposure for higher ATP yield per glycosyl unit oxidized (De Zwaan 1983, Demers & Guderley 1994). Evidence of valve closure and the resulting reduced activity and metabolic demand can be observed in this study by comparing the initial heart rates of tidepool and tidally exposed mussels at ambient temperature (13°C; Table 2). On average, tidepool mussels had an initial heart rate of 18.74 bpm, whereas the heart rate of tidally exposed mussels was 8.81 bpm — less than 50% of tidepool mussels, indicating reduced activity and baseline metabolic costs or active depression of metabolic processes during air exposure (Connor & Gracey 2012, Lesser 2016). Furthermore, while there was a large difference in the maximum heart rate between tidepool and tidally exposed mussels, tidally exposed mussels, on average, exhibited a larger proportional increase in heart rate from initial levels over the course of the thermal ramp (~2.82 fold) than tidepool mussels (~2.55 fold). Taken together, this suggests that tidally exposed mussels in this study may have lower metabolic demands during thermal stress as well as a more efficient mechanism of meeting that demand, which could enable the maintenance of cardiac performance and extension of upper thermal tolerance limits. Further evidence of metabolic depression being used as an energy saving strategy in tidally exposed *M. californianus* can be observed by comparing the glycogen usage between

the 2 low tide habitats. In tidepool mussels, glycogen declined with increasing temperatures, reflecting the breakdown of energy reserves to meet metabolic demand and fuel the Hsp/Hsc70 response. In contrast, tidally exposed mussels maintained similar levels of glycogen throughout the thermal ramp, indicating reduced reliance on energy stores during thermal stress or a suppression of enzyme activity related to glycogenolysis (De Zwaan 1983). This supports previous research that indicates metabolic rate depression is an energy conservation strategy (Marshall et al. 2011, Hui et al. 2020).

4.2. Thermal regime

Mussels acclimated to predictable and unpredictable thermal stress can prolong optimal cardiac performance, as evidenced by having an elevated final BPT in comparison to mussels acclimated to no heat, complementing previous research that acclimation to elevated temperatures increases upper thermal tolerance (e.g. Schaefer & Ryan 2006, Oliver & Palumbi 2011, Kern et al. 2015, Schoepf et al. 2015, Cheng et al. 2018). These results differ from our previous findings with winter-acclimated mussels, where only mussels in the unpredictable regime had higher BPT than control mussels (Nancollas & Todgham 2022). These contrasting results suggest that increases in BPT could be dependent upon a specific temperature threshold in their prior thermal history, or it could be regulated by specific seasonal characteristics. In the previous study (Nancollas & Todgham 2022), predictable mussels were warmed to 20°C every daytime low tide, and unpredictable mussels experienced a different max temperature within the range of 13–28°C. The increases in final BPT exhibited by predictable mussels in the current study suggests that exposure to predictable thermal fluctuations to 20°C was not enough to increase final BPT, but 25°C was, indicating that temperatures between 20–25°C represent a crucial threshold for modulating thermal tolerance.

Acclimation to thermal stress increased final BPT, but only acclimation to an unpredictable thermal regime increased FLT, suggesting that a specific element of the unpredictable thermal regime is a key driver for increasing how long the mussels can continue to beat past final BPT. The predictable and unpredictable regimes were designed to have the same mean temperature over the course of acclimation (25°C), but differed in other elements, such as prior thermal history, thermal variability, and unpredictability. In our previous study (Nancollas & Todg-

ham 2022), acclimation to an unpredictable thermal regime increased BPT, but not FLT, suggesting that thermal unpredictability alone is not the factor that increased FLT in the current study. It is possible that the increased FLT exhibited by mussels in the unpredictable regime is due to the larger thermal range experienced by mussels in the unpredictable treatments over the course of the acclimation period and providing an additional thermal safety margin between final BPT and FLT. Just as predictable thermal fluctuation can increase thermal performance in comparison to a constant mean temperature, increases in thermal range around the same mean temperature can also increase thermal tolerance (Schaefer & Ryan 2006, Kern et al. 2015, Schoepf et al. 2015, 2022, Salinas et al. 2019). Even though mussels from unpredictable treatments were not experiencing a higher degree of thermal variability each day of the acclimation period, experiencing a higher maximum temperature at some point in their recent thermal history may be enough to initiate physiological mechanisms to extend FLT. Indeed, Moyon et al. (2020) has recently shown that *M. californianus* acclimated to 14°C acquire improved heat tolerance when exposed to a single sublethal bout of heat stress (30 or 35°C for 2 h) up to 3 wk prior to exposure to extreme heat stress (40°C for 2 h). Similarly, heat hardening increases heat tolerance, antioxidant defence, and metabolic remodelling in *M. galloprovincialis* (Georgoulis et al. 2021, 2022). Taken together, these results could suggest that the way in which mussels respond to thermal stress is nuanced, and the magnitude of temperature experienced in their thermal history and predictability or repeatability of exposure may convey potentially different physiological enhancements to thermal stress. This could suggest that modulations on BPT may depend on regular exposure to a threshold temperature (most likely between 20 and 25°C), whereas FLT may be modulated by magnitude of temperature (>30°C) in recent thermal history.

