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Hot Days and Heatwaves: How Environmental Complexity Shapes the Physiological Responses of Mussels to Climate Change

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# Hot Days and Heatwaves: How Environmental Complexity Shapes the Physiological Responses of Mussels to Climate Change

By

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# ABSTRACT

Rocky intertidal systems have been highlighted as potential early indicators for the impacts of climate change due to the simultaneous fluctuation of multiple environmental parameters caused by the predictable movement of the tide. A central prediction of recent climate change models is that there will be an increase in temperature variation and unpredictability, which will increase the probability of extreme hot days and multi-day heatwave events. Sessile intertidal organisms already experience a high degree of thermal unpredictability due to the combined effects of solar radiation and tidal movement. Most of our understanding of thermal physiology of intertidal organisms is based on experiments that have typically focused on exposing intertidal organisms to constant or predictable fluctuations in temperature when organisms are submerged in water, rather than unpredictable fluctuations in temperature that are more representative of the natural environment. How thermal unpredictability operates in intertidal habitats with different media, such as tidepool (submerged in water) or tidally-exposed (circatidal exposure of air and water) habitats to shape organismal performance is poorly understood, especially when coupled with other important determinants of performance, such as food availability. The overall goal of my dissertation was to understand how key aspects of environmental variation in the rocky intertidal interact to influence thermal performance in the California mussel (*Mytilus californianus*). I examined how intertidal habitat, thermal predictability and food availability shaped the performance of *M. californianus* to both a single, lethal thermal stress event (extreme hot day), as well as a sublethal multi-day thermal stress event (heatwave) and measured physiological performance through metabolic and biochemical analyses. I found that intertidal habitat, thermal predictability, food availability and the degree of thermal stress (lethal or sublethal; single or repeated thermal stress events) all interacted in nuanced ways to shape the thermal performance of *M. californianus*. It also became apparent that mussels tailor their physiological responses to predictable signals in their environment. In rocky intertidal systems, the most predictable environmental signal is the transition between immersion and emersion due to the movement of the tide. Here, I found that acclimation to a tidal cycle of immersion and emersion played a fundamental role in shaping physiological responses in mussels and was the predominant driver for regulating thermal performance to both lethal and sublethal thermal stress. When the predictable signal of air exposure was absent, as occurs in tidepool habitats, mussels became more dependent on thermal predictability and food availability to modulate thermal performance. Overall, my PhD research indicates that incorporating realistic environmental conditions into experimental design is crucial for understanding how future climate change scenarios will influence the physiological performance of intertidal mussels. By having a better understanding of how the complexity of the environmental signal impacts mussel performance, we can provide much more ecologically realistic predictions of the vulnerability of mussel populations to climate change.

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# CHAPTER 1

## Introduction

A central question of environmental biology is how organisms interact with their environment across temporal scales (Pinsky et al., 2019) and understanding the mechanisms underlying 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 become 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 it is also responsible for an increase in 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; Arias et al., 2021; Guo et al., 2018; IPCC, 2022; Stillman, 2019) and heatwave events (Angélil et al., 2017; IPCC, 2022). The majority of research to date investigating the link between climate change and organismal performance has focused on mean environmental conditions over time (Baldwin et al., 2019; Drake et al., 2017), yet temperature variability and extreme events can substantially impact organismal physiology and ultimately survival (Buckley and Kingsolver, 2012).

The rocky intertidal has become a model ecosystem for assessing how changes in temperature can affect physiological performance in marine ectotherms, due to the large fluctuation in temperature that occurs with the movement of the tide. Transition from the submerged, marine environment at high tide, to a terrestrial environment at low tide brings about a number of changes in an organism's thermal environment. For sessile intertidal organisms, such as mussels and barnacles, exposure to air during low tide brings about not just changes between ocean and air temperature, but also rapid increases in body temperature due to solar radiation from the sun. The complex interaction between air temperature and solar radiation, coupled with other external factors such as wind and cloud cover can result in sessile intertidal organisms experiencing unpredictable fluctuations in temperature on a daily basis.

Though thermal fluctuation during air exposure at low tide is an inherent characteristic of daily life in the intertidal zone, most of our knowledge of the thermal performance in intertidal organisms is where experiments have typically focused on steady state or predictable increases in temperature when organisms are submerged in water, rather than unpredictable variation during air exposure, which is more representative of the natural environment resulting from the ebb and flow of the tide. Recent acknowledgment of the importance of environmental complexity in rocky intertidal 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). One of the key takeaways of these studies is that in adult organisms, individuals 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), suggesting that some aspect of air exposure may confer enhanced tolerance (Bjelde and Todgham, 2013; Drake et al., 2017; Huang et al., 2015; Jones et al., 2009; Roberts et al., 1997; Stillman and Somero, 2000; Tagliarolo and McQuaid, 2016). It is thought this could be because the transition between the marine environment at high tide to the terrestrial environment at low tide is the most predictable environmental fluctuation in the intertidal zone, and thus 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).

Given the interest in trying to better align environmental temperatures with experimental temperature manipulations, there has been an increasing number of studies exposing organisms to fluctuating temperatures. Exposure to predictable fluctuating thermal environments has been shown to increase thermal tolerance (da Silva et al., 2019; Drake et al., 2017; Feldmeth et al., 1974; Kern et al., 2015; Oliver and Palumbi, 2011; Otto and Rice, 1974; Schaefer and Ryan, 2006; Threader and Houston, 1983; Vafeiadou et al., 2018; Vajedsamiei et al., 2021), and intertidal species exposed to predictable thermal fluctuations can be more tolerant to 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 occurs stochastically on multiple complex time scales (hours to years) (Burggren, 2019; Dillon et al., 2016) and thus, predictable cycles often do not accurately capture the thermal environment *in situ*. There can be a risk of 'overpredicting' the degree of responses to temperature change if the temperatures used are unrealistic. Therefore, studies with predictable temperature change may not be suitable for investigating 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).

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 thermal history 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; Madeira 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 gastropods and corals that have shown evidence of 'preparative defence' (Dong et al., 2008) or cellular 'frontloading' (Barshis 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), and anaerobic demands can often 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 activity of enzymes 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).

Due to the integrative effects of temperature on other abiotic and biotic parameters, there has been growing need to examine the combined effects of temperature with other environmental variables on the fitness of organisms in order to predict how current and future patterns of weather and climate are likely to affect species distribution patterns in marine environments (Gunderson et al., 2016; Schneider et al., 2010; Todgham and Stillman, 2013). Along with temperature, food availability has been shown to be among the most important determinant of survival, growth and reproduction for a wide array of organisms (Menge et al., 2008; Schneider et al., 2010; Sokolova, 2013). Low food availability has been shown to exacerbate the effects of thermal stress (Matzelle et al., 2015; Sarà et al., 2011; Schneider et al. 2010) by decreasing upper thermal limits (Cheng et al., 2018) and survival at high temperatures (Fitzgerald-Dehoog et al., 2012; Schneider et al. 2010). Climate change induced warming is linked to reductions in food availability through alterations in phytoplankton abundance, composition, or distribution. As the base of the food web, phytoplankton play an essential role in the health of the marine ecosystem, and changes in abundance or composition can have repercussions for marine community structure (Boyce et al., 2010). On a global scale, evidence suggests that phytoplankton biomass has declined over the last few decades, with some studies reporting up to a 40% decline (Boyce et al., 2010) coupled with changes in community composition (Hays et al., 2005; Tortell et al., 2002). On a regional scale, the phytoplankton response to warming is much more complex, especially in upwelling regions (Xiu et al., 2018). In a warming ocean, upwelling seasons are predicted to begin earlier, last longer, and intensify more at higher latitudes on upwelling coasts, homogenizing upwelling gradients within those regions (Wang et al., 2015). However, increased stratification may reduce nutrients and increase temperature in upwelled waters (Wang et al., 2015), which may decrease phytoplankton biomass (Rost et al., 2008). More immediate and localized alterations in food availability from climate change events, such as heatwaves, has also been reported, with marine heatwaves temporarily causing rapid alterations to community composition (Areteaga and Rousseaux, 2023).

The California mussel, *Mytilus californianus* is a dominant intertidal species that ranges from Alaska (USA) to Baja California (Mexico) along the west coast of North America (Lockwood and Somero, 2011). *M. californianus* is a foundational species and ecosystem engineer, aggregating into large beds that provide microhabitat for hundreds of species (Suchanek, 1979;1992) and are considered critical for biodiversity conservation as one of the world's most diverse temperate systems (Smith et al., 2006; Suchanek, 1992). In addition to the important ecological role of mussel beds, mussels provide a number of ecosystem services such as biofiltration, nutrient cycling and food provisioning, which are estimated to be worth billions of dollars per year (Ferriera and Bricker, 2016; Vaughn, 2018). Due to its important ecological role and distribution range, *M. californianus* has become a key model organism for understanding the effects of climate change, and there is a wealth of research exploring its physiological tolerances and underlying mechanisms (Braby and Somero, 2006; Buckley et al., 2001; Connor et al., 2011; Jimenez et al., 2016; Lockwood and Somero, 2011; Logan et al., 2012; Menge et al., 2008; Schneider et al., 2008; 2010; Somero, 2010; Tomanek et al., 2012). Mussels have been shown to be particularly susceptible to stochastic weather and climate events, such as heatwaves, and *M. californianus* populations have experienced large declines (>60%) and mass mortalities as a result of climate change induced effects (Harley et al., 2008; Helmuth et al., 2006; Helmuth et al., 2016; Smith et al., 2006; Sorte et al., 2017). Recent advancements in biomimetic sensors and the development of 'Robomussels' (Choi et al., 2019; Fitzhenry et al., 2004; Helmuth et al., 2016) have shown that body temperature of mussels vary dramatically depending on position on the shore and microhabitat conditions experienced, and that temperatures experienced by mussels are much more unpredictable than originally thought (Choi et al., 2019). However, we currently know little about how thermal unpredictability shapes the physiological performance of mussels to tolerate heat stress, and how stochasticity integrates into a multi-stressor framework.

The overall goal of my PhD research was to understand how key aspects of the intertidal environment interact to influence thermal performance in the California mussel. This begins with Chapter 2, where I explore how natural unpredictable fluctuations of temperature shapes the physiological performance of mussels within a tidal framework. Here, I focused on measuring a number of metabolic and biochemical metrics to understand the physiological mechanisms underpinning changes in thermal performance, focusing on cardiac performance, cellular stress mechanisms, energy storage, anaerobic capacity and life history tradeoffs in the form of growth. In Chapter 3 I incorporate more environmental complexity by considering how habitat medium (air or water for tidally-exposed vs. tidepool mussels, respectively) may interact with thermal unpredictability to influence thermal performance under low and high food availability. Here, I streamlined my focus to cardiac performance, cellular stress mechanisms and energy stores to try and understand how different elements of a mussel’s environment alter mechanistic levels of performance. Finally, in Chapter 4, I continue investigating the interactions between habitat, thermal predictability and food availability but focus my attention on how they shape sublethal, multi-day thermal stress events, such as heatwaves. By having a better understanding how the complexity of the environmental signal impacts mussel performance, we can provide much more ecologically realistic predictions of the vulnerability of mussel populations to climate change.

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# CHAPTER 2

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

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# 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 also had lower gill malate dehydrogenase activity and succinate content, which could reflect increased metabolic capacity. Our results suggest that incorporating key elements of the intertidal environment complexity are important considerations for determining how species will respond to climate warming.

# INTRODUCTION

A central question of environmental biology is how organisms integrate environmental signals and 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, 2022; Stillman, 2019) and heatwave events (Angélil et al., 2017; IPCC, 2022). 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.

## MATERIALS 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 lead to progressive warming in subsequent day time low tides; 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 (≤ 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 (n= 12 per treatment) 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. Twelve 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, 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 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 (n=20 for each treatment) 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 was analysed with the 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 (Lenth 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 of 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 2).

### 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 Hsp/Hsc70, 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%), which was statistically similar to the air treatment.

### 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 $\text{protein}^{-1}$ $\text{min}^{-1}$) 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 $\text{protein}^{-1}$ $\text{min}^{-1}$). 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, $F_{(4,37)} = 9.105$, $P = <0.0001$; 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. We also predicted that due to the stochastic nature of thermal variability that occurs in nature, mussels acclimated to the unpredictable regime would have the most similar physiological performance to field mussels. 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 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) in comparison 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 this 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. Further investigation is needed to tease apart how each of these environmental elements influences thermal performance in mussels.

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. As *M. californianus* typically does not gape during air exposure, daytime low tide often results in a shift to anaerobic metabolism to tolerate thermal stress. Consequently, 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 than constant regimes at the same mean temperature (Kern et al., 2015; Ruel and Ayres, 1999; Schafer and Ryan, 2006; Williams et al., 2012) and acclimation to fluctuating thermal regimes can result in lower routine metabolic rate in comparison to constant conditions with the same mean temperature (Dame and Verberg 1978; Dong and Dong, 2006; Marshall et al., 2021; Tian et al., 2004; Verheyen and Stoks, 2019; Widdows, 1976). 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). For example, in *M. edulis*, warm acclimation results in lower oxygen consumption and filtration rates at a given temperature in comparison to cold-acclimated mussels (Widdows, 1971). This indicates increased metabolic efficiency, which is likely reflected in MDH activity and succinate content. 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). 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. It is interesting that there was no difference between predictable and unpredictable treatment for MDH and succinate in the mantle considering there was a significant difference in glycogen content which would indicate differences in energy usage. This could perhaps be due to the hypothesised difference in reproductive investment vs. glycogen storage suggested above between predictable and unpredictable treatments, resulting in equal metabolic demand in the mantle tissue and thus similar MDH activity and succinate content. Analysis of proxies for reproduction or enzymes more associated with glycolysis and gluconeogenesis may shed more light on the specific metabolic dynamics between the predictable and unpredictable in the mantle tissue.

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. Growth rates typically increase with submergence time in Mytilus sp. (Pannella 1976, Buschbaum & Saier 2001) as calcification cannot occur in air (Tagliarolo et al., 2012). Even when calcification rates are higher during in immersion in intertidal species, daily growth rates are reduced (Tagliarolo et al., 2012). Therefore, it is likely that reduced growth in the tidal treatments is due to limited submersion and thus calcification time. Conversely, 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 Hsp/Hsc70 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). Therefore, this could explain why we found no expression in the thermal treatments as we took samples just before daytime low tide. 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 Hsp/Hsc70 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 or 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). A fruitful 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 more accurately 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, and thermal variability) specifically shapes physiological performance, especially under hotter (summer) conditions. 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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## TABLES

**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 2.** 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

# FIGURES

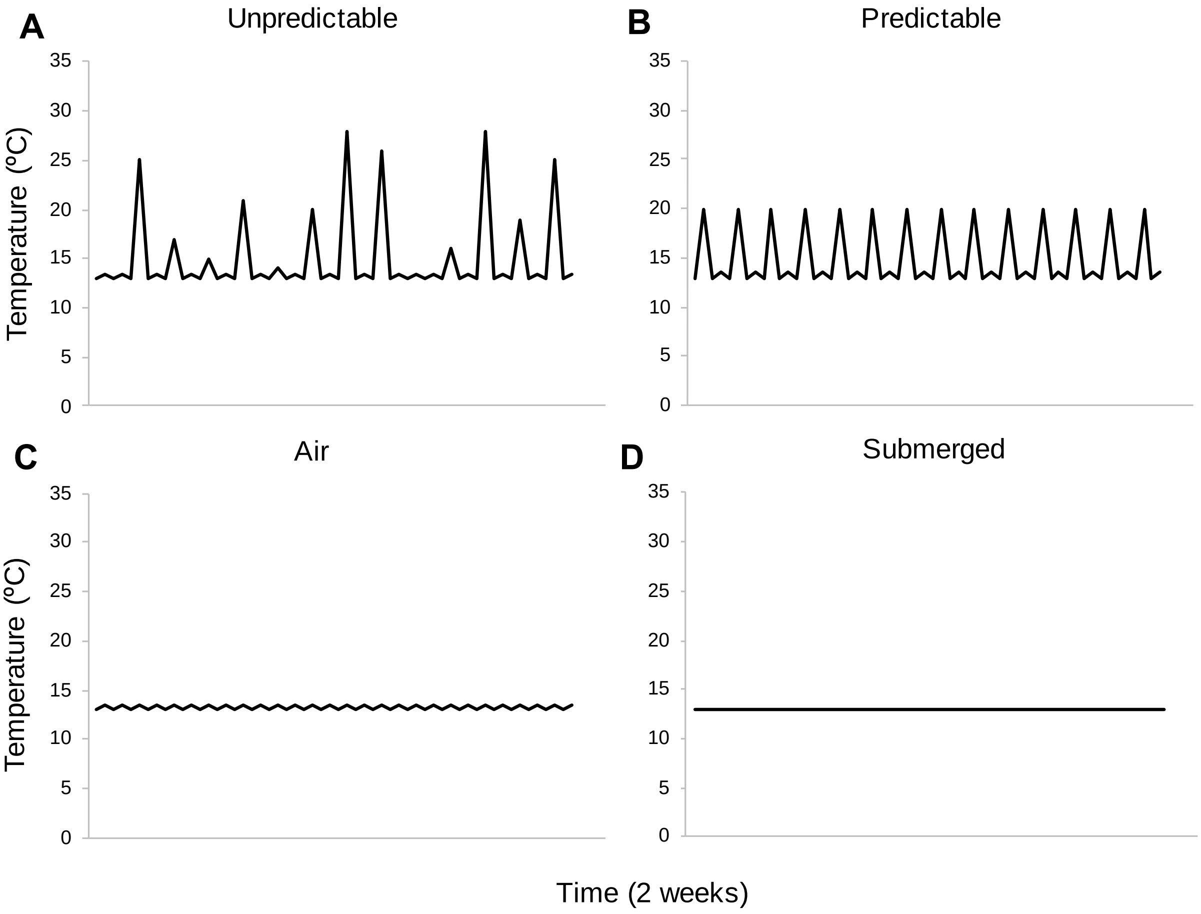

**Figure 1.** Temperature profiles for each acclimation treatment over the 2-week acclimation period. (A) Unpredictable treatment: circatidal regime and varying aerial temperatures during each daytime low tide. (B) Predictable treatment: circatidal regime with consistent, predictable warming to 20°C 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).

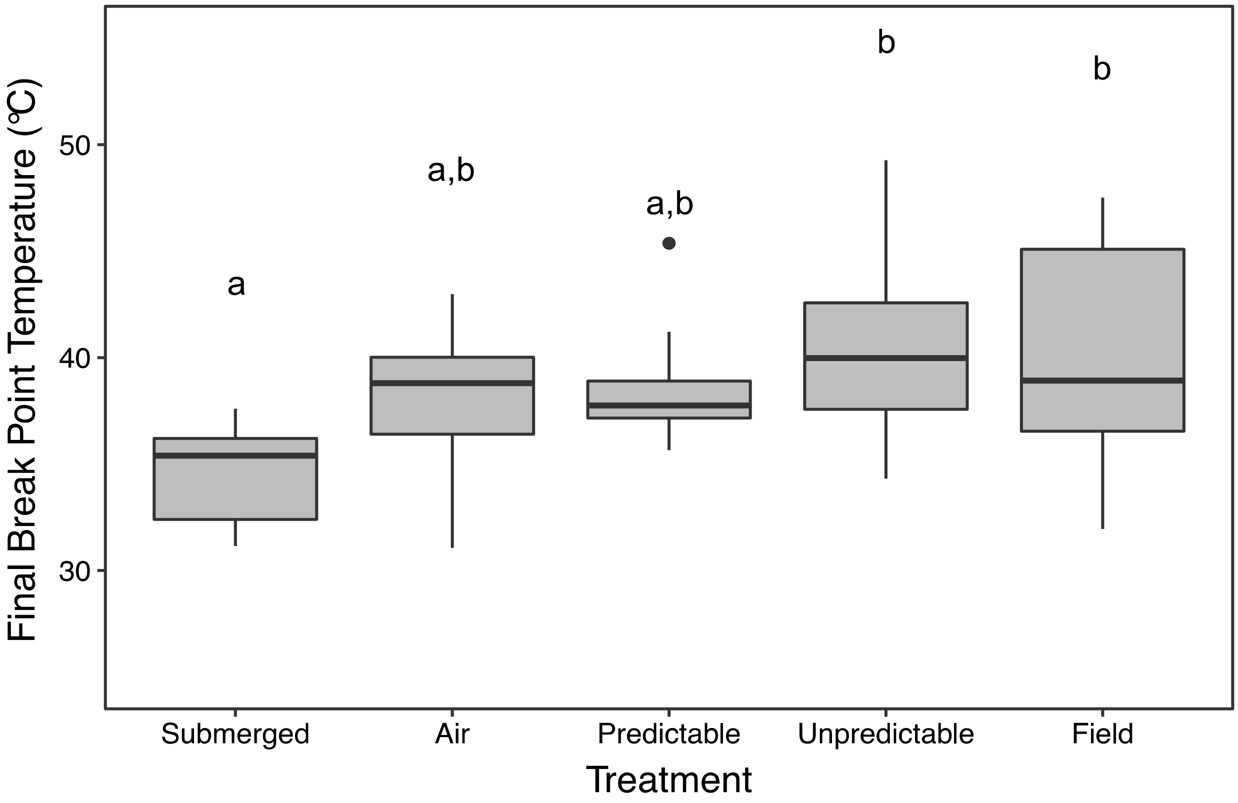

**Figure 2.** Final break point temperature in heart rate for *Mytilus californianus* individuals from submerged (n = 11), air (n = 11), predictable (n = 11), unpredictable (n = 12) and field (n = 12) acclimation treatments measured during an acute thermal ramp (increase of 6°C an hour for six hours) 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, $P < 0.05$).