Mussels acclimated to unpredictable thermal stress had elevated baseline glycogen levels, and in tidepool mussels had a greater depletion during thermal stress which likely contributed to the increased FLT seen in mussels acclimated to unpredictable thermal stress, suggesting that energy roles play an important role in tolerating unpredictable conditions. These results align with our previous research (Nancollas & Todgham 2022), where mussels acclimated to unpredictable thermal regimes had the highest glycogen content just before low tide. At the time, we speculated that this was to prepare for the anticipated stress of low tide conditions. While this was indeed the case in tidepool

mussels, tidally exposed mussels maintained baseline levels throughout the course of the acute thermal ramp. This raises the question: Why maintain elevated levels of glycogen if not to use during periods of stress? One possible explanation is that glycogen was reserved for tolerating the repercussions of the low tide period during re-immersion. Metabolic depression during emersion often results in the suppression of many energy demanding pathways, and maintenance of glycogen stores during metabolic depression has been reported for molluscs (De Zwaan & Putzer 1985, Isani et al. 1995, Picard et al. 2014), due to the suppression of enzymes involved in glycogenolysis (De Zwaan & Putzer 1985). Upon re-immersion and return to aerobic metabolism, intertidal organisms often experience high metabolic demand or 'oxygen debt' in order to re-establish physiological homeostasis, which is exacerbated when coupled with thermal stress (Hofmann & Somero 1995, Tomanek & Somero 2000, Schill et al. 2002, Dudognon et al. 2013, Zhang et al. 2014, Ivanina et al. 2016). Glucose levels in *M. californianus* exhibit a circatidal rhythm with spikes during re-immersion (Connor & Gracey 2012), and evidence of high rates of both aerobic and anaerobic component of metabolism have been reported during reimmersion (Zurburg et al. 1982, De Zwaan 1983, Nicchitta & Ellington 1983). It is possible that when re-immersed, breakdown of glycogen reserves is needed to fuel the elevated energy demand, and mussels from unpredictable treatments have elevated levels due to the potential of experiencing elevated thermal stress in comparison to other treatments. Investigation into glycogen usage during different periods of the tidal cycle and how it interplays with temperature would aid in our understanding of how energy homeostasis is regulated.

4.3. Food availability

Surprisingly, food availability had little effect on thermal performance of *M. californianus* in this study. Previous studies have indicated that low food availability can exacerbate the effects of thermal stress, leading to lower upper thermal tolerance and reduced survival (Dahlhoff & Menge 1996, Dahlhoff et al. 2001, Dahlhoff 2004, Fitzgerald-Dehoog et al. 2012, Matzelle et al. 2015, Cheng et al. 2018). In our study, we predicted similar effects, where mussels under low food regimes would have reduced energy resources to tolerate acute thermal stress, resulting in lower upper thermal limits. Contrary to our prediction, low food availability did not influence the majority of our metrics measured or upper thermal tolerance. Low

food availability did have an effect on the baseline levels of Hsp/Hsc70, as well as changes in Hsp levels during the acute thermal ramp, which was different between tidepool vs. tidally exposed mussels.