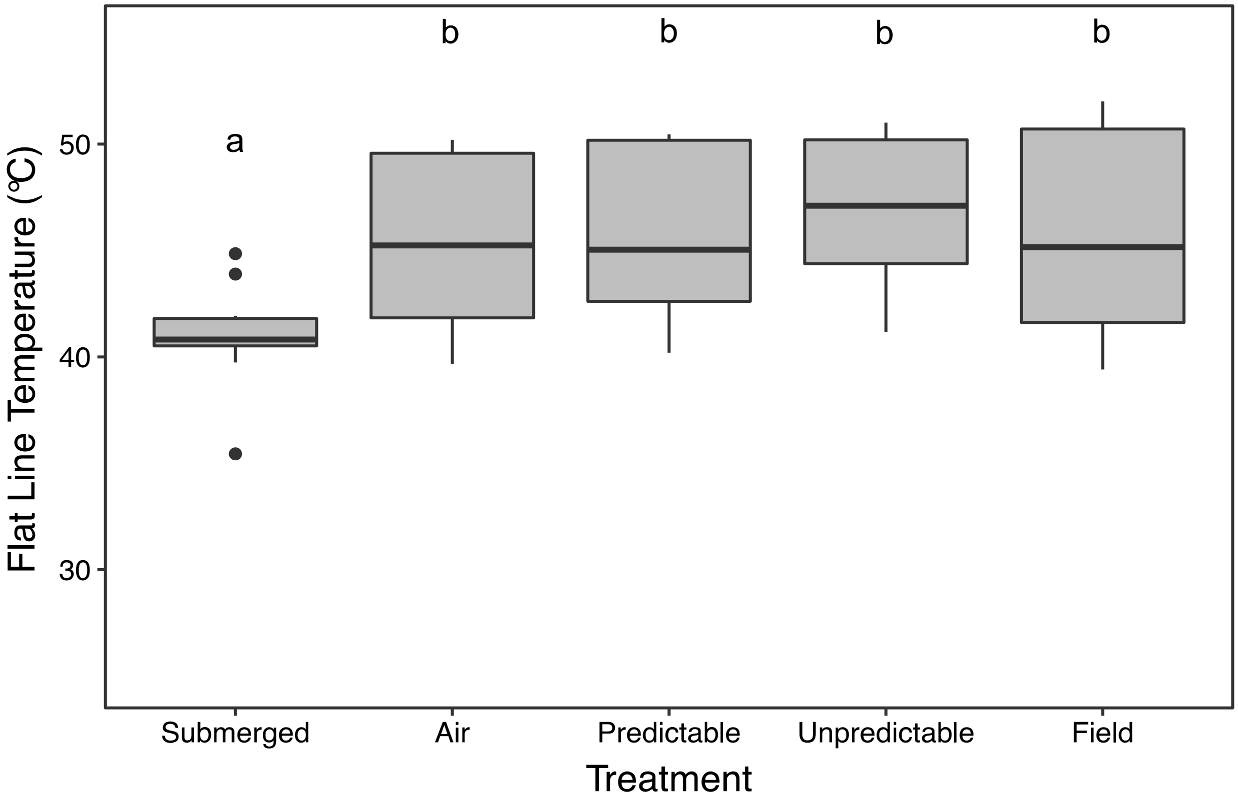

**Figure 3.** Flat line temperature in heart rate for *Mytilus californianus* individuals from submerged (n = 11), air (n = 11), predictable (n = 11), unpredictable (n = 12) and field (n = 12) acclimation treatments measured during an acute thermal ramp (increase of 6°C an hour for six hours) in air. 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).

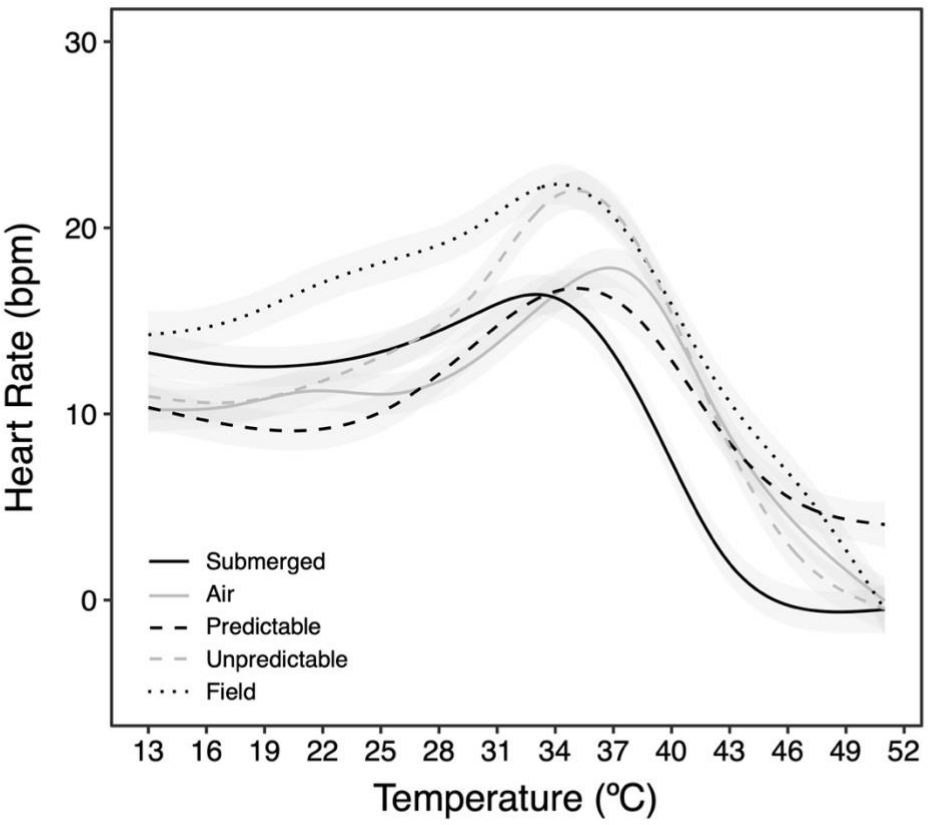

**Figure 4.** Generalized additive mixed modelling (GAMM) for heart rates of *Mytilus californianus* individuals from submerged (n = 11), air (n = 11), predictable (n = 11), unpredictable (n = 12) and field (n = 12) acclimation treatments measured during an acute thermal ramp (increase of 6°C an hour for six hours) in air. Statistical differences are reported in Table 2.

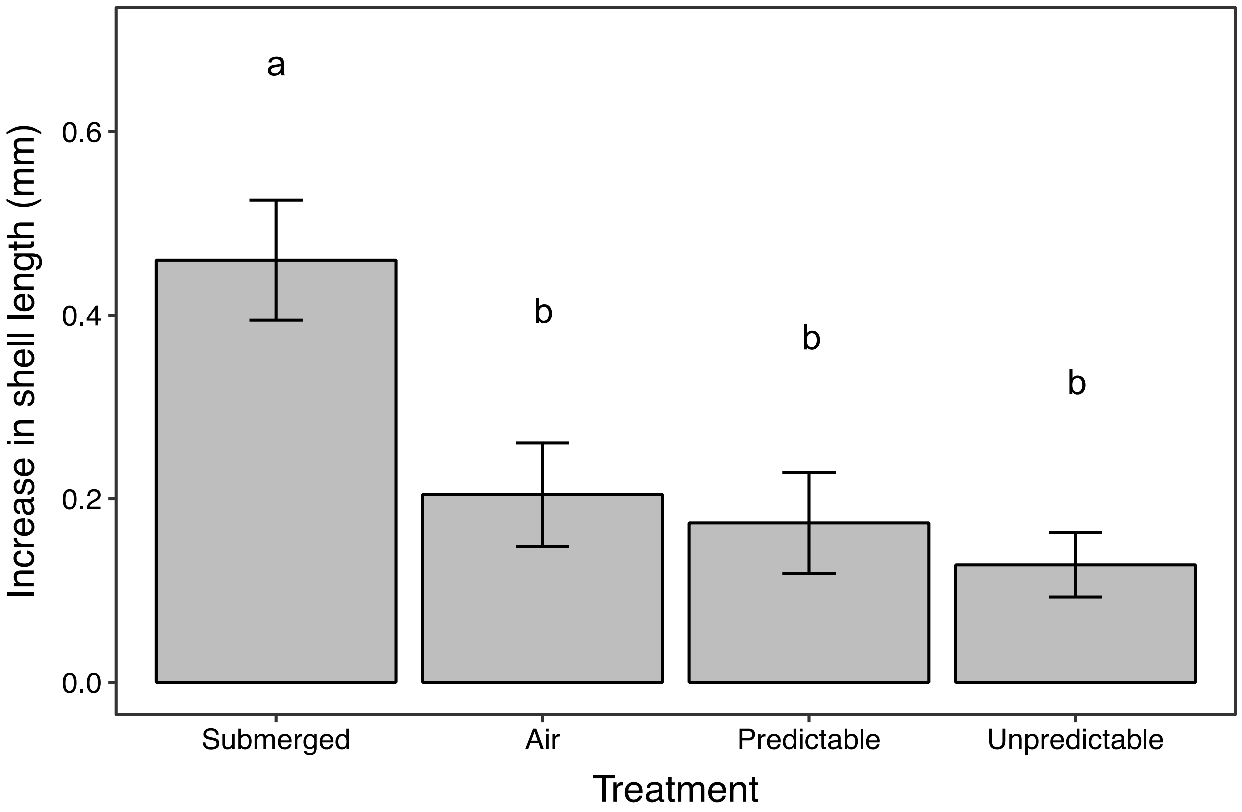

**Figure 5.** Increases in shell length (mm) of *Mytilus californianus* during the 2-week acclimation period in air (n = 19), predictable (n = 18), submerged (n = 17) and unpredictable (n = 20) acclimation treatments. Each bar represents the mean (± S.E.M). Differences in letters represents significant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

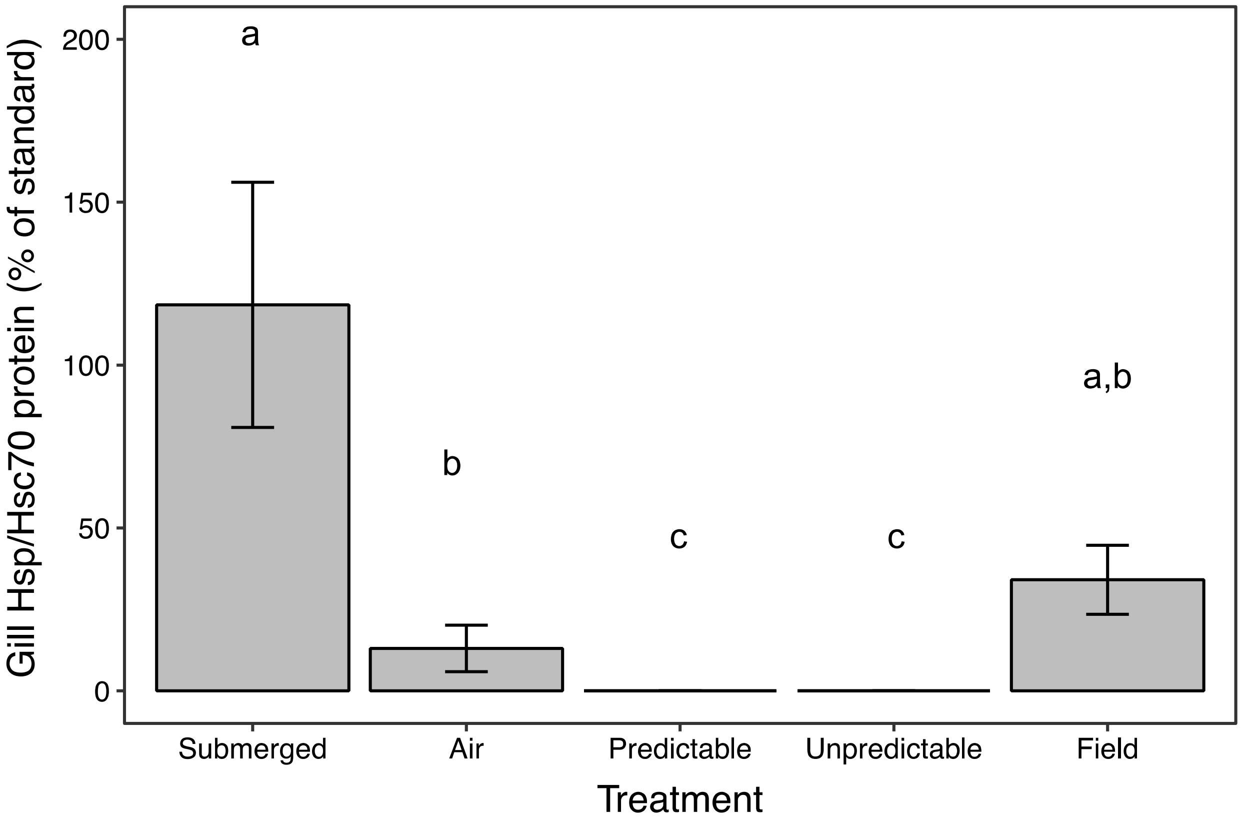

**Figure 6.** Relative levels of Hsp/Hsc70 protein in the gill tissue sampled just before daytime low tide in *Mytilus californianus* individuals from submerged (n = 8), air (n = 9), predictable (n = 7), unpredictable (n = 9) and field (n = 9) acclimation treatments. Protein levels are shown as relative values based on band intensities standardised with the level of Hsp/Hsc70 in a gill tissue sample from the submerged group that had shown positive expression of Hsp/Hsc70 (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).

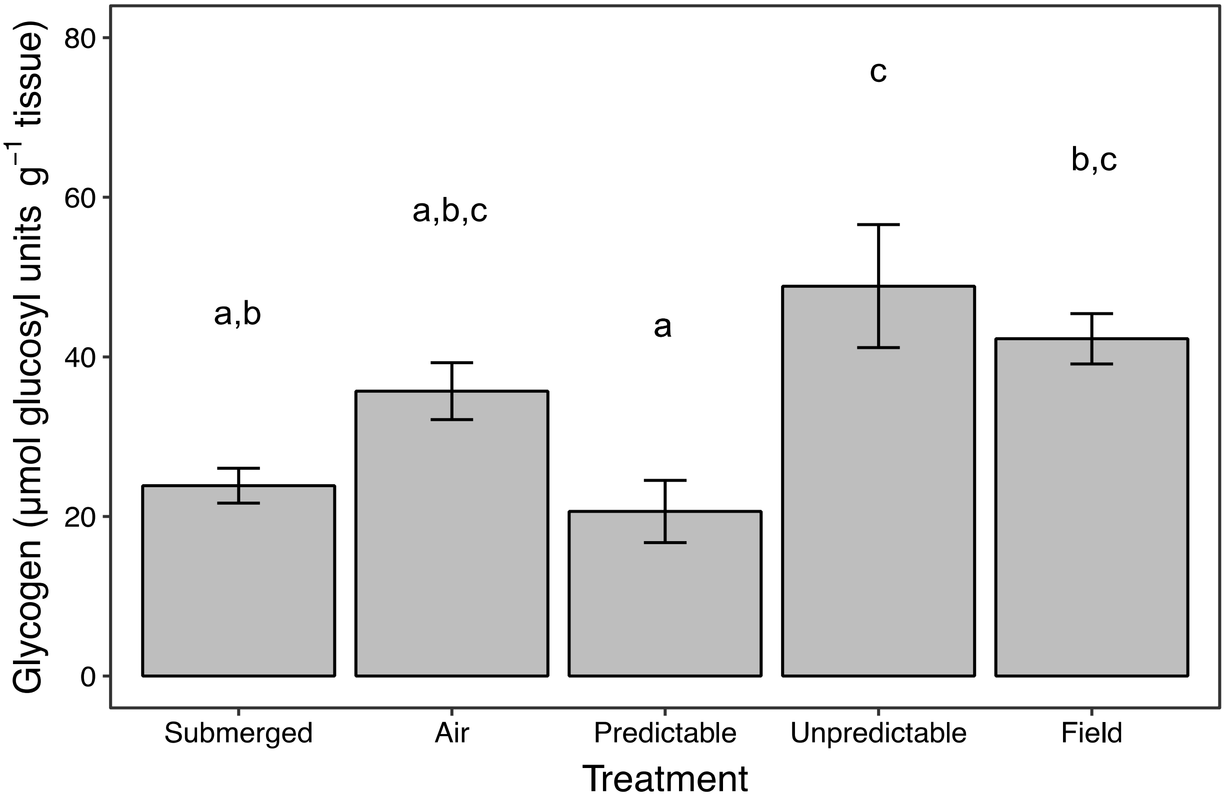

**Figure 7.** Glycogen content of mantle tissue sampled just before daytime low tide in *Mytilus californianus* individuals from submerged (n = 8), air (n = 9), predictable (n = 7), unpredictable (n = 9) and field (n = 9) acclimation treatments. Each bar represents the mean (± S.E.M). Differences in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

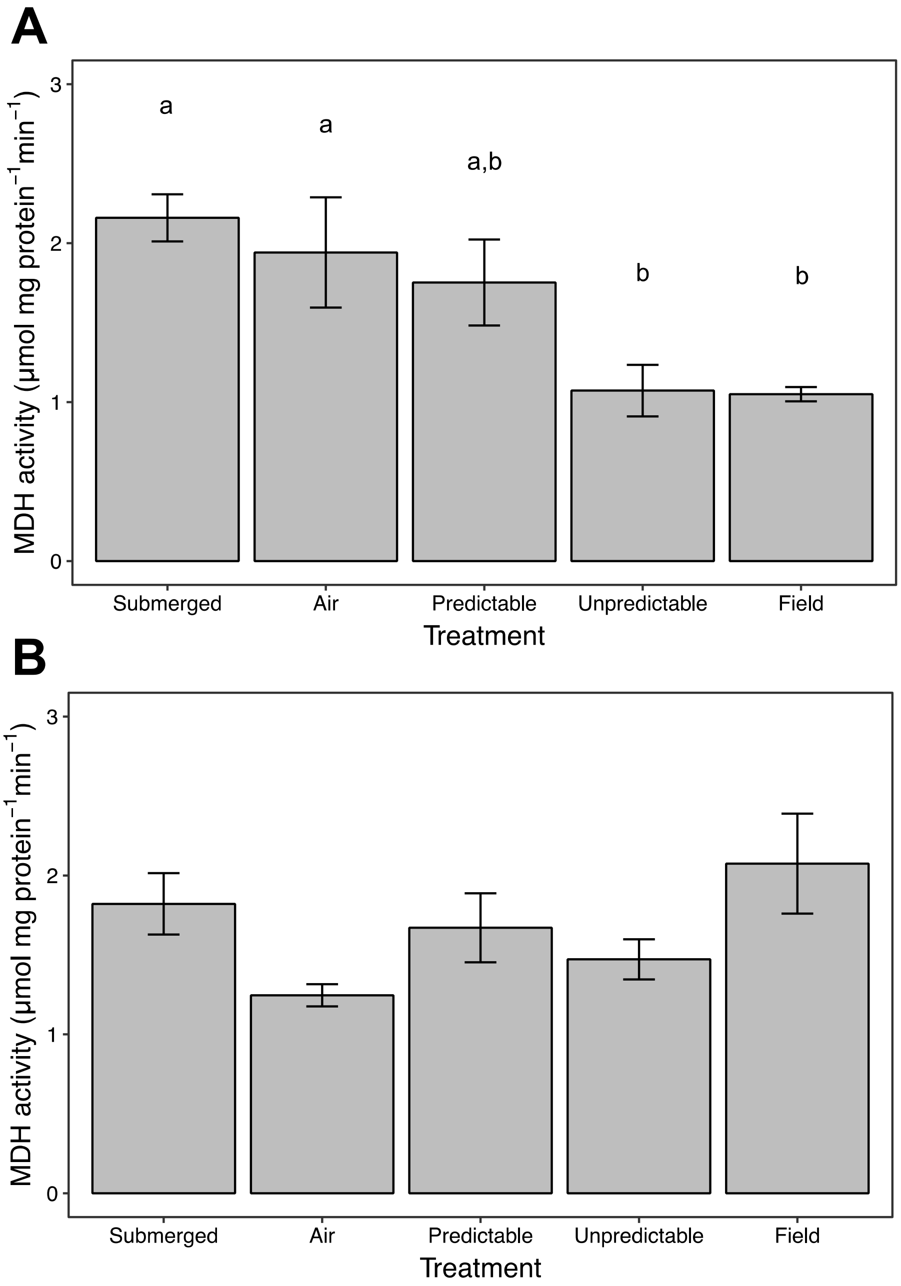

**Figure 8.** MDH activity of gill (A) and mantle (B) tissue sampled just before daytime low tide in *Mytilus californianus* individuals from submerged (n = 8), air (n = 9), predictable (n = 7), unpredictable (n = 9) and field (n = 9) acclimation treatments. Each bar represents the mean (± S.E.M). Differences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

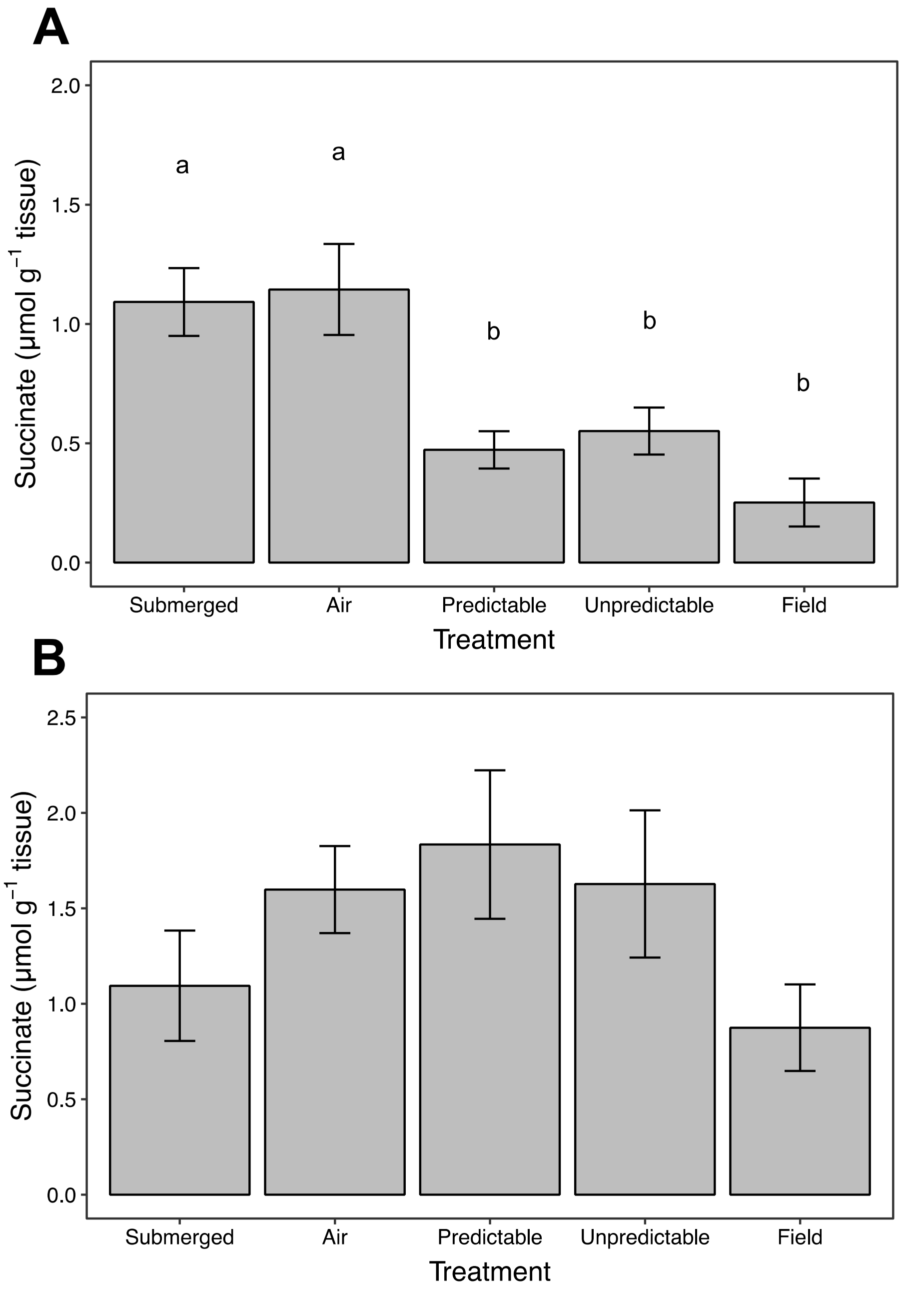

**Figure 9.** Succinate content of gill (A) and mantle (B) tissue sampled just before daytime low tide in *Mytilus californianus* individuals from submerged (n = 8), air (n = 9), predictable (n = 7), unpredictable (n = 9) and field (n = 9) acclimation treatments. Each bar represents the mean (± S.E.M). Differences in letters represents significant differences between acclimation treatments (one-way ANOVA, Tukey's HSD, *P* <0.05).

# CHAPTER 3

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

## ABSTRACT

Recent climate change models indicate that there will be an increase in thermal unpredictability and in the frequency and intensity of hot days. Many sessile intertidal organisms live close to their upper thermal limits and experience a high degree of thermal unpredictability due to combined effects of solar radiation and tidal movement. How thermal unpredictability operates in intertidal habitats with different media, such as tidepool (always submerged in water) or tidally-exposed (circatidal exposure to air) environments is poorly understood, especially when coupled with other important determinants of performance, such as food availability. Understanding how these factors interact to influence performance under increasing temperatures is essential towards understanding the effects of climate change on intertidal communities. 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 *Mytilus californianus* in two habitat treatments: tidepool (submerged in water) and tidally-exposed (circatidal exposure to air and water). Mussels were warmed at a rate of 6.5°C/hour for 6 hours in their respective habitat medium (water or air). Heart rate was monitored continuously, and tissues samples were taken for glycogen and Hsp/Hsc70 determination just before the start of the thermal ramp (baseline: 13°C) and at 20°C, 30°C and 40°C. We found that habitat medium was the largest driver for shaping thermal performance, as tidepool mussels exhibited 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 from the 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 organisms that experience thermal stress in a tidepool habitat may be more suspectable to climate induced increases in temperature, but thermal unpredictability may aide in increasing performance.

## 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 (Burggren, 2019; Kroeker et al., 2016; 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. Intertidal organisms often live close to their upper thermal limits, and climate-induced increases in temperature are predicted to have severe consequences for intertidal community composition (Helmuth et al., 2002; Somero, 2002; Somero, 2005; Stillman and Somero, 2000). 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; Somero, 2005; Stillman, 2002). 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; Hui et al., 2020; Zhang et al., 2014), prior thermal history (e.g Pasparakis et al., 2016; Schoepf et al., 2022; Tomanek and Somero, 1999), thermal variability (e.g Han et al., 2020; Marshal et al., 2021; Oliver and Palumbi, 2011), as well as interaction with other environmental variables (e.g Kelly et al., 2016; Pagnini et al, 2014; Todgham and Stillman, 2013) and thermal environment (e.g Bjelde et al., 2013; Stillman and Somero, 1996; Tagliarolo and McQuaid, 2015). Investigation into the complexity of the thermal signal has resulted in an increase in experiments incorporating thermal fluctuation within a tidal 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; Nancollas and Todgham, 2022; Paganini et al., 2014; Vajedsamiei et al., 2021; Widdows and Shick, 1985; Yin et al., 2017). Such studies have revealed that exposure to predictable fluctuating environments can increase thermal tolerance in comparison to constant conditions with the same mean temperature (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; Vajedsamiei et al., 2021), suggesting thermal fluctuation provides complexity that is important for structuring thermal tolerance. While predictable fluctuating regimes are more representative of the intertidal environment, they are still not accurately capturing the unpredictable element of *in situ* thermal fluctuation and risk under or over predicting the degree of physiological responses. This is particularly concerning, as recent climate change models predict an increase in temperature variation and unpredictability accompanied by an increase in the frequency and intensity of extreme temperatures (Angélil et al., 2017; Guo et al., 2018; Arias et al., 2021; Stillman, 2019). Currently, 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 physiological homeostasis during acute thermal stress, including metabolic modulation (Dahlhoff et al, 2001; Hui et al, 2020; Marshall et al., 2011) and the initiation of cellular stress response mechanisms, such as heat shock proteins (Hsps). Cardiac function has been shown to be important in establishing thermal tolerance limits (Dong et al., 2022), and the close linkage between cardiac performance and metabolism in ectotherms (Frederich and Pörtner, 2000) makes heart rate an effective indicator in molluscs. Activation 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 is crucial for intertidal organisms to survive stressful low tide periods (Ivanina et al, 2008; Sokolova et al., 2012; Somero, 2002). The induction and magnitude of the inducible heat shock response can be altered by acclimation conditions (Buckley et al., 2001; Dong et al., 2008; Han et al., 2013; Hofmann and Somero, 1995; Madeira 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), 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) or cellular 'frontloading' (Barshis et al., 2013; Collins et al., 2021). As our understanding of how the thermal environment informs the cellular stress response evolves, it is important to understand how exposure to representative thermal patterns may shape the thermal stress response, linking 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 tidepools that are isolated during low tide remain constantly submerged, but also experience stochastic fluctuations in temperature and other environmental variables (Helmuth and Hoffman, 2001; Nakano and Iwama, 2002). The habitat medium (air or water) in which intertidal organism experiences thermal stress can dictate different metabolic profiles. Some organisms, such as gastropods and crabs, are able to maintain comparable metabolic performance in air and water (Bjelde et al., 2013; Huang et al., 2015; McMahon, 1990; Stillman and Somero, 1996;), 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 (Fly et al., 2012; McMahon et al., 1991; Sokolova and Pörtner, 2001; Tagliarolo et al., 2012, Tagliarolo and McQuaid., 2016; Nancollas and McGaw, 2021a). Interestingly, regardless of the metabolic strategy utilized during air exposure, growing evidence indicates that upper thermal tolerance is elevated when thermal stress occurs in air vs water for adult intertidal organisms, suggesting that some aspect of air exposure confers enhanced tolerance (Bjelde and Todgham, 2013; Huang et al., 2015; Jones et al., 2009; Tagliarolo and McQuaid, 2016). Typically, organisms that are emersed during low tide experience higher maximum temperatures and variability than their tidepool counterparts, and we are beginning to understand that when combined with air exposure, thermal unpredictability can have complex effects on thermal tolerance that may be correlated to metabolic profile (Drake et al., 2017; Nancollas and Todgham, 2022). However, we still know little about how it shapes performance in an aquatic setting. While tidepools may not experience quite the same degree of thermal variability as tidally-exposed habitats, thermal fluctuation can still exceed 20°C within a day and can be equally as unpredictable (Nakano and Iwama, 2002). Understanding how natural, unpredictable thermal patterns shapes performance in tidepool habitats will be imperative to understand how climate change may shape intertidal assemblages. Furthermore, direct comparison of how thermal unpredictability influences performance in aquatic and aerial settings will provide us with a more mechanistic understanding of metabolic performance in this dynamic environment for animals found across different habitats.

Along with temperature, food availability has been shown to be among the most important determinant 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 (Feder and Hofmann, 1999; 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 (Boyce et al., 2010; Hays et al., 2005; Henson et al., 2021; Rost et al., 2008; Tortell et al., 2002; Wang et al., 2015, Xui et al., 2018). Our current understanding of how temperature interacts with food availability to inform physiological performance is largely based on laboratory studies acclimating organisms to different mean temperatures with various food levels. Such studies indicate that low food availability can exacerbate the effects of thermal stress (Dahlhoff et al., 2001; Dahlhoff, 2004; Dahlhoff and Menge, 1996; Matzelle et al., 2015; Sarà et al., 2014; Schneider et al. 2010) by decreasing upper thermal limits (Cheng et al., 2018) and survival at high temperatures (Fitzgerald-Dehoog et al., 2012; Schneider et al. 2010). Intertidal organisms already experience reduced feeding opportunities due to being isolated from the ocean during low tide in comparison to their subtidal counterparts (Dahlhoff and Menge, 1996; Tagliarolo et al., 2012) and rely on the subsequent high tide period to replenish energy stores and mitigate any damage caused by the stressful low tide period (Dahlhoff and Menge, 1996; Tagliarolo et al., 2012). 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 and Todgham, 2022), suggesting that declines in food availability could have detrimental effects on physiological performance in intertidal mussels. An understanding of how unpredictable fluctuating temperatures interact with food availability to modulate physiological performance, and whether this interaction is uniform across tidepool and tidally-exposed habitats, will be crucial for understanding how intertidal assemblages will respond to climate-induced changes in their environment.

The primary objective of this study was to investigate how varying levels of thermal predictability interact with food availability in tidepool and tidally-exposed habitats to shape the thermal performance of the California mussel, *Mytilus californianus*. *Mytilus californianus* occur in both tidepool and tidally-exposed habitats along much of the West coast of North America, and experiences unpredictable fluctuations in temperature on a daily basis. *Mytilus californianus* is a foundational species and ecosystem engineer, but populations have experienced large declines (>60%) and mass mortalities as a result of climate change-induced effects (Harley et al., 2008; Helmuth et al., 2006; Helmuth et al., 2016; Smith et al., 2006; Sorte et al., 2017). In this study, we examined how acclimation to various combinations of habitat, thermal predictability and food availability shaped cardiac performance, the cellular stress response (Hsp/Hsc70) and energy reserves (glycogen content) 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 intertidal habitats to shapes performance during acute stress will be paramount for predicting how climate change will affect intertidal communities.

## 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 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, 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

Mussels were weighed, measured, labelled, and randomly divided between one of twelve different acclimation treatments and were held under these conditions for 3 weeks. The twelve acclimation treatments consisted of three different temperature regimes (unpredictable: U; predictable: P; no heat: N) and two levels of food (high: H, low: L) within two different habitat types: tidepool (always submerged: S) and tidally-exposed (circatidal exposure to air and water: A) (Figure 1A). Experimental tanks and treatment design were modelled off the methods outlined in Nancollas and Todgham (2022), with several modifications. For the six tidally-exposed treatments, tanks were built to simulate natural intertidal conditions by replicating semidiurnal 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 microcontrollers (Arduino YUN, Adafruit, New York, NY, USA; Drake et al., 2017; Miller and Long, 2015) and oscillated based on a semidiurnal tidal pattern. Tanks were flowthrough where incoming seawater was first passed through a 30μm sand filter and then 5μm filter to remove biological matter, including natural food sources. For the six 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 habitat type, three temperature regimes occurred, which differed in degree of thermal stress and predictability: unpredictable, predictable, no heat stress (Figure 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 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 two ‘robomussel’ temperature loggers (Maxim Integrated Products, Dallas, TX, USA): one embedded on the rock next to tidally-exposed *M. californianus*, and one in a tidepool (size: L:1m, W: 0.6m, D: 0.2m) surrounded by *M. californianus* in Bodega Marine Reserve. Each robomussel continuously monitored temperature every 10 minutes from May 2020 to October 2020. Assessment of the logger data revealed that the tidally-exposed robomussel and the tidepool robomussel 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°C to 36°C whereas tidepool temperatures ranged from 13°C 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 (Figure A1).

For the unpredictable treatments (U), mussels experienced varying temperatures within the range of 13-34°C during daytime low tide (Figure 1B). This temperature profile mirrored a 3-week 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-month logger period (25°C) and included the maximum temperature (34°C). Mussels in the predictable treatments (P) 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 weeks as the unpredictable treatment, but in a predictable manner. As a control, a final thermal regime with no heating (N) 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 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. 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 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. 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 six 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* 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 hour 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 (H) were fed 100,000 cells $mL^{-1}$ (~10 μg Chl $L^{-1}$) whereas low food treatments (L) were fed 10,000 cells $mL^{-1}$ (~1 μg Chl $L^{-1}$), which equated to 50 cells/g mussel wet weight and 5 cells/g mussel wet weight respectively. These food levels were chosen to represent the average high and low levels of chlorophyll observed near Bodega Bay, CA (data access: boon.ucdavis.edu; coastwatch.pfeg.noaa.gov/infog/MW_chla_las.html).

Each of the treatments had two 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 m (YSI Incorporated, Yellow Springs, OH, USA) while API saltwater test kit (API, Chalfont, PA, USA). 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.

## Heart rate

To understand how habitat, thermal predictability and food availability influenced the cardiac performance of *Mytilus californianus* during thermal stress, heart rate was measured in six mussels from each of the twelve treatments during an acute thermal ramp starting at 13°C (ambient ocean temperature) and increasing at rate of 6.5°C $h^{-1}$ for six hours. 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 six tidepool treatments were warmed in water and mussels from the six tidally-exposed treatments were warmed in air. Heart rate was monitored for each individual (n = 6 per treatment) as described by Nancollas and 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 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. Similarly to Tagliarolo and McQuaid (2015) and Nancollas and Todgham (2022), 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.

### Cardiac performance analysis

Cardiac performance was analysed following methods previously described (Bjelde and Todgham, 2013; Nancollas and 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 break point temperatures (BPT) 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 and

Somero, 1996), including mussels (Tagliarolo and McQuaid, 2015). Final BPT was calculated as described elsewhere (Bjelde and Todgham, 2013, Drake et al., 2017; Nancollas and Todgham, 2022). Briefly, individual mussel heart rate (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.

### Mussel tissue sampling

To understand how 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 day-time low tide, ambient temperature ~13°C). Tissues samples were also taken at 20°C, 30°C and 40°C (see Figure 2 for diagrammatic representation of sampling) during the acute thermal ramp to understand how 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 six tidepool treatments at the 40°C sampling point. Gill tissue was used to assess cellular stress mechanisms (Hsp/Hsc70 protein) and mantle tissue was used to quantify energy stores (glycogen content). Mussels were removed from treatment tanks and gill and mantle tissue were dissected quickly (i.e. under 20s). 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 Nancollas and Todgham (2022). Briefly, 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) and Western blot analysis as described by Nancollas and Todgham (2022). Briefly, 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), with a prestained molecular marker (PageRuler, Thermo Fisher Scientific) in one lane and an internal standard in the second lane to calibrate protein expression within and among gels. The internal standard was pooled gill samples from 40°C submerged mussels that had shown positive expression of Hsp/Hsc70. Proteins were separated by SDS-PAGE and transferred using the semi-dry transfer method. Transfer membranes were then blocked in 2% bovine serum albumin (BSA) in Tween-20 Tris-buffered saline (TTBS), rinsed, and 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 in TTBS and then soaked in horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1706516, Thermo Fisher Scientific) diluted 1:5000 in 2% BSA in TTBS. Membranes were then washed in TTBS, 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) 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 intensity of the internal standard.