The lack of influence of food availability on upper thermal limits could be due to a few reasons. One possibility is that the food available in our low food group was not low enough to be metabolically limiting, and this is indicated by the comparable levels of glycogen seen between the high and low food groups. We chose our food levels based on the average high and low chlorophyll levels found in Bodega Bay, and the same food levels have been used by other studies using live algae (Hettinger et al. 2013). Many studies that have investigated the interaction between temperature and low food availability have typically looked at their interaction in relation to growth or survival by incorporating longer (>8 wk) acclimation times (Schneider & Helmuth 2007, Schneider et al. 2010, Fitzgerald-Dehoog et al. 2012). Food availability may only play an important role in thermal performance when low food conditions persist over a longer period of time (>8 wk) or when prior thermal history (i.e. acclimation temperature) is 30°C or above for multiple subsequent days, a conclusion also surmised by Vasquez & Tomanek (2019). Indeed, we have found the role of food availability to play a much larger role in thermal performance when mussels experience multiple subsequent days of sublethal heat stress (35°C) (S. J. Nancollas & A. E. Todgham unpubl. data). This indicates that current low levels of food in Bodega Bay are likely sufficient to maintain upper thermal limits to acute hot days but may impact thermal performance and survival if mean conditions are >30°C or mussels are exposed to a multi-day heatwave event. Investigation into how low food availability may affect other important metrics such as antioxidant defence or energy regulation may provide a more comprehensive understanding of the short-term effects of low food.

Acclimation to a low food regime appears to limit the ability of mussels to produce preparatory levels of Hsp/Hsc70 and alters the induction response during the acute thermal ramp, suggesting that low food may modulate the cellular stress response. Similar results were found in whelks and gastropods, where starvation led to the reduced ability to mount an Hsp70 response (Dahlhoff et al. 2002, Jeno & Brokordt 2014). Acclimation to a low food regime also altered how Hsp/Hsc70 was induced during the acute thermal ramp and exhibited low tide habitat specific responses. In tidepool treatments, acclimation to low food appeared to result in an earlier induction of Hsp/Hsc70, with mussels acclimated to a low food regime elevat-

ing Hsp/Hsc70 at 20°C (largely driven by the tidepool predictable low food treatment) but then returning to baseline levels for the rest of the acute thermal ramp. In contrast, tidally exposed treatments were able to maintain a small, but persistent increase over the course of the thermal ramp, largely driven by low baseline levels, especially by the tidally exposed, unpredictable, low treatment. There have been few studies that have investigated how low food affects the Hsp/Hsc70 response, so the mechanism behind the low tide habitat-specific differences presented here remains unknown. It is possible that acclimation to low food regimes could reduce the induction temperature and/or magnitude of responses through the interaction between energy pathways and the cellular stress response (Vasquez & Tomanek 2019). A more targeted investigation into how low food shapes the energetic pathways associated with the cellular stress response is needed to elucidate this association.

5. CONCLUSIONS

In conclusion, *Mytilus californianus* incorporates information from a number of abiotic and biotic signals to inform thermal performance. From our results, it is clear that experiencing acute thermal stress in aquatic or aerial setting results in different strategies for tolerating thermal stress. Importantly, tidepool mussels are already regularly experiencing temperatures that exceed their breakpoint temperature due to climate induced warming, and the striking difference in performance of mussels in different low tide habitats suggests that increases in acute hot days may more negatively affect mussels in aquatic habitats. Further increases in temperature could have severe consequences for tidepool community composition and function as well as the rocky intertidal more broadly. Here, the thermal pattern was based on a tidepool that contained *M. californianus* that was a representative size of *M. californianus* containing tidepools on this and nearby shores, but tidepools of different sizes will have different thermal properties and thus should be considered when interpreting these results. Similarly, the assessment of Hsc/Hsp70 and glycogen were analysed in gill and mantle tissue, respectively, and analysis of these metrics in other tissues is needed in order to provide a more comprehensive understanding of how tidally exposed and tidepool mussels differ in response to thermal stress.

Acclimation to unpredictable fluctuations in temperature, which intertidal organisms are typically exposed to *in situ*, appears to increase upper thermal

tolerance by extending the upper temperature limit for maintaining cardiac function, suggesting that intertidal organisms may be more tolerant to increases in temperature than originally thought. However, we know little about the physiological trade-offs that may come with enhanced tolerance and the apparent need to maintain high energy stores. Further investigation into how elevated thermal tolerance affects life history traits such as reproduction will be important for understanding the long-term community effects of the predicted increase in thermal unpredictability. Lastly, while low food availability did not significantly impact upper thermal tolerance to acute thermal stress, it did appear to affect the preparatory levels and subsequent induction of Hsp/Hsp70 during thermal stress. Therefore, low food availability could affect the ability of organisms to mount a sufficient cellular stress response, which could have critical consequences for tolerating repeated sublethal thermal events, like a heatwave. Moving forward, investigation into the contrasting metabolic strategies and energy pathways exhibited by tidepool and tidally exposed mussels and how this relates to informing thermal performance would help understand more holistically how intertidal organisms that exist on the rocky shore and/or tidepools will perform in future climate change scenarios.

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