### Glycogen content

Glycogen content was measured as described by Nancollas and Todgham (2022), 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.

## Statistical analysis

All data sets were analysed using R (R Development Core Team, 2008) 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, 2019).

### *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 3ur et al. (2009) and Angilletta et al. (2013). 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 factor. 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., 2013) libraries in R.

Separate linear mixed effect models (LME) using the REML method were fit using the nlme package with Final Break Point Temperature (BPT), Flat line temperature (FLT) and Maximum heart rate as the response variables with habitat, thermal regime, and food availability as fixed effects, and acclimation tank included as a random effect. As tidepool treatments exhibited two 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 (Barton, 2015). The model 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.

### *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 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 linear mixed effect models were created that included the fixed factors habitat, thermal regime, and food availability as well as sampling temperature (4 levels: baseline [13°C], 20°C, 30°C, 40°C; Figure 2), 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 boxcox transformation was implemented.

## RESULTS

### Cardiac performance

*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 (Figure 3, Table 1). Temperature sensitivity of heart rate during the acute thermal ramp was significantly affected by 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 two distinct breakpoints. 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 increased performance in comparison to mussels acclimated to no heat treatments ($P < 0.05$).

*Breakpoint temperatures*

Tidepool mussels exhibited two distinct breakpoints, the first occurring between 26.51 ± 1.35°C and 28.72 ± 0.35°C, which also coincided with the maximum heart rate, and the second (and final) BPT occurring between 36.89 ± 0.6°C and 40.34 ± 0.25°C. In contrast, the majority of tidally-exposed mussels exhibited a single BPT between 40.2 ± 1.11°C and 45.19 ± 0.45°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 breakpoint temperature (BPT) than mussels acclimated to no heat treatments (Tukey HSD, $P < 0.05$).

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 habitat ($F_{(1,60)} = 67.011$, $P = <0.0001$; Figure 4), with tidally-exposed treatments having higher final breakpoint temperature (BPT) in comparison to tidepool treatments. There was also a significant effect of thermal regime ($F_{(2,60)} = 9.485$, $P = 0.0034$; Figure 4), where predictable and unpredictable treatments had significantly higher BPT than mussels acclimated to no heat treatments ($P < 0.05$). There was no effect of food availability on final BPT ($F_{(1,60)} = 0.378$, $P = 0.5503$), 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 (Figure 4).

### *Flatline temperature*

The best statistical model included all fixed factors without their interactions. We found that there was a significant effect of habitat ($F_{(1,67)} = 109.52$, $P = <0.0001$; Figure 5) with tidally-exposed treatments having higher FLT than tidepool treatments. The FLT for tidepool treatments ranged from 40 ± 0.12°C to 42.28 ± 0.22°C whereas the FLT of tidally-exposed treatments were much more variable, ranging from 43.28 ± 0.38°C to 47.7 ± 0.36°C. There was also a significant effect of thermal regime ($F_{(2,67)} = 10.55$, $P = 0.0008$; Figure 5), where the unpredictable treatments had significantly higher FLT than mussels acclimated to no heat and predictable treatments (Tukey HSD, $P < 0.05$). There was no effect of food availability on FLT ($F_{(1,67)} = 1.99$, $P = 0.1748$).

*Maximum heart rate*

The best statistical model included all fixed factors with their interactions. Maximum heart rate was significantly different between habitat ($F_{(1,60)} = 103.794$, $P = <0.0001$; Table 2), where tidepool mussels had much higher maximum heart rates than tidally-exposed mussels. In tidepool mussels, maximum heart rates ranged from 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 ($F_{(2,60)} = 0.5199$, $P = 0.6049$) or food availability ($F_{(1,60)} = 0.055$, $P = 0.8155$).

## Cellular stress mechanisms and energy stores

### *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 ($F_{(2,60)}$ = 6.054, $P$ = 0.0167) significantly impacted baseline Hsp/Hsc70 levels but there was also a significant interaction between habitat and thermal regime ($F_{(2,60)}$ = 4.152, $P$ = 0.0461). 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. 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 effected the baseline Hsp/Hsc70 levels ($F_{(1,60)}$ = 10.16, $P$ = 0.0023), 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 habitat, food availability, and sampling temperature with their interactions, as thermal regime did not contribute significantly to the trend. Both habitat ($F_{(1,235)}$ = 8.527, $P$ = 0.0038; Figure 6) 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 habitat, food availability and sampling temperature ($F_{(3,235)}$ = 3.794, $P$ = 0.011). With post hoc analysis, it was revealed 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, $P < 0.05$). In comparison, low food tidepool treatments saw an increase between baseline (13°C) levels and 20°C (Tukey HSD, $P < 0.05$), largely driven by the tidepool, predictable, low (SPL) 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, $P < 0.05$)

*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 ($F_{(2,60)} = 3.597$, $P = 0.0335$), with mussels from unpredictable treatments having elevated glycogen levels over mussels acclimated to no heat treatments (Tukey HSD, $P < 0.05$), but there was no effect of habitat ($F_{(1,60)} = 0.1436$, $P = 0.7061$) or food availability ($F_{(1,60)} = 0.7256$, $P = 0.3977$) on baseline (13°C) levels.

For assessment of glycogen stores during the acute thermal ramp, the best statistical model included habitat, thermal regime, and sampling temperature with their interactions, as food availability did not contribute significantly to the trend. Habitat ($F_{(1,235)} = 17.095$, $P = <0.001$; Figure 7) and sampling temperature ($F_{(3,235)} = 5.069$, $P = 0.002$; Figure 7) had significant effect on glycogen levels during the thermal ramp, but there was also a significant interaction between 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°C and 40°C (Tukey HSD, $P < 0.05$). The tidepool, unpredictable, high (SUH) 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 (159.78 ± 15.01 µmol glucosyl units $g^{-1}$) and resulting in the lowest glycogen levels at 40°C (46.35± 6.28 µmol 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 ($F_{(2,235)} = 3.93$, $P = 0.021$), with mussels from unpredictable treatments having significantly different glycogen levels in comparison to mussels acclimated to no heat treatments.

## 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. thermal environment, predictability, interaction with other stressors) integrate to modulate thermal tolerance. In our study we were interested to see how habitat, thermal predictability and food availability interacted to shape the physiological performance of *Mytilus californianus* exposed to an acute thermal ramp. We predicted that 1) mussels acclimated to a tidepool habitat of constant submergence would have a lower thermal tolerance than those tidally-exposed; 2) mussels subjected to an unpredictable thermal regime would be able to tolerate a large temperature range by maintaining high levels of energy stores, and an elevated cellular stress response leading to an increased thermal tolerance 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. Our results suggest that habitat medium was the largest driver for modulating physiological responses during an acute thermal ramp. Tidepool mussels exhibited higher heart rates with multiple breaks, but lower final BPT and FLT, even with greater glycogen usage and elevated cellular stress response, suggesting that thermal stress in an aquatic medium is more stressful than in air. Thermal regime also had a pronounced impact on thermal performance with mussels acclimated to predictable and unpredictable regimes having elevated baseline Hsp/Hsc70 levels and elevated BPT. 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. Surprisingly, food availability had little impact on cardiac performance or energy stores, but did affect baseline and subsequent induction of Hsp/Hsc70, which had habitat specific effects. Taken together, our results indicate that 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.

## Habitat

The cardiac performance and thermal sensitivity of mussels acclimated to tidepool and tidally-exposed habitats were markedly different, suggesting that habitat medium 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, but a lower 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 is higher during emersion in comparison to immersion (Jones et al., 2009; Bjelde and Todgham, 2013; Huang et al., 2015; Tagliarolo and McQuaid, 2016). These differences suggest that the interaction of air exposure and increasing temperatures may confer enhanced stress tolerance in mussels. The mechanism behind this enhanced tolerance is most likely related to potential capacity limitations on tidepool mussels during warming coupled with contrasts in metabolic efficiency and metabolic strategy to tolerate thermal stress between tidepool and tidally-exposed mussels. Cardiac activity is often used as an indicator of metabolism in molluscs, especially for species that do not gape and consume oxygen during emersion, like *Mytilus californianus* (Braby and Somero, 2006; Collins et al., 2020, Zhang et al., 2014). Cardiac breakpoint temperature is often described as the temperature where heart rate reaches its maximal value and then falls relatively rapidly as temperature continues to rise (Braby and Somero, 2006; Logan et al., 2012; Wang et al., 2019; Dong et al, 2022), and is considered an indication of the onset of aerobic capacity limitation resulting in suboptimal performance (Dong et al., 2022; Sokolova and Pörtner, 2003). In the current study, tidepool mussels have two distinct break points, 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 tidepool mussels reach capacity limitations and are experiencing suboptimal performance after 29°C. This is comparable with the critical temperature found in other *Mytilus sp* experiencing aquatic warming at a similar rate (Braby and Somero, 2006; Tagliarolo and McQuaid 2015). Suboptimal performance is characterized by an increased reliance on anaerobic metabolism (due to the inability of the heart to provide adequate oxygen for the rising demand) 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 and 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. These data suggest that tidepool mussels were experiencing cardiac capacity limitation and reliance on anaerobic metabolism, confirming a shift into their suboptimal performance range. Typically, continued performance in this range is time limited and can quickly progress into lethal temperatures (FLT) unless further metabolic modifications occur (Dong et al., 2022; Sokolova et al., 2012). In the case of tidepool mussels, individuals are able to extend this performance range by performing metabolic arrest whereby mussels actively depress heart rate for a short period of time in an effort to conserve energy (Bjelde and Todgham, 2013; Hui et al., 2020). This allows 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 mollusc species during a thermal ramp (Helm and Trueman, 1967; Trueman and Lowe, 1971; Bayne et al., 1976; Braby and Somero, 2006; Marshall et al., 2011; Bjelde and Todgham, 2013; Zhang et al., 2014; Tagliarolo et and McQuaid, 2015; Bjelde and Todgham, 2015; Pasparakis et al., 2016; Wang et al., 2019; Hui et al., 2020). While tidepool mussels are 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 suboptimal performance range in comparison to tidally-exposed mussels ultimately resulted in a lower Final BPT and FLT 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 metabolic strategy utilized to tolerate thermal stress coupled with increased metabolic efficiency. When emersed, *Mytilus californianus* does not typically exhibit gaping behaviour (Widdows and Shick, 1985; Gracey et al., 2016) and oxygen concentrations in the mantle cavity can quickly drop and anaerobic metabolism commences at the onset of hypoxia (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 (Hui et al., 2020). Consequently, intertidal organisms acclimated to cyclical regime of periodic air exposure exhibit a number of physiological modifications that promote metabolic efficiency to reduced metabolic demand during emersion (Nancollas and McGaw, 2021a,b; Demurs and Gurdley, 1994; deZwaan, 1983; Widdows and Shick, 1985). For example, *M. edulis* acclimated to cyclical air exposure reduced ammonia excretion, which enabled more scope for growth (Widdows and Shick, 1985) and the mussels metabolized alternative substrates during air exposure for higher ATP yield (deZwaan, 1983), which allowed anaerobic metabolism to be 50% more efficient in intertidal mussels in comparison to submerged mussels (Demurs and Gurdley, 1994). Evidence of these strategies 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, suggesting reduced baseline metabolic costs or active depression of metabolic processes during air exposure (Connor and Gracey 2012). 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 cardiac capacity 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 two 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 (deZwaan, 1983). This supports previous research that indicates metabolic rate depression is an energy conservation strategy (Marshall and McQuaid, 2011, Hui et al., 2020).

The capacity for tidally-exposed mussels to modulate metabolism in this way could be facilitated by the fact that tidally-exposed treatments always experience warming coupled with an additional environmental signal that is predictable: air exposure. While *M. californianus* show impressive circadian regulation (Connor and Gracey, 2011; Connor and Gracey, 2019), the transition from aquatic to aerial environments is thought to be one of the strongest environmental cues regulating thermal performance in intertidal organisms (Lockwood et al., 2015). Air exposure and thermal stress often initiate comparable compensation pathways such as oxidative stress and anaerobic metabolism (Elowe and Tomanek, 2021; Lockwood et al., 2015) and it may be possible that the onset of air exposure in tidally-exposed treatments provides advance warning of anticipated stress that provides tidally-exposed mussels time to make metabolic adjustments to better tolerate thermal stress. Without a predictable environmental signal to forewarn tidepool mussels of impending thermal stress, tidepool mussels may not have sufficient time to make metabolic adjustments which leads to increased thermal sensitivity.

## 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 thermal stress, complementing previous research that acclimation to elevated temperatures increases upper thermal tolerance (Cheng et al., 2018; Giomi et al., 2016; Kern et al., 2015; Oliver and Palumbi, 2011; Schaefer and Ryan, 2006; Schoepf et al., 2015). These results differ to our previous findings with winter-acclimated mussels, where only mussels in the unpredictable regime had higher BPT than control mussels (Nancollas and 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 and 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 BPT exhibited by predictable mussels in the current study suggests that exposure to predictable thermal fluctuations to 20°C was not enough to increase BPT but 25°C was, indicating that temperatures between 20-25°C represents a crucial threshold for modulating thermal tolerance.

Acclimation to thermal stress increased 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 upper thermal tolerance. 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 and Todgham, 2022), acclimation to an unpredictable thermal regime increased BPT, but not FLT, suggesting that thermal unpredictability alone is not the factor that increase FLT in the current study. In our previous study, we discussed the potential of immediate thermal history (last few days) providing a heat hardening response to the increase in cardiac performance, as the days before the acute thermal stress were some of the warmest days in acclimation (28°C, 18°C, 25°C, where acclimation average was 20°C and maximum was 28°C). In the current study, the immediate thermal history prior to the acute thermal ramp was warm, but not hot (28°C, 19°C, 24°C, where acclimation average was 25°C and maximum was 34°C), and on average was cooler than the predictable regime, suggesting that immediate thermal history was likely not the main driving force behind the increased FLT. Therefore, the most likely contributor for increasing FLT in the unpredictable treatments is the larger thermal range experienced by mussels in the unpredictable treatments over the course of the acclimation period. Just as predictable thermal fluctuation can increase thermal performance in comparison to a constant mean temperature (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; Vajedsamiei et al., 2021), increases in thermal range around the same mean temperature can also increase thermal tolerance (Kern et al., 2015; Otto and Rice, 1974, Shaefer and Ryan, 2006; Schoepf et al., 2015; 2022; Salinas et al., 2018). 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 increase FLT. Moyen 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°C or 35°C for 2hr) up to 3 weeks prior to exposure to an extreme heat stress (40°C for 2hr). Interestingly, Moyen et al., (2020) showed that mussels exposed to a single sublethal bout of heat stress at 25°C did not enhance heat tolerance in comparison to control mussels (14°C). As mussels from the unpredictable treatments were the only mussels to be subjected a temperature above 30°C during the acclimation period, this could explain why they had elevated FLT and why we did not see an increase in FLT in mussels from the predictable treatments in comparison to mussels held under no thermal stress. 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 depend on regular exposure to a threshold temperature (most likely between 20-25°C), whereas FLT may be modulated by magnitude of temperature (>30°C) in recent thermal history.

The mechanism behind increased BPT in predictable and unpredictable treatments could be in part, at least for tidepool mussels, due to elevated baseline Hsp/Hsc70, which also exhibits a comparable temperature threshold response. For tidepool mussels, predictable and unpredictable treatments had higher baseline Hsp/Hsc70 in comparison to the no thermal stress treatment, indicating that that acclimation to predictable or unpredictable fluctuating thermal regimes produce a preparatory response in tidepool mussels, which has previously been observed in warm-adapted molluscs (Dong et al., 2008). Similar to BPT, the preparatory response of Hsp/Hsc70 may appear to be activated once a certain temperature threshold is repeatedly reached during prior thermal history, as acclimation to predictable warming from 13°C-20°C or unpredictable warming in the range of 13°C-28°C had an absence of preparatory Hsp/Hsc70 responses (Nancollas and Todgham, 2022). Studies on the induction of Hsp70 production in *M. californianus* in response to thermal stress has shown that Hsp70 synthesis in the gill on average does not occur until 23°C in mussels acclimated to similar temperature as this study (13°C) (Buckley et al., 2001; Roberts et al., 1997; Halpin et al., 2004). This suggest that changes in preparatory cellular stress mechanisms and Final BPT may be linked, and could aid in protecting the integrity of cardiac proteins to elevated temperatures. However, this is clearly not the only mechanism contributing to upper thermal tolerance as while tidally-exposed mussels generally exhibited a similar trend of predictable and unpredictable mussels having elevated baseline

Hsp/Hsc70 over mussels acclimated to no thermal stress, this was not significant. From the metrics measured in this study, it is not possible to identify the specific mechanism underlying thermal performance enhancements in tidally-exposed predictable and unpredictable mussels, though it could be related to further adjustments in metabolic efficiency. Warm acclimation has been shown to modify energy metabolism in *Mytilus sp.* with an upregulation of proteins enhancing energy regulation, which have been linked to increase in upper thermal tolerance (Fields et al., 2006; Anestis et al, 2010). Similarly, acclimation to thermal fluctuations within a tidal framework have indicated an increase in metabolic efficiency through reductions in baseline gill succinate content and reduction in baseline gill MDH activity, particularly in mussels acclimated to an unpredictable thermal regime, when compared to mussels acclimated to a tidal cycle with no thermal stress (Nancollas and Todgham, 2022). Investigating how thermal predictability shapes energy dynamics during a thermal ramp may help in further understanding the role of thermal predictability in shaping upper thermal tolerance.

Mussels acclimated to unpredictable thermal stress had elevated baseline glycogen levels, and in tidepool mussels had a greater depletion during thermal stress which like contributed to the increased FLT seen in unpredictable mussels, suggesting that energy roles play an important role in tolerating unpredictable conditions. These results align with our previous research (Nancollas and 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 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 been reported for molluscs (deZwaan and Putzer, 1985; Isani et al., 1995; Picard et al., 2014) due to the suppression of enzymes involved in glycogenolysis (deZwaan and Putzer, 1985). Upon re-immersion and return to aerobic metabolism, intertidal organisms experience high metabolic demand or "oxygen debt" in order to re-establish physiological homeostasis which is exacerbated when coupled with thermal stress (Hofmann and Somero, 1996; Tomanek and Somero, 2000; Schill et al., 2002; Clark et al., 2008; Zhang et al., 2014; Dudognon et al., 2013; Ivanina et al 2016,). Glucose levels in *M. californianus* exhibit a circatidal rhythm with spikes during re-immersion (Connor and Gracey, 2012), and evidence of high rates of both aerobic and anaerobic metabolism have been reported during reimmersion (Zurburg et al., 1982; Nicchitta and Ellington, 1983; DeZwaan, 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 aide in our understanding of how energy homeostasis is regulated.

### 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 et al., 2001; Dahlhoff, 2004; Dahlhoff and Menge, 1996; Schneider et al.

2010, Sarà et al., 2012; Matzelle et al., 2015; Cheng et al., 2018; Fitzgerald-Dehoog et al., 2012). In our study, we predicted similar effects, where mussels under low food regimes would have reduced 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 and upper thermal tolerance, but 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 habitat specific.

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, CA and the same food levels have been used by other studies using live algae (Hettinger et al., 2014). 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 weeks) acclimation times (Schneider et al., 2010; Fitzgerald-Dehoog et al., 2012; Schneider and Helmuth, 2007). Indeed, even though Cheng et al. (2018) reported a lower breakpoint temperature in warm acclimated (30°C) *Perna perna* when acclimated to low food regime in comparison to high food, heart rate was the same between the food treatments across all temperatures (15-35°C), and food availability had no effect on cold acclimated (16°C) mussels BPT. Similarly, there was only an effect of food availability on survival when *M. galloprovincialis* and *M. trossulus* were acclimated to hot (30°C) conditions for 8 weeks, and not to cool (20°C) or warm (25°C) conditions (Schneider et al., 2010). This suggests that food availability may only play an important role in thermal performance when low food conditions persist over a longer period of time (>8 weeks) or when prior thermal history (i.e. acclimation temperature) is 30°C or above for multiple subsequent days, a conclusion also surmised by Vasquez and 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) (Nancollas and Todgham, manuscript in preparation). This indicates that current low levels of food in Bodega Bay are 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.

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 a Hsp70 response (Dahlhoff et al., 2002; Jeno and Brokordt, 2014). Production of Hsp/Hsc70 is energetically costly, so it is reasonable that investment into preparatory levels may not be possible under low food regimes. However, this was not due to not having sufficient energy resources, as glycogen levels were comparable to high food treatments, suggesting that maintenance of high glycogen stores was more beneficial than an elevated preparatory Hsp/Hsc70 response. This strategy seems advantageous as the breakdown of glycogen can fuel a number of different metabolic pathways depending on the severity of the stress (including inducible Hsp70 production), and can be utilized to fuel reproductive efforts during optimal environmental conditions, rendering it a more flexible mechanism to tolerate the dynamic intertidal environment (Sokolova et al., 2012). Acclimation to a low food regime also altered how Hsp70 was induced during the acute thermal ramp, and exhibited 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 elevating Hsp/Hsc70 at 20°C but then returning to baseline levels for the rest of the acute thermal ramp. This relationship was mainly driven by the tidepool, predictable, low (SPL) treatment exhibiting a spike in Hsp/Hsc70 at 20°C. The SPL treatment also had reduced cardiac performance during the acute thermal ramp and the lowest maximum heart rate, and the earlier spike in Hsp/Hsc70 levels could indicate that this combination of treatment factors (predictable thermal stress plus low food in a submerged setting) was the most stressful. 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 (AUL) treatment. There have been few studies that have investigated how low food affects the Hsp/Hsc70 response, so the mechanism behind the habitat-specific differences presented here remains unknown. Low food availability has been shown to modulate energy pathways that are also associated with the cellular stress response (Vasquez et al., 2019), and acclimation to a variety of conditions can alter the threshold temperature that induces Hsp70 or the magnitude of Hsp70 expression (Buckley et al., 2001, Dietz and Somero, 1992, Tomanek and Somero, 2002). Therefore, it is possible that acclimation to low food regimes could reduce the induction temperature and/or magnitude of responses. A more targeted investigation into how low food shapes the energetic pathways associated with the cellular stress response is needed to elucidate this association.

## Conclusion

In conclusion, *M. 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. Mussels exposed to aquatic warming are more thermally sensitive and maintain cardiac performance during increasing temperatures through multiple breaks in cardiac function combined with increased cellular stress response and depletion of energy stores. In contrast, mussels exposed to aerial warming undergo metabolic depression and likely utilize metabolic enhancements to increase upper thermal tolerance. Importantly, tidepool mussels are already regularly experiencing temperatures that exceed their capacity limitations, and the striking difference in performance from mussels in different habitat media (i.e. air) suggests that increases in acute hot days will more negatively affect mussels in aquatic habitats, suggesting further increases in temperature could have severe consequences for tidepool community composition and function as well as the rocky intertidal more broadly. Acclimation to unpredictable fluctuations in temperature, as intertidal organisms are typically exposed to *in situ*, appears to increase upper thermal tolerance, 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 this effects 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 an 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 how intertidal organisms will perform in future climate change scenarios.

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# TABLES

**Table 1.** ANOVA table results of the GAMM analysis, displaying the effect to 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 twelve treatments (n = 6 for each treatment) during the acute thermal ramp (6.5°C $h^{-1}$). Treatments names combine habitat (S: tidepool; A: tidally-exposed) with thermal regime (N: no thermal stress; 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) value for Temperature.

| **Predictor factor** | **df** | ***F*-value** | ***P*-value** |
|---|---|---|---|
| Habitat | 2 | 14.073 | 0.000181 |
| Thermal regime | 1 | 4.098 | 0.01671 |
| Food | 1 | 0.054 | 0.816544 |
| Habitat*Thermal regime | 2 | 1.269 | 0.281453 |
| Habitat*Food | 2 | 0.095 | 0.758218 |
| Food*Thermal regime | 1 | 1.887 | 0.151815 |
| Habitat*Thermal regime*Food | 2 | 0.861 | 0.422742 |
| **s(Temperature)** | **edf** | ***F*-value** | ***P*-value** |
| 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 |

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

| **Acclimation treatment** | **IHR** | **MHR** | **# 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 |

# FIGURES

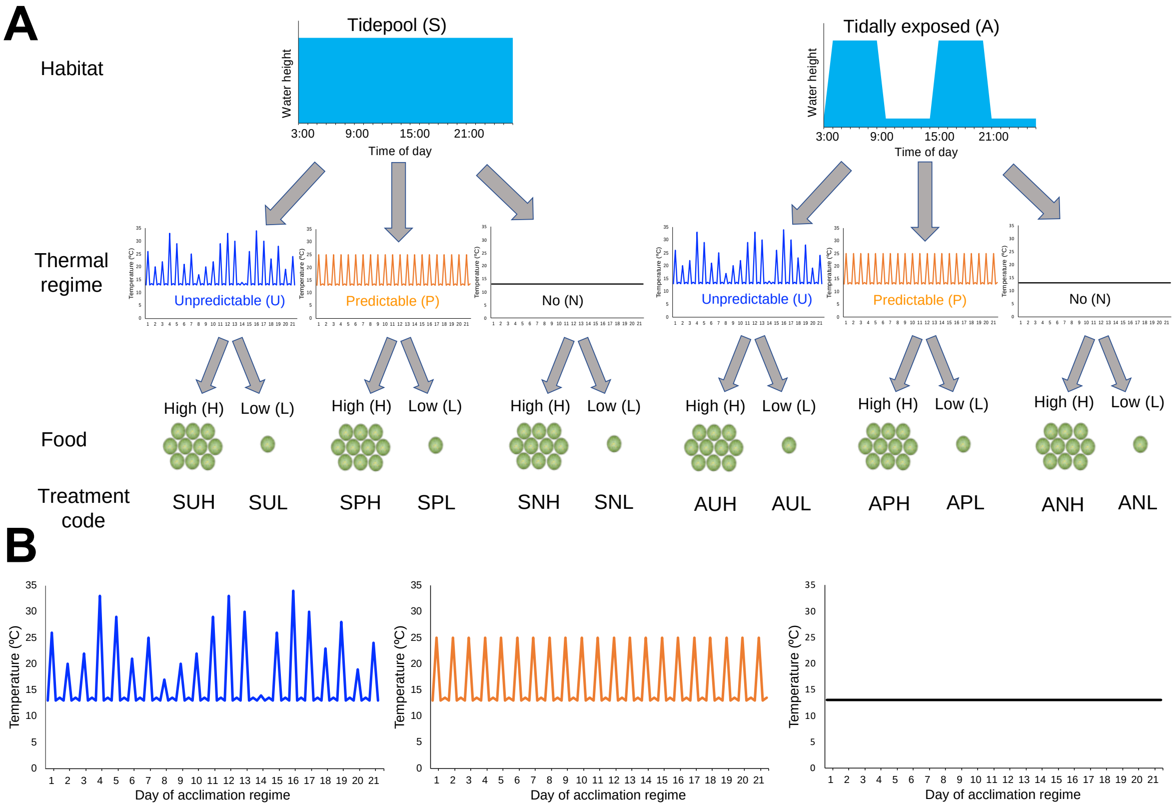

**Figure 1.** A: Schematic of the twelve treatments for acclimation. *Mytilus californianus* were acclimated for 3 weeks to either tidepool (S) or tidally-exposed (A) treatments combined with either unpredictable (U), predictable (P) or no (N) thermal stress and high (H) or low (L) food availability for a total of twelve 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 three thermal regimes incorporated during acclimation. Unpredictable (blue) mussels were warmed to a different max temperature during daytime low tide, and this profile

mimicked a 3-week period of max temperatures from the ‘master’ logger profile. Predictable (orange) mussels were warmed to the same max temperature every daytime low tide (25°C) which was the average of the max temperatures in the unpredictable regime. Finally, no (black) thermal stress, where mussels were not warmed during daytime low tide and experienced ambient temperatures (13°C).

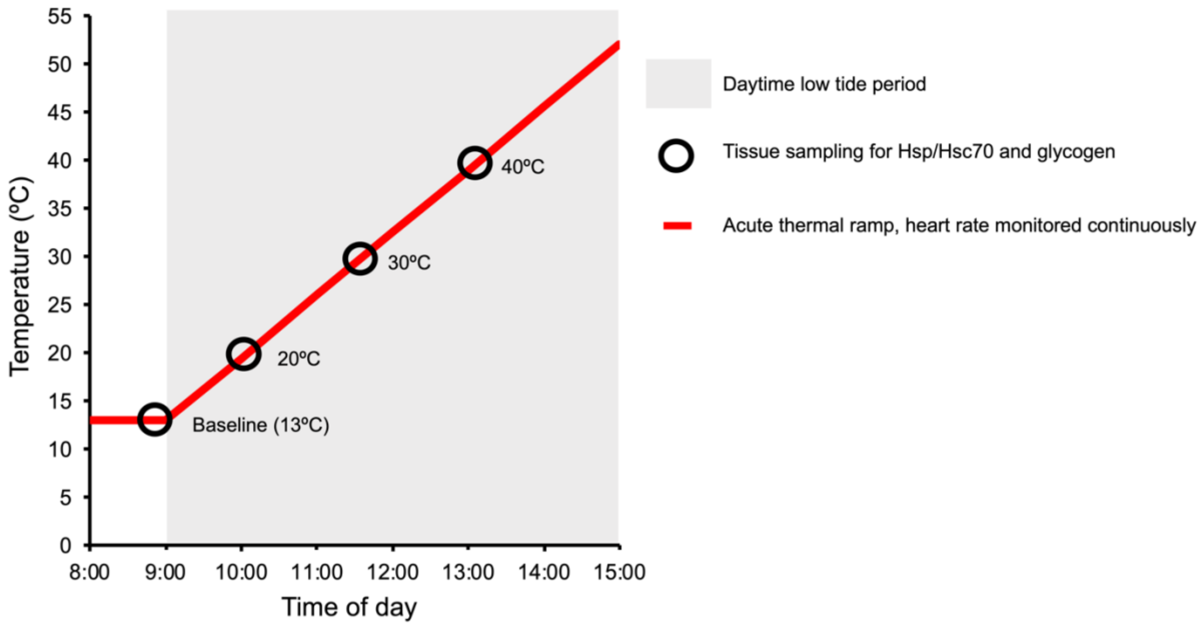

**Figure 2.** Diagrammatic schematic of the acute thermal ramp experiment. The acute thermal ramp coincided with the time in which daytime low tide occurred during acclimation (9 am - 3 pm, grey shaded area). During daytime low tide, tidepool mussels were submerged whereas tidally-exposed mussels were exposed to air. At the initiation of the daytime low tide period (9 am) the acute thermal ramp would begin, increasing the temperature at a rate of 6.5°C/hr as represented by the red line. During the acute thermal ramp, heart rate was monitored continuously over the entire warming period (13°C-52°C; 9 am – 3 pm), and this data was used to assess cardiac performance during the acute thermal ramp by creating thermal performance curves, as well as assessing Final Breakpoint temperature (BPT), Flatline temperature (FLT) and Maximum Heart rate (MHR). Tissue samples for Hsp/Hsc70 and glycogen analysis were taken at 4 different sampling temperatures, as indicated by the black circles. The first sample (baseline) was taken just before the daytime low tide period at ambient temperature (13°C) and was used to understand preparedness for anticipated period of stress. During the acute thermal ramp, tissues

samples were also taken at 20°C, 30°C and 40°C, and were used with the baseline (13°C) sample to understand how Hsp/Hsc70 and glycogen levels changed as temperature increased.

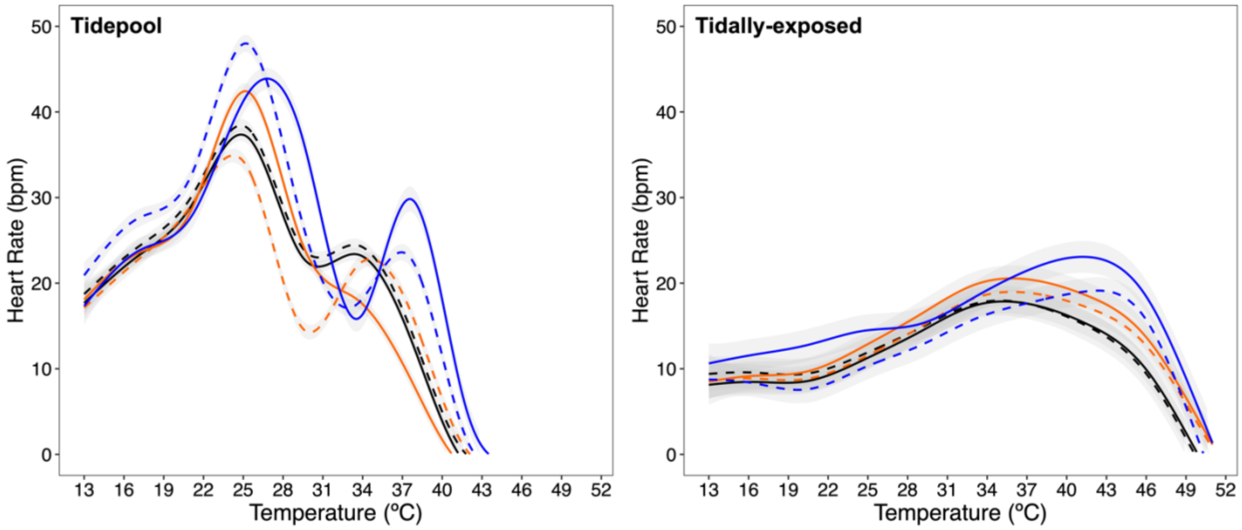

**Figure 3.** Thermal performance curves produced by GAMM for heart rates throughout the acute thermal ramp for mussels from the tidepool and tidally-exposed treatments. Unpredictable treatments are in blue, predictable in orange and no thermal stress in black. Solid lines represent high food treatments and dashed lines represent low food. All treatments (n = 6 per treatment) were subjected to an acute thermal ramp from 13°C 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.

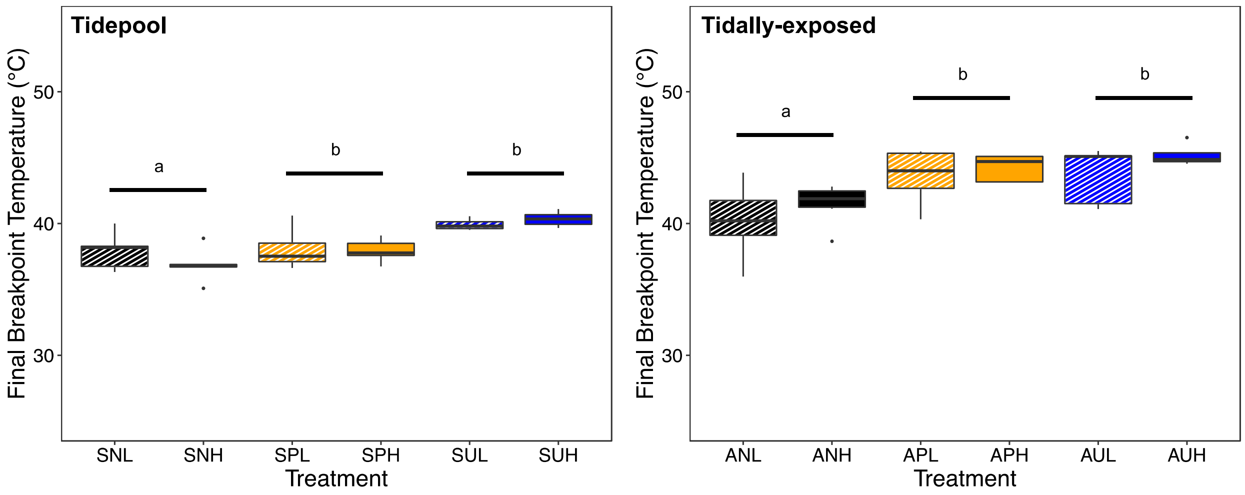

**Figure 4.** Final breakpoint temperatures for *Mytilus californianus* from tidepool (S) and tidally-exposed (A) treatments. Unpredictable (U) treatments are in blue, predictable (P) in orange and no (N) thermal stress in black. Solid plots represent high food (H) treatments and plots with diagonal lines represent low food (L) treatments. All treatments (n = 6 per treatment) were subjected to an acute thermal ramp from 13°C 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 times IQR. Points beyond the whiskers are outliers. Different letters represent a significant difference between thermal regimes (Tukey HSD, $P < 0.05$). There was no significant difference between low and high food treatments (LME, $P > 0.05$).

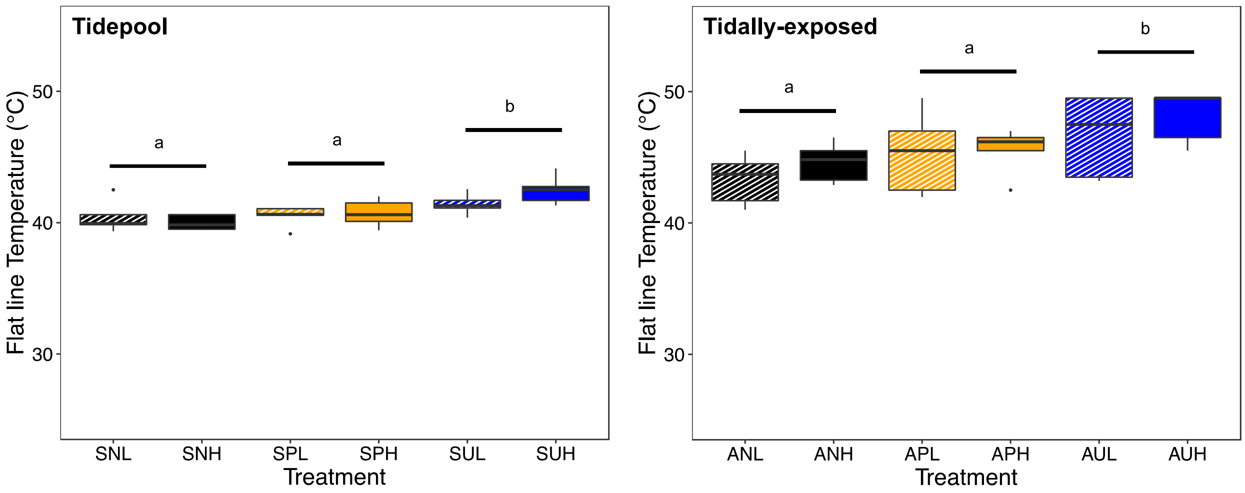

**Figure 5.** Flat line temperatures for *Mytilus californianus* from tidepool (S) and tidally-exposed (A) treatments. Unpredictable (U) treatments are in blue, predictable (P) in orange and no (N) thermal stress in black. Solid plots represent high food (H) treatments and plots with diagonal lines represent low food (L) treatments. All treatments (n = 6 per treatment) were subjected to an acute thermal ramp from 13°C 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 times IQR. Points beyond the whiskers are outliers. Different letters represent a significant difference between thermal regimes (Tukey HSD, $P < 0.05$). There was no significant difference between low and high food treatments (LME, $P > 0.05$).

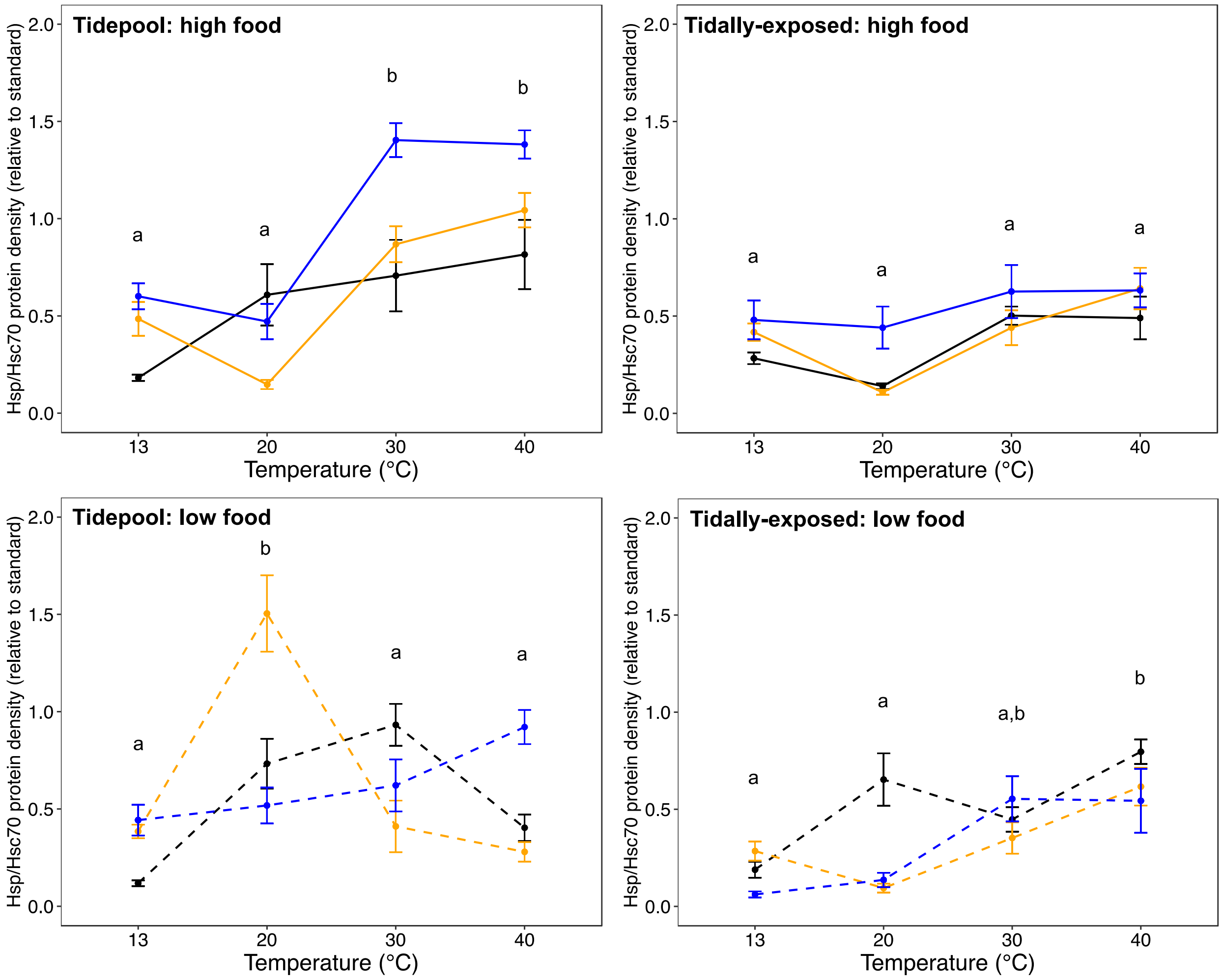

**Figure 6.** Hsp/Hsc70 of *Mytilus californianus* gill tissue taken just before the daytime low tide (baseline:13°C) and at 20°C, 30°C and 40°C during an acute thermal ramp. Due to a statistically significant interactive effect between habitat, food availability and sampling temperature, plots have been separated out into tidepool high food (top left) and low food (bottom left) treatments, as well as and tidally-exposed high food (top right) and low food (bottom right) treatments. In all cases, unpredictable treatments are in blue, predictable in orange and no thermal stress in black. For continuity with other plots, solid lines represent high food treatments and dashed lines represent low food treatments. All treatments (n = 6 per treatment) were subjected to an acute thermal ramp from 13°C 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 ± SEM. Different letters represent a significant difference in levels at different sampling temperatures (Tukey HSD, $P < 0.05$). There was no significant difference in thermal regime.

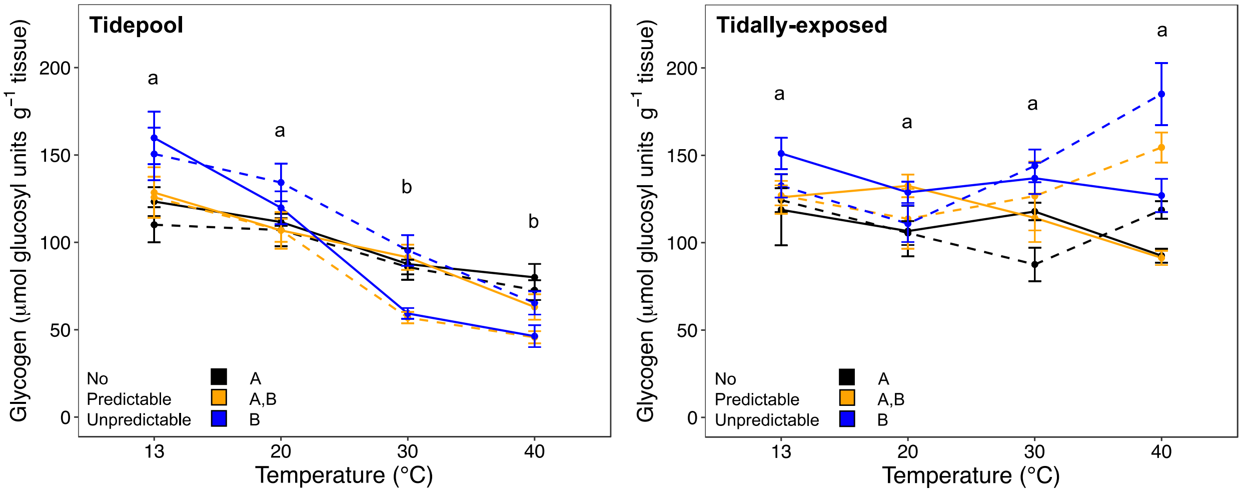

**Figure 7.** Glycogen content of *Mytilus californianus* mantle tissue taken just before the daytime low tide (baseline:13°C) and at 20°C, 30°C and 40°C during an acute thermal ramp from tidepool and tidally-exposed treatments. Unpredictable treatments are in blue, predictable in orange and no thermal stress in black. Solid lines represent high food treatments and dashed lines represent low food treatments. All treatments (n = 6 per treatment) were subjected to an acute thermal ramp from 13°C 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 were warmed in air. Values are mean ± SEM. Different lower-case letters represent a significant difference in levels at different sampling temperatures (Tukey HSD, $P < 0.05$). Different upper-case letters represent a significant difference between thermal regimes. There was no significant difference in food availability (LME, $P > 0.05$).

# APPENDIX

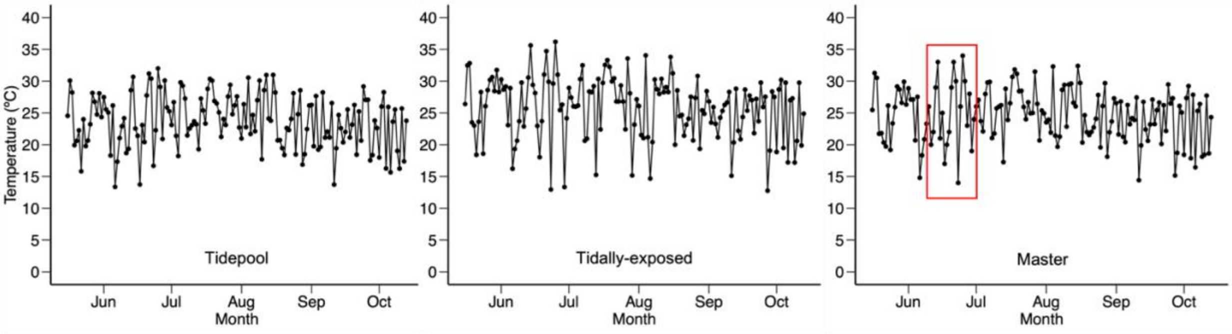

**Figure A1.** Maximum temperatures for each day from the robomussels situated in a tidepool (left) and attached to tidally-exposed rock (centre). Temperature data from both the tidepool and tidally-exposed logger were combined to create the master temperature profile (right). The red outline on the master profile represents the section on logger data that was use to inform the unpredictable thermal regime.

# CHAPTER 4

## Heatwaves in the intertidal zone: How habitat, thermal predictability and food availability structure the physiological responses of the California mussel to repeated thermal stress events

### ABSTRACT

A central prediction of recent climate change models is that there will be an increase in thermal unpredictability and heatwave events. Most of our understanding of intertidal thermal physiology is based on single, acute (often lethal) thermal events. Less is known about how intertidal organisms may respond to sublethal repeated thermal stress events, such as heatwaves. Importantly, we know little about how aspects of an organism's environment (habitat medium, thermal predictability, food availability) may interact to influence thermal performance during heatwaves. Here, we examined how acclimation to different levels of thermal predictability (no heat, predictable, unpredictable) with high and low food availability influence performance during a three-day simulated heatwave in *Mytilus californianus* in two habitat treatments: tidepool and tidally-exposed. The heatwave event consisted of an initial baseline day, where no warming occurred, followed by three heatwave days where mussels were warmed to 35°C each daytime low tide in their respective medium (water or air), followed by a half day recovery. Heart rate was recorded continuously during the heatwave event as a proxy for metabolism and tissues samples were taken just before the start and at the end of daytime low tide each day to assess levels of cellular stress and energy stores. We found that habitat was the strongest driving factor for shaping physiological performance in *Mytilus californianus* and dictated how both food availability and thermal history interacted to influence mussel performance. Tidepool mussels exhibited increased thermal sensitivity, with higher maximum heart rates and breaks in cardiac performance during warming on the heatwave days. Performance of tidepool mussels was also strongly regulated by food availability where low food resulted in increased thermal sensitivity and maximum heart rates, but also an increase in post warming recovery time and Hsp/Hsc70 responses coupled with a decline in energy stores over the course of the heatwave. In contrast, tidally-exposed mussels exhibited no breaks in cardiac function during warming, and maintained baseline levels Hsp/Hsc70 and glycogen throughout the heatwave event, but performance during warming was more regulated by thermal history rather than food availability. Our results suggest that the predicted increases in heatwave events from climate change will not have a uniform effect in tidepool and tidally-exposed habitats which could have severe consequences for intertidal community composition.

## INTRODUCTION

Temperature is one of the most important abiotic factors affecting the distribution and physiological performance of organisms in the intertidal zone (Helmuth et al., 2006; Liao et al., 2021; Miller et al., 2009; Stillman and Somero, 1996). A central prediction of climate change models is that there will not only be an increase in the global mean temperature and number of extreme hot days, but there will also be an increase in thermal unpredictability and multi-day thermal stress events, such as heatwaves (Angeliel et al, 2017; Arias et al., 2021; Coumou and Rahmstorf, 2012; Frolicher et al., 2018; Guo et al., 2017; Oliver et al., 2018; Stillman, 2019). Heatwaves are predicted to increase in severity, frequency, and duration as a consequence of climate change, and extreme heatwaves are already triggering mass mortality events for a variety of organisms (Harley, 2011; Jones et al., 2018; Piatt et al., 2020; Roberts et al, 2019).

The rocky intertidal zone has often been considered a model ecosystem for assessing the effects of climate warming due its dynamic, unpredictable, and often extreme fluctuations in temperature due to the rise and fall of the tides. As intertidal organisms live in an environment that fluctuates between aquatic and terrestrial with the movement of the tide, they are vulnerable to atmospheric heatwaves during daytime low tide. The majority of our understanding of how intertidal organisms respond to thermal stress events is based on exposure to single, acute (typically lethal) thermal stress events in order to understand an organism's upper thermal limits and the mechanisms that define those limits (Somero, 2002; Tomanek and Helmuth, 2002). From such studies, we know that intertidal organisms rely on key physiological mechanisms in order to tolerate acute warming episodes, including metabolic regulation and protection of physiological homeostasis through the initiation of cellular stress response mechanisms (Dong et al., 2022; Hofmann and Todgham, 2010; Hui et al., 2020; Pörtner, 2002; Somero, 2012). In intertidal molluscs, metabolic regulation of cardiac function has been shown to be important in establishing upper thermal limits (Dahlhoff et al, 2001; Dong et al.,2022; Hui et al, 2020; Marshall et al., 2011), and cellular stress mechanisms such as Hsp/Hsc70 can aide in maintaining protein integrity during thermal stress and can also increase upper thermal limits (Buckley et al., 2001; Dong et al., 2008; Han et al., 2013; Hofmann and Somero, 1995; Madeira 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). Both metabolic modulation and initiation of cellular stress mechanisms can be energetically costly, and maintaining sufficient energy stores has been shown to be important for surviving stressful low tide periods (Ivanina et al, 2008; Sokolova et al., 2012; Somero, 2002). Understanding how these mechanisms work together to influence upper thermal limits during acute, lethal increases in temperature has provided valuable context for an organism’s upper thermal tolerance; however, intertidal organisms almost exclusively experience thermal stress in a sublethal, often consecutive manner. Therefore, understanding physiological responses to repeated sublethal thermal events will likely be more ecologically relevant for understanding how intertidal organisms will respond to climate change (Dong et al., 2022; Sarà et al 2011; Tagliarolo et al., 2016).

While still in its infancy, our knowledge on the effects of atmospheric heatwaves on intertidal organisms indicate that heatwaves can produce cumulative physiological costs through the depletion of energy stores, as well as increases in oxidative and cellular stress mechanisms (He et al., 2022a; He et al., 2022b; Virgin and Schiel, 2023; Xu et al., 2023), which can produce long term fitness costs (He et al., 2022a; He et al., 2022b; Leung et al., 2019; Xu et al., 2023) that can eventually lead to death (Leung et al., 2019; Xu et al., 2023). To date, most heatwave research has been done with intertidal organisms acclimated to constant environmental conditions, and we know little about how important aspects of an organism’s environment, such as habitat medium, predictability of thermal history, and food availability may influence the physiological responses to heatwaves.

During daytime low tide, the complex landscape of the rocky intertidal zone can result in conspecifics experiencing unpredictable thermal stress in both aquatic (e.g. tidepools) and terrestrial (e.g tidally-exposed to air) environments. Growing evidence suggests that the medium (air or water) in which thermal stress occurs can dictate the metabolic responses of intertidal organisms. Some intertidal organisms are able to maintain comparable metabolic performance in air and water (Bjelde and Todgham., 2013; Huang et al., 2015; McMahon, 1990; Stillman and Somero, 1996), whereas other species will metabolically depress in air in order to conserve energy and minimize the desiccation risk that can often co-occur with air exposure (Fly et al., 2012; McMahon et al., 1991; Nancollas and McGaw, 2021; Sokolova and Pörtner, 2001; Tagliarolo et al., 2012, Tagliarolo and McQuaid, 2016). Regardless of the metabolic strategy utilized during air exposure, evidence indicates that in adult individuals, upper thermal tolerance is elevated when thermal stress occurs in air vs water for intertidal organisms, suggesting that some aspect of air exposure may confer enhanced tolerance (Bjelde and Todgham, 2013; Huang et al., 2015; Jones et al., 2009; Tagliarolo and McQuaid, 2016). However, this enhanced tolerance is likely dependent on the degree of desiccation stress (humidity of air), as well as organisms' size and life stage (Jurgens et al., 2016).

Prior thermal history has been shown to significantly influence thermal performance and it has been well established that organisms acclimated to warmer temperatures often have higher upper thermal limits and/or experience reduced physiological costs associated with responding to elevated temperatures (Barry et al., 1995; Somero, 2005; Stillman, 2002). Recent studies have shown that intertidal organisms acclimated to a predictable fluctuating thermal regime can exhibit elevated thermal tolerance in comparison to organisms exposed to constant conditions with the same mean temperature (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; Vajedsamiei et al., 2021). Intertidal organisms typically experience unpredictable temperature fluctuations due to daily shifts in environmental variables, such as solar radiation, wind speed and precipitation (Burggren, 2019; Helmuth et al., 2006) and we are beginning to understand how unpredictable temperature fluctuations may influence performance to a single acute thermal event, such as an extreme hot day (Drake et al., 2017; Nancollas and Todgham, 2022). With the predicted increase of thermal unpredictability as a consequence of climate change (Angeliel et al, 2017; Arias et al., 2021; Guo et al., 2017), understanding how thermal unpredictability influences responses to heatwaves will be important to accurately understand the physiological resilience of intertidal organisms to climate change.

Along with temperature, food availability has been highlighted as an important factor for maintaining physiological performance during thermal stress (Sokolova et al., 2012). 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 (Boyce et al., 2010; Hays et al., 2005; Henson et al., 2021; Rost et al., 2008; Tortell et al., 2002; Wang et al., 2015). Marine heatwaves especially have been shown to produce rapid and drastic alterations to community composition (Areteaga and Rousseaux, 2023). Sufficient food availability has been shown to be crucial for organismal fitness traits such as growth, reproduction, and overall survival during chronic thermal stress (Huey and Kingsolver, 2019, Sarà et al., 2011; Schneider et al., 2010), and predictions from field and empirical studies have indicated that maintaining sufficient energy resources will be crucial for tolerating climate change (Huey and Kingsolver, 2019; Sarà et al., 2011). Despite this, little is known about how food availability influences more mechanistic levels of performance such as metabolism and cellular stress mechanisms during a heatwave, and how food availability may interact with thermal history in different habitats.

Intertidal mussels are foundational species and important ecosystem engineers that aggregate in dense beds in the rocky intertidal zone, in both tidepool and tidally-exposed environments. Mussel beds provide a number of crucial ecological functions such as increasing habitat complexity, providing settlement substrate, wave protection, and shelter for a number of other intertidal organisms (Fly et al., 2015). Mussel beds also play an important role in buffering thermal extremes for organisms and juvenile mussels that reside within the mussel matrix (Jurgens and Gaylord, 2016). The California mussel, *Mytilus californianus* occurs along much of the West coast of North America, and experiences unpredictable fluctuations in temperature on a daily basis. *Mytilus californianus* populations have experienced large declines (>60%) as a result of climate change-induced effects (Harley, 2008; Helmuth et al., 2006; Helmuth et al., 2016; Smith et al., 2006; Sorte et al., 2017), and more recently, mass-mortality events as a result of heatwaves (Galil et al., 2022, He et al., 2022; Seuront et al., 2019). In this study, we examined how acclimation to variations in thermal predictability and food availability in both aquatic and air-exposed low tide habitats influence cardiac performance, the cellular stress response (Hsp/Hsc70) and energy reserves (glycogen content) during a simulated heatwave event. We predicted that: 1) mussels acclimated to tidepool treatments would be more susceptible to repeated thermal stress than tidally-exposed mussels; 2) mussels acclimated to an unpredictable thermal regime would tailor their physiology to be able to tolerate potentially high levels of thermal stress and thus would maintain elevated energy reserves and incur reduced physiological costs during the heatwave; and 3) mussels subjected to low food availability would have reduced resources to tolerate multiple consecutive thermal stress events, and thus would have a reduced performance as the heatwave progressed. With the predicted increases in thermal unpredictability, and heatwave events forecasted by climate change models, understanding how thermal predictability interacts with food availability in different habitats to influence performance during heatwaves will be paramount for predicting how climate change will affect intertidal communities.

# MATERIALS 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 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, 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

Acclimation conditions were the same as described in Chapter 3. To summarize, mussels were randomly divided between one of twelve different acclimation treatments and were held under these conditions for 3 weeks. The twelve acclimation treatments combined three different temperature regimes (unpredictable: U; predictable: P; no: N heat stress) and two levels of food (high: H, low: L) within two different habitat types: tidepool (always submerged: S) and tidally-exposed (circatidal exposure to air and water: A) (Figure 1A). The six tidally-exposed treatments were built to simulate natural intertidal conditions by replicating semidiurnal circatidal changes in water height, which was manipulated using Arduino microcontrollers (Arduino YUN, Adafruit, New York, NY, USA; Drake et al., 2017; Miller and Long, 2015) and oscillated based on a semidiurnal tidal pattern. This means that tidally-exposed mussels experienced two 6 hour periods of emersion each day – daytime low tide (9am-3pm) and night-time low tide (9pm-3am), each of which was followed by a 6 hour period of immersion at high tide (3pm-9pm; 3am-9am).

For the six tidepool treatments, mussels were held in identical conditions as tidally-exposed treatments, but experienced no air exposure, and were always held in submerged conditions.

Thermal stress in the intertidal typically occurs during daytime low tide, where solar radiation combines with air temperatures to elevate mussel body temperature. As such, temperature manipulation occurred during each daytime low tide and was performed in the appropriate corresponding medium based on habitat - tidepool mussels were warmed in water and tidally-exposed treatments were warmed in air. Temperature profiles for regimes were based off daytime low tide data from two 'robomussel' temperature loggers (Maxim Integrated Products, Dallas, TX, USA): one embedded on the rock next to tidally-exposed *M. californianus*, and one in a tidepool (size: L:1m, W: 0.6m, D: 0.2m) surrounded by *M. californianus* in Bodega Marine Reserve. 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 (Figure A1). For the unpredictable treatments (U), mussels experienced varying temperatures within the range of 13-34°C during daytime low tide (Figure 1B). This temperature profile mirrored a 3-week 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-month logger period (25°C) and included the maximum temperature (34°C). Mussels in the predictable treatments (P) 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 throughout the 3 weeks as the unpredictable treatment, but in a predictable manner. As a control, a final thermal regime with no heating (N) 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. Temperature manipulation was achieved via Arduino microcontrollers, which 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 heating system. For tidally-exposed treatments, the heating system was 150 W ceramic heat lamps, and for tidepool treatments was an 150 W aquarium heater. 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 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 fed live *Nannochloropsis* 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 hour 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 (H) were fed 100,000 cells $mL^{-1}$ (~10 µg Chl $L^{-1}$) of phytoplankton whereas low food treatments (L) were fed 10,000 cells $mL^{-1}$ (~1 µg Chl $L^{-1}$), which equated to 50 cells/g wet weight and 5 cells/g wet weight respectively. These food levels were chosen to represent the average high and low levels of chlorophyll observed near Bodega Bay, CA (data access: boon.ucdavis.edu; coastwatch.pfeg.noaa.gov/infog/MW_chla_las.html).

Each of the treatments had two 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 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). Mortality was low in all tanks (< 1 %) and if occurred, food was adjusted to maintain consistent per mussel density across tanks.

**Heatwave event**

After acclimation, mussels from the 12 treatments were subjected to a simulated heatwave event. Currently, there is no universal definition of a heatwave and definition of heatwave intensities and durations vary by region. The IPCC defines a heatwave as "a period of abnormally hot weather that typically lasts 2 or more days". Here, our understanding was that a heatwave is a weather event where organisms experience extreme, but sublethal temperatures across multiple sequential days. The simulated heatwave event started with an initial baseline (B) day, where no warming occurred, and was followed with three subsequent heatwave days (HW1, HW2, HW3) followed by half day for recovery (R) (See Figure 2 for schematic representation). During the three heatwave days, mussels were warmed from ambient conditions (~13°C) to 35°C during daytime low tide, at a warming rate of 3.67°C $hr^{-1}$. 35°C was chosen as it corresponds with the hottest temperature recorded by our *in situ* robomussels, but still below the lethal limit of *M. californianus* (which is ~40°C for submerged individuals; see Chapter 3). In line with acclimation conditions and the temporal thermal ramps seen *in situ*, on the heatwave days, mussels were warmed during the daytime low tide period only (9am-3pm). Temperature was ramped using the Arduino microcontroller - heat lamp/bar system described previously. Habitat conditions from the acclimation treatments were maintained throughout the heatwave event, where tidepool mussels remained constantly submerged and tidally-exposed mussels experienced a semidiurnal cyclical exposure of air and water during the heatwave event, and timings of tidal transition were synchronous with the acclimation period. After the daytime low tide period, tanks were flushed with fresh seawater, which quickly returned tank temperature to ambient conditions within 15 minutes of the end of low tide, to replicate the fast reprieve from high tide seen *in situ*. Tanks were continually flushed for a further hour to allow for post warming recovery from daytime low tide conditions before tanks were fed according to their acclimation treatment (Low food: 10,000 cells mL$^{-1}$, High food: 100,000 cells mL$^{-1}$).

### Heart rate and cardiac performance analysis

To understand how habitat, thermal predictability and food availability influenced cardiac performance of *Mytilus californianus* during a heatwave, heart rate was measured in six mussels from each of the twelve treatments continuously during the entire heatwave event. Heart rate was monitored for each individual as described by Nancollas and Todgham (2022), which was based on non-invasive technique introduced by Depledge et al. (1996) and modified by Burnett et al. (2013). 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 LDA, Leiria, Portugal), digitized 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 heatwave event that was also recorded through the LabChart software. Similar to Tagliarolo and McQuaid (2015) and Nancollas and Todgham (2022), preliminary tests showed that the heart rate signals stabilized 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. Heart rate signal was converted to beats min$^{-1}$ using the LabChart 8.0 software and used in the following cardiac performance analysis.

### Cardiac performance analysis

Several metrics were used to assess cardiac performance to the multi-day heatwave event. Firstly, we assessed whether acclimation to the twelve treatment combinations produced a difference in baseline cardiac responses in mussels, independent of temperature change. To do this, we analysed the heart rate of each mussel during daytime low tide on the baseline day (9am-3pm; Figure 2) when no heating occurred. To assess how mussels from each acclimation treatment responded to thermal stress on the heatwave days, overall temperature sensitivity of heart rate for each heatwave day was examined using thermal performance curves, using methods from Bjelde and Todgham, 2013 and Nancollas and Todgham, 2022. We also measured maximum heart rate for each individual mussel on each of the three heatwave days as a measure of cardiac capacity, which was defined as the highest heart rate recorded during the heat ramp for each individual. As tidepool mussels exhibited cardiac breaks during warming on the heatwave days, we also determined breakpoint temperature (BPT) for tidepool mussels, which is defined as the temperature at which cardiac performance starts to decline and coincided with maximum heart rate. BPT was calculate by plotting individual mussel heart rate (beats min$^{-1}$) against temperature and the intersection point (BPT) was determined using the segmented package in R. Tidally-exposed individuals did not experience a breakpoint in cardiac performance, so BPT was not calculated for these 6 treatments. Finally, in addition to measures of cardiac performance during the warming period, we also analysed the post warming recovery of heart rate of mussels after each warming period, to understand if mussels incurred a metabolic debt during this period (characterized here as deviation from baseline heart rate), and whether these responses varied depending on days of consecutive heat stress. This was done by analysing cardiac responses in the 60 minute portion between when temperature had returned to ambient (13°C) conditions after warming (3:15 pm) and when feeding begins (4:15pm). Post warming recovery was determined for each heatwave day, and was assessed in terms of deviation from baseline responses, where baseline here was the same 60 minute portion of cardiac responses on the initial baseline day (3:15-4:15 pm on baseline day) (Figure 2B).

## Mussel tissue sampling

To understand how habitat, thermal predictability, and food availability influenced cellular stress levels and energy stores during the heatwave event, tissue samples (gill and mantle) were dissected from mussels (n = 6) from each treatment at two different time points on each day of the heatwave event: just before the start of the daytime low tide period (samples B, H1, H2, H3, R), and at the end of the low tide period (samples: BT, H1T, H2T, H3T), for a total of 9 different sampling stages (See figure 2). The exception was the recovery day, which only had 1 tissue sampling stage just before daytime low tide. The aim of these sampling stages was to capture preparatory cellular stress and energy levels just before a period of anticipated stress (just before daytime low tide) and levels at the end of the anticipated stress period (at the end of low tide). Gill tissue was used to assess cellular stress mechanisms (Hsp/Hsc70 protein) and mantle tissue was used to quantify energy stores (glycogen content). Mussels were removed from treatment tanks and gill and mantle tissue were dissected quickly (i.e. under 20s). 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 Nancollas and Todgham (2022). Briefly, 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) and Western blot analysis as described by Nancollas and Todgham (2022). Briefly, 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), with a prestained molecular marker (PageRuler, Thermo Fisher Scientific) in one lane and an internal standard in the second lane to calibrate protein expression within and among gels. The internal standard was pooled gill samples from 40°C submerged mussels that had shown positive expression of Hsp/Hsc70. Proteins were separated by SDS-PAGE and transferred using the semi-dry transfer method. Transfer membranes were then blocked in 2% bovine serum albumin (BSA) in Tween-20 Tris-buffered saline (TTBS), rinsed, and 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 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. Membranes were then washed in TTBS, 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) 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 intensity of the internal standard.

### Glycogen content

Glycogen content was measured as described by Nancollas and Todgham (2022), 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.

### Statistical analysis

All data sets were analysed using R (R Development Core Team, 2008) 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. Factors incorporated for specific analyses are stated below, but in general, the three acclimation factors (habitat, thermal regime, food availability) were considered as fixed effects. Other fixed effects depended on the metric measured and analysis undertaken, but for heart rate analysis ‘heatwave day’ refers to the heatwave days (HW1, HW2, HW3), and ‘temperature’ refers to the temperatures during the daytime low tide warming on the heatwave days (13-35□C). For tissue analysis “heatwave stage” refers to the 9 tissue sampling stages (B, BT, H1, H1T, H2, H2T, H3, H3T, R). Acclimation tank was incorporated as a random effect for all models, as was the ID of the individual mussels for heart rate analyses. For all analyses, a stepwise approach was taken for model creation where separate models were created that included various combinations of the relevant 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 (Barton, 2015). The model with the lowest AICc was deemed the most appropriate and used in analysis, and the final model choice is stated in the results section. Unless stated otherwise, an *anova* table was generated for each model to obtain statistical differences. Relevant post hoc tests were performed using the Tukey HSD method with the package emmeans (Lenth, 2019).

### *Cardiac performance*

To assess for any baseline differences in cardiac performance between acclimation treatments, we used a linear mixed effect model (LME) with habitat, thermal regime and food availability as fixed effects. Acclimation tank was incorporated as a random effect as was Mussel ID to account for repeated measures. To compare for differences in thermal sensitivity of heart rates between acclimation treatments during warming on heatwave days, we used generalized additive mixed modeling (GAMM) due to the nonlinear responses exhibited following Zuur et al. (2009) and Angilletta et al. (2013). Day of the heatwave was found to be highly interactive with treatment combinations, so three separate GAMMs were created for each of the three heatwave days (HW1, HW2, HW3) with habitat, thermal regime and food availability 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 factor. As heatwave day was highly interactive with the original GAMM model, to understand how heatwave day influenced heart rate within specific treatments, 12 separate GAMM analyses were run for each of the twelve treatment combinations, where day of the heatwave (HW1, HW2, HW3) was the fixed predictor factor and identity of each mussel was included as a random effect. All GAMMs 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., 2013) libraries in R. Maximum heart rate during the heatwave was analysed with a LME using the lme4 package (Bates et al., 2015) where heatwave day, habitat, thermal regime, and food availability were considered as fixed effects. Acclimation tank and mussel ID were also included as a random effect. Maximum heart rate data was transformed using a boxcox transformation using the package “bestnormalize”. For tidepool mussels, BPT was also assessed using a LME where heatwave day, thermal regime and food availability were incorporated as fixed effects and acclimation tank and mussel ID was incorporated as a random effect to account for repeated measures. BPT was transformed using a square root transformation using the package “bestnormalize”. To assess the cardiac performance during post warming recovery, a separate LME was run for each of the 12 acclimation treatments, where day was a fixed effect with 4 levels (Baseline, HW1, HW2, HW3), acclimation tank and mussel ID and time (minutes) were incorporated as random effects.

### *Gill Hsp/Hsc70 and mantle glycogen*

For Hsp/Hsc70, we originally ran a Linear Mixed Model (LME) that included the fixed factors habitat, thermal regime, and food availability from the acclimation treatments as well as heatwave stage to understand if Hsp/Hsc70 changed over the course the heatwave. The output of the four-way model detailed that habitat was highly significant across all models but also had significant interactions with all other factors, indicating that tidepool and tidally exposed mussels were responding to the simulated heatwave differently. Consequently, we decided to run two separate LMES for tidepool and tidally exposed treatments to better understand how food availability, thermal regime and heatwave stage impacted performance for tidepool and tidally exposed mussels, and we ran a separate LME with just habitat as a fixed factor to confirm differences between habitats. Hsp/Hsc70 data was transformed using the package 'bestnormalize' where a boxcox transformation was implemented. Glycogen content was analysed with a LME where heatwave day, habitat, thermal regime, and food availability were considered as fixed effects. Acclimation tank was also included as a random effect.

## RESULTS

### Cardiac performance

#### *Thermal performance during heatwave warming*

During ambient periods of immersion (13°C) tidally-exposed mussels exhibited comparable heart rates to those of tidepool mussels (~15 bpm) but when emersed, heart rates almost halved (~8 bpm) in the tidally-exposed mussels. This was reflected in difference in heart rates during daytime low tide on the baseline day between tidepool and tidally-exposed mussels (LME, $F_{(1,48)} = 293.7$, $P = <0.0001$; Figure 3).The heart rates of mussels from all acclimation treatments were significantly influenced by temperature increase, which was consistent across the heatwave days (Figure 3, Table 1). The most notable driver of cardiac thermal performance across all heatwave days was habitat (Figure 3, Table 1), as mussels from tidepool and tidally-exposed treatments displayed very different cardiac responses during the warming periods of the heatwave event. Regardless of the day in the heatwave, 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 breaks in cardiac function. In contrast, heart rates of tidally-exposed mussels were characterized by a much more gradual increase with temperature increase and did not experience breaks in cardiac function. There was also an interaction between habitat and food availability (Table 1; Figure 3) on day 1 (HW1) and day 2 (HW2), where low food tidepool treatments exhibited increased thermal sensitivity in comparison to high food tidepool treatments, but this relationship disappeared by day 3 (HW3) (Table 1; Figure 3). In contrast, there was no difference in thermal sensitivity between low and high food groups during any of the three heatwave days in the tidally-exposed treatments. Thermal regime also played a significant role on thermal sensitivity, but the relationship differed depending on day (Table 1; Figure 3). On HW1, mussels acclimated to no heat had significantly lower thermal sensitivity than those in predictable and unpredictable treatments (Tukey HSD, $P$ = <0.0001, $P$ = 0.0192, respectively). On HW2, predictable treatments demonstrated elevated performance above mussels acclimated to no heat and unpredictable treatments (Tukey HSD, $P$ = 0.0001, $P$ = 0.0005, respectively). On HW3, the influence of thermal regime returned to that of HW1, with mussels acclimated to no heat having lower thermal sensitivity to that of predictable and unpredictable treatments (Tukey HSD, $P$ = <0.0001, $P$ = 0.0042, respectively).

### *Treatment responses to consecutive thermal stress events*

The way in which mussels responded to consecutive heatwave days was highly dependent on the specific treatment combination. When analysed separately to see how mussels from each of the twelve treatments responded to three days of consecutive thermal stress, we predominantly found that performance was dependent on the specific treatment combination, but there were some general patterns related to thermal regime that were consistent (Table 2; Figure

3. The most consistent pattern was that regardless of habitat or food availability, whereby mussels acclimated to unpredictable treatments had consistent performance and sensitivity across the 3 heatwave days (Table 2, Figure 3), showing no difference in cardiac performance during warming between HW1, HW2 and HW3 (Table 2, Figure 3). Similarly, mussels acclimated to no heat generally increased performance on HW1 in comparison to HW2 (Table 2, Figure 3), but this was not able to be sustained by HW3, resulting in performance to reverting back to day 1 performance (Table 2, Figure 3). In contrast, multi-day performance from mussels from predictable regimes also seemed to be influenced by food availability. The SPL and APL treatments exhibited heightened thermal sensitivity and maximum performance during HW1 and HW2 (Table 2, Figure 3) but then sensitivity was reduced during HW3. Comparatively, mussels from the SPH and APH treatment were able to increase performance for each subsequent heatwave day (Table 2, Figure 3).

### *Cardiac capacity*

The best model for maximum heart rate included habitat, thermal regime, food availability, and day of heatwave and their interactions. Habitat significantly affected maximum heart rate, with tidepool mussels having significantly higher heart rates than tidally-exposed (LME, $F_{(1,40)}$ = 139.4, $P$ = <0.0001; Table 3), but there was also a significant interaction between habitat and food availability (LME, $F_{(1,40)}$ = 6.02, $P$ = 0.0186, Table 3) and habitat and thermal regime (LME, $F_{(2,40)}$ = 3.702, $P$ = 0.0313). Maximum heart rate of tidepool mussels was influenced by food availability, with low food treatments having higher maximum heart rates than high food treatments (Tukey HSD, $P$ = <0.0001), whereas maximum heart rate of tidally-exposed treatments was more influence by thermal regime, with mussels acclimated to no heat treatments having significantly lower maximum heart rates than predictable and unpredictable treatments (Tukey HSD, $P = 0.037$, $P = 0.021$; respectively).

*Breakpoint temperature in tidepool mussels*

The best statistical model included thermal regime, food availability and their interaction. Thermal regime significantly impacted BPT (LME, $F_{(2,89)} = 18.4537$, $P = <0.0001$; Table 3), but there was also a significant interaction between thermal regime and food availability (LME, $F_{(2,89)} = 6.437$, $P = 0.0025$). Post hoc analysis revealed that this was because the BPT of SUH mussels was elevated above SPH and SNH mussels (Tukey HSD, $P = 0.0065$ and 0.0006; respectively), and SUL and SPL, was elevated above SNL (Tukey HSD, $P = 0.0173$; $P = 0.0019$; respectively) and SPL was elevated above SPH (Tukey HSD, $P = 0.0017$. The lowest BPT was observed in SNH mussels on HW1 and HW2, with an average of 23.9 ± 0.5 and the highest BPT was observed on in SPL mussels on HW2, with an average of 28.07 ± 0.72.

*Post warming responses to consecutive thermal stress events*

Similarly to responses during the warming period, recovery of heart rate during the post warming period (3:15-4:15pm) was highly dependent on the specific treatment combination as well as day of the heatwave, but there were general similarities found amongst treatments. For tidepool mussels, post warming recovery of heart rate was strongly influenced by food availability (Table 4; Figure 4). Once temperature was returned to ambient conditions, high food treatments (SNH, SPH, SUH) exhibited quick return of heart rate to baseline conditions across all three heatwave days, with no significant deviation from baseline responses (Table 4; Figure 4). For low food treatments, SNL exhibited deviation from baseline on all three heatwave days

(Table 4, Figure 4). SPL and SUL treatments were able to able to quickly return to baseline conditions after the HW1, but then deviated from baseline after HW2 and HW3 (Table 4; Figure 4). For tidally exposed mussels, recovery was more complex, and appeared to be more regulated by thermal regime with some influence from food availability. Mussels acclimated to no heat treatments generally experienced elevated heart rate at the start of the post warming recovery period, resulting in significant deviation from baseline responses for HW1 and HW2 but then maintained baseline responses on HW3 (Table 4, Figure 4). In contrast, the heart rates of predictable and unpredictable treatments during the post warming recovery period were significantly lower than baseline responses across all three heatwave days (Table 4, Figure 4).

### Gill Hsp/Hsc70 protein levels

Tidepool and tidally-exposed mussels responded differently to the heatwave simulation (LME, $F_{(1,617)}$ = 15.319, $P = 0.0007$; Figure 5). For tidepool mussels, the best statistical model incorporated food availability and heatwave stage (9 levels: B, BT, H1, H1T, H2, H2T, H3, H3T, R; see Figure 2) with their interaction, as thermal regime did not contribute significantly to the results. There was a significant effect of heatwave stage (LME, $F_{(8,282)}$ = 6.514, $P = <0.0001$) but also a significant interaction of food availability and heatwave stage (LME, $F_{(8,282)}$ = 2.013, $P$ = 0.0452). Post hoc analysis revealed this was due to low food groups increasing over time and becoming significantly elevated over baseline on H2, H2T, H3 and H3T (Tukey HSD, $P$ = 0.0364; $P = 0.0271$; $P = 0.0439$; $P = 0.0402$, respectively; Figure 5). In contrast tidepool high food groups peak during recovery, where Hsp/Hsc70 levels are significantly elevated above baseline (Tukey HSD, $P = 0.0062$; Figure 5). For tidally-exposed mussels, the best model incorporated food availability and heatwave stage without their interaction. There was a significant effect of food availability (LME, $F_{(1,287)}$ = 17.409, $P = <0.0001$; Figure 5) where high food treatments has significantly higher Hsp/Hsc70 levels than low food treatments. There was also a significant effect of heatwave stage (LME, $F_{(8,279)} = 4.915$, $P = <0.0001$; Figure 5), where initially a tidal effect was observed, with Hsp/Hsc70 levels elevated just before daytime low tide (H1, H2, H3, R) in comparison to baseline end of daytime low tide (BT) (Tukey HSD, $P = 0.0028$; $P = 0.0021$; $P = 0.013$, $P = 0.0019$), but levels were also elevated above BT at H3T (Tukey HSD, $P = 0.0257$) (Figure 5).

**Mantle glycogen levels**

The best statistical model included habitat, thermal regime, food availability and heatwave stage as fixed factors and their interaction. There was a significant effect of food availability (LME, $F_{(1,12)} = 18.92$, $P = 0.0260$; Figure 6) but there was also a significant interaction of habitat, food availability and heatwave stages (LME, $F_{(8,464)} = 2.1137$, $P = 0.0332$; Figure 6). This was due to the glycogen levels of low food tidepool treatments declining as the heatwave event progressed, with glycogen levels being significantly lower than baseline levels from stage H2T onwards (Tukey HSD, H2T: $P = 0.0342$; H3: $P = 0.0436$, H3T: $P = 0.0103$, R= $P = 0.0241$; Figure 6). In contrast, high food tidepool treatments as well as all tidally-exposed treatments saw no change in glycogen levels throughout the duration of the heatwave event. There was also a significant effect of thermal regime (LME, $F_{(2,12)} = 18.927$, $P = 0.0002$), where mussels acclimated to the unpredictable treatments had elevated glycogen in comparison to predictable and no heat acclimation treatments (Tukey HSD, $P = 0.0126$; $P = 0.0032$, respectively).

# DISCUSSION

Heatwaves are predicted to increase in frequency, severity, and duration as a consequence of climate change, yet we know little about how intertidal organisms physiologically respond to repeated sublethal but high thermal stress events. Furthermore, it is important to understand how key aspects of an individual's environment (medium in which stress occurs, thermal history, food availability) may interact to influence physiological performance and response to a heatwave event. Our results suggest that habitat (i.e. tidepool vs. tidally-exposed location during low tide) was the largest driver for modulating physiological responses during a heatwave event, and dictated how both food availability and thermal history interacted to structure mussel performance. Tidepool mussels exhibited increased thermal sensitivity, with higher maximum heart rates and breaks in cardiac performance during warming on the heatwave days, resulting in Hsp/Hsc70 elevated above baseline towards the end of the heatwave event. Taken together, these results suggest that experiencing repeated thermal stress in an aquatic habitat is more stressful than experiencing repeated thermal stress in air.  The performance of tidepool mussels was also strongly regulated by food availability where low food resulted in increased thermal sensitivity and maximum heart rates, but also an increase in post warming recovery time and Hsp/Hsc70 responses coupled with a decline in energy stores over the course of the heatwave. These results suggest that tidepool low food groups struggled to maintain performance towards the end of the heatwave. In contrast, tidally-exposed mussels coped much better overall with no breaks in heart rate function during warming, and maintenance of lower levels of Hsp/Hsc70 and energy stores throughout the heatwave event. Furthermore, physiological performance of tidally-exposed mussels appeared to be more dependent on thermal history rather than food availability. Taken together, our results indicate that thermal physiology of *Mytilus californianus* is complex, regulated by several different environmental signals, and that these signals interact differentially to affect physiological performance.

### Habitat

Whether mussels are tidally exposed during low tide had a pronounced effect on the way that *M. californianus* responded to a simulated heatwave event, suggesting that whether mussels are in water or air plays an important role in modulating physiological performance during repeated sublethal thermal stress events. During warming, tidepool mussels exhibited higher thermal sensitivity and performance with higher maximum heart rates but also experienced breaks in cardiac performance as temperatures were increased, suggesting capacity limitations. In contrast, heart rates of tidally-exposed mussels were less sensitive to warming, did not reach as high a maximum heart rate, and exhibited no breaks in cardiac performance. The mechanism behind these contrasting responses exhibited by the two different habitat treatments is likely due to the metabolic strategy utilized to tolerate thermal stress in water vs. in air, coupled with differences in metabolic efficiency as we observed in Chapter 3. Tidepool mussels experienced elevated cardiac performance during warming, but this performance appeared to be capacity limited as evidence by the breaks in heart rate simultaneously occurring with maximum heart rate, which is considered an indication of the onset of aerobic capacity limitation resulting in suboptimal performance (Dong et al., 2022; Sokolova and Pörtner, 2003). Depending on the degree of thermal stress, continued performance after this breakpoint in this range is time-limited and can quickly progress into lethal temperatures unless further metabolic modifications occur (Dong et al., 2022; Sokolova et al., 2012). In the case of tidepool mussels, individuals are able to extend this performance range to higher temperatures by performing metabolic suppression whereby mussels actively depress heart rate for a short period of time in an effort to conserve energy (Hui et al., 2020). This allows 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 mollusc species during a thermal ramp (Bayne et al., 1976; Bjelde and Todgham, 2013; Bjelde et al., 2015; Braby and Somero, 2006; Helm and Trueman, 1967; Hui et al., 2020; Marshall et al., 2011; Pasparakis et al., 2016 ; Tagliarolo and McQuaid, 2015; Trueman and Lowe, 1971; Wang et al., 2019; Zhang et al., 2014;). This is evident here, where after the break, tidepool mussels were able to increase thermal sensitivity and cardiac performance again, exhibited by the rise in heart rate until the end of the warming period.

In contrast, tidally-exposed mussels exhibited a very different metabolic strategy to tolerate thermal stress with lower thermal sensitivity and maximum heart rates. This was likely due to tidally-exposed mussels experiencing thermal stress in air rather than in water, as emersion (independent of temperature change) elicits a reduction in metabolic rate. This is supported by significantly lower heart rates on the baseline day where no warming occurred when compared to the tidepool group (i.e. ~50% reduction in heart rate in air). During emersion, *M. californianus* does not typically exhibit gaping behaviour (Connor and Gracey, 2020; Widdows and Shick, 1985), and therefore without a mechanism to refresh oxygen levels, internal oxygen concentrations quickly drop when the valves are closed and anaerobic metabolism commences at the onset of hypoxia (Bayne et al., 1976). In order to tolerate these conditions, mussels suppress their metabolism in order to reduce energy demands and increase reliance on anaerobic metabolism (Hui et al., 2020) during the emersion period as well as limit desiccation. When emersion is coupled with thermal stress, tidally-exposed organisms are likely able to maintain metabolic depression and exhibit low thermal sensitivities and maximum heart rates in comparison to tidepool mussels. This strategy enables tidally-exposed mussels to circumvent early breaks and capacity limitations in cardiac performance that are exhibited by tidepool mussels, and is likely facilitated by increased metabolic efficiency. Due to the frequent exposure to emersion and thus the need to depress metabolism daily, intertidal organisms acclimated to a cyclical regime of periodic air exposure exhibit a number of physiological modifications that promote metabolic efficiency to reduced metabolic demand during emersion (Demurs and Gurdley, 1994; deZwaan, 1983; Nancollas and McGaw, 2021a,b; Widdows and Shick, 1985). This can include reduction of metabolic baseline costs (Widdows and Shick, 1985), as well as increased anaerobic metabolism efficiency (Demurs and Gurdley, 1994; deZwaan, 1983;).

The capacity for tidally-exposed mussels to modulate metabolism in this way could be facilitated by the fact that tidally-exposed treatments predictably experience warming coupled with an additional environmental signal that is predictable: air exposure. While *Mytilus californianus* show impressive circadian regulation (Connor and Gracey, 2011; Connor and Gracey, 2020), the transition from aquatic to aerial environments is thought to be one of the strongest environmental cues regulating thermal performance in intertidal organisms (Lockwood et al., 2015). Air exposure and thermal stress often initiate comparable compensation pathways such as oxidative stress and anaerobic metabolism (Elowe and Tomanek, 2021; Lockwood et al., 2015) and it may be possible that the onset of air exposure in tidally-exposed treatments provides advance warning of anticipated stress that provides tidally-exposed mussels time to make metabolic adjustments to better tolerate thermal stress. Although anecdotal, possible evidence of this can observed in the gradual rise in heart rates of tidally-exposed mussels from predictable and unpredictable treatments during the baseline daytime low tide, where no warming occurred, that is not exhibited by mussels from predictable and unpredictable tidepool treatments. Without a predictable environmental signal to forewarn tidepool mussels of impending thermal stress, tidepool mussels may not have sufficient time to make anticipatory metabolic adjustments, which leads to increased thermal sensitivity. More investigation is needed to tease apart the role of air exposure in structuring thermal performance of intertidal organisms. Certainly, as the relative humidity of air can vary during low tide in tidally-exposed habitats, rise in desiccation risks as humidity decreases may limit the enhanced thermal tolerance observed here in tidally-exposed mussels when exposed to thermal stress in air. Similarly, it is possible that the difference observed here between the cardiac responses of tidepool and tidally-exposed mussels could also be due to difference in the thermal dynamic properties of water and air. While the robomussels described here have been shown to exhibit a strong correlation with the internal temperatures of live mussels in both air and water (Tagliarolo and McQuaid, 2016), it is possible that internal body temperatures of the tidepool and tidally-exposed mussels could have differed within this study. Investigation into how the physical attributes of air exposure specifically structure thermal performance could provide us with more insights into the limitations of physiological performance in air.

### Food availability

One of the most striking results found in this study was the difference in cardiac performance between low and high food treatments in tidepool mussels. The relationship between food availability and temperature has typically been examined in terms of its effect on performance metrics related to an organism's fitness, such as growth (Sinclair et al., 2016), reproduction (Huey and Kingsolver, 2019) and survival (Schneider et al., 2010). In all such instances, there has been a strong relationship that with increasing temperatures, low food availability produces reduced performance in comparison to high food availability. Therefore, we anticipated that low food availability would result in a reduced cardiac performance, represented by lower maximum heart rates and a shift in the shape of the thermal performance curve with reduced performance over the course of the heatwave event. Surprisingly, we found that low food treatments, especially the tidepool predictable thermal regime treatment (SPL), had higher maximum heart rates and greater thermal sensitivity - but this came with longer post warming recovery times coupled with increased cellular stress and a reduction in energy stores as the heatwave progressed, indicative of cumulative physiological costs (He et al., 2022b). Interaction of temperature with other environmental conditions, such as ocean acidification and hypoxia, have been shown to increase the thermal sensitivity of cardiac performance in intertidal molluscs (Minuti et al., 2021; Wang et al., 2018), so it is reasonable that thermal sensitivity here can be affected by food availability in tidepool mussels. Interestingly, this effect of food availability on thermal sensitivity in tidepool mussels was not observed in mussels when exposed to a faster, lethal heating rate (6.5°C $hr^{-1}$; Chapter 3), suggesting the way in which food availability influences thermal performance is associated with the degree of thermal stress. Rates of warming has been shown to significantly impact the thermal performance in a number of intertidal species (Cereja, 2020; Leij et al., 2022; Moyen et al., 2019; Nguyen et al., 2011; Peck et al., 2009) and have indicated that slower rates of warming can result in decreased thermal tolerance. Currently, studies have largely focused on the effect of warming rate on upper thermal limit metrics, such as flatline temperature (FLT), but our results suggest the rate of warming may also influence cardiac responses to sublethal temperatures as well.

While increased thermal sensitivities at rapid warming rates can be an advantageous strategy to meet the rapid increases in metabolic demand, high thermal sensitivities at lower, sublethal warming rates could indicate a reduced capacity to metabolically compensate for rising temperatures (Chapperon et al., 2016; Farrell et al., 2016; Ghaffari et al., 2019), resulting in increased strain on physiological systems. This is evident by the post warming recovery of heart rate after each daytime low tide warming period, where mussels in the tidepool low food treatments experienced significant deviation from baseline heart rate suggesting a metabolic debt that needed to be repaid after thermal stress (Huang et al., 2015; Hui et al., 2020; Shick et al., 1988; Virgin and Schiel, 2023; Yin et al., 2017). Further evidence of a physiological cost in low food tidepool mussels was observed with the rise in Hsp/Hsc70 levels and decline in glycogen levels as the heatwave progressed, suggesting that low food tidepool mussels are experiencing cumulative physiological costs of repeated thermal stress events and are unable to restore physiological homeostasis prior to the next thermal stress event under low food regime. This seems to be particularly evident in tidepool mussels from the no heat (SNL) and predictable (SPL) low food treatments suggesting that acclimation to predictable temperatures in their recent thermal history may limit the capacity of mussels to modulate their physiology to temperatures beyond their prior thermal history. Evidence suggests that organisms that reside in predictable (vs, unpredictable) environments, tailor their physiology towards maximizing aspects of organismal fitness, such as growth and reproduction, and thus may have narrow thermal performance breadths and reduced physiological plasticity to tolerate unexpected periods of increased thermal stress (Deutsch et al. 2008; Fischer et al., 2009; 2011; Gilchrist 1995; Huey and Kingsolver 1993; Rohr et al. 2018; Stillman 2003; Tewksbury et al. 2008; Wang et al., 2020).

While food availability was a strong driver for physiological performance in tidepool mussels, physiological performance of tidally-exposed mussels appeared to be more regulated by thermal history, and the influence of food availability was predominantly limited to difference in levels of Hsp/Hsc70. Acclimation to a low food regime appeared to limit the ability of tidally-exposed mussels to produce elevated levels of Hsp/Hsc70, suggesting that low food coupled with periodic air exposure may modulate the cellular stress response at a mechanistic level. Similar results were found in whelks and gastropods, where starvation led to the reduced ability to mount a Hsp70 response (Dahlhoff et al., 2002; Jeno and Brokordt, 2014). Production of Hsp/Hsc70 is energetically costly, so it is reasonable that investment in sufficient constitutive or inducible levels may not be possible under low food regimes. However, this was not due to not having sufficient energy resources, as glycogen levels were comparable to high food treatments, suggesting that maintenance of high glycogen stores was more beneficial than recruiting a significant Hsp/Hsc70 response in tidally-exposed mussels. This strategy seems advantageous as the breakdown of glycogen can fuel a number of different metabolic pathways depending on the severity of the stress (including inducible Hsp70 production), and can be utilized to fuel reproductive efforts during optimal environmental conditions, rendering it a more flexible mechanism to tolerate the dynamic intertidal environment (Sokolova et al., 2012). Tidally-exposed mussels also exhibited an interesting tidal pattern of Hsp/Hsc70 during most of the heatwave event, with elevated levels just before the daytime low tide period largely driven by the predictable and unpredictable treatments, suggesting a preparatory response to anticipated period of thermal stress. Similar results have been observed in intertidal limpets (Dong et al., 2011; Wang et al., 2020) and corals (Barshis et al., 2013), indicating that anticipatory responses may be more important for tolerating sublethal stress. As tidally-exposed mussels tolerate the warming periods via metabolic depression, it is not surprising that there are predominantly low levels of Hsp/Hsc70 during the warming period (though levels do increase towards the end of the heatwave event). Intertidal organisms which exhibit metabolic depression during warming may wait until re-immersion to initiate restorative cellular mechanisms when exposed to sublethal thermal stress (Hofmann and Somero, 1995; Marshall et al., 2011). Further investigation into how environmental drivers may influence the temporal pattern of Hsp/Hsc70, and how food availability may mechanistically alter the production of Hsp/Hsc70 could provide useful insights in understanding future effects of climate change on thermal tolerance mechanisms.

## Thermal regime

The influence of thermal regime on the performance of mussels during the simulated heatwave event was largely evident in differences in cardiac performance during warming, and appeared to exert more regulation over the performance of tidally-exposed mussels than food availability. Regardless of habitat or food availability, acclimation to unpredictable and predictable thermal stress increased cardiac thermal performance during warming periods. In tidepool mussels, this increased performance was characterized by an increase in breakpoint temperature, whereas in tidally-exposed mussels this was reflected in increased maximum heart rate. These results compliment previous research that demonstrate that acclimation to elevated temperature increases thermal tolerance (Cheng et al., 2018; Giomi et al., 2016; Kern et al., 2015; Oliver and Palumbi, 2011; Schaefer and Ryan, 2006; Schoepf et al., 2015). In tidepool mussels, BPT was predominantly influenced by thermal regime, where predictable and unpredictable mussels had elevated BPT above mussels acclimated to no heat treatments. The average BPT of predictable and unpredictable mussels was between 26 and 28°C, very similar to the average temperatures of their acclimation regimes, which suggests increases in BPT are predominantly a function of thermal acclimation and may be tailored around mean environmental temperatures. Interestingly, the BPT found here is similar to the BPT found in *M. californianus* subjected to acute temperatures (Chapter 3), indicating that unlike overall thermal sensitivity, BPT may be a more fixed component of cardiac performance that is modulated by acclimatory effects and less affected by warming rate, which parallels similar findings in intertidal mussels (Dong et al., 2022; Moyen et al., 2019).

Thermal regime appeared to be the predominant driver behind performance differences in tidally-exposed mussels, influencing performance during warming but also post warming recovery responses. Tidally-exposed mussels acclimated to no heat (ANL and ANH), exhibited lower cardiac capacity during warming and different post warming recovery responses in comparison predictable and unpredictable mussels. During post warming recovery, ANL and ANH mussels exhibited elevated heart rate early in the recovery period that diminished by third heatwave day. Metabolic depression during thermal stress, as exhibited here by tidally exposed mussels, can often results in an oxygen dept upon re-immersion in oxygen saturated seawater (Bayne et al.,1976; McMahon et al., 1991; Widdows and Shick, 1988; Yin et al., 2017;). The size of the metabolic dept is typically related to the severity of thermal stress coupled with the thermal tolerance/capacity of the individual (Hui et al., 2020; Scanes et al., 2014; Virgin and Schiel, 2023). As ANL and ANH mussels had limited capacity to increase cardiac performance during thermal stress, evidenced by the lower maximum heart rates, this could have resulted in increased metabolic debt to be paid immediately upon re-immersion, resulting in elevated heart rates to restore physiological homeostasis, possible via oxidative stress mechanisms. In contrast, tidally-exposed treatments acclimated to predictable and unpredictable regimes generally appear to maintain some form of metabolic depression during the post warming recovery period, with cardiac performance generally lower than baseline across the majority of the heatwave days. While it is unclear what is driving this response, it could be possible that increased performance during the warming period could aide in reducing physiological costs during re-immersion, allowing mussels to wait for the influx of food during the feeding period before initiating any necessary repair mechanisms (Bayne et al., 1988; May et al., 2021). Investigation into mechanisms underlying energy regulation during post warming recovery would help explain this response.

Not only did thermal regime influence how mussels responded to a period of warming, it was also the predominant driver for how mussels tailored their physiological responses to repeated thermal stress events. Each of the twelve acclimation treatments exhibited their own nuanced responses to the repeated nature of thermal stress, indicating that habitat medium, thermal history, and food availability all contributed to structure multi-day thermal performance, but the clearest overlying trends were largely associated with thermal history. Firstly, mussels acclimated to no heat treatments were able to increase cardiac performance from HW1 to HW2, but this was unable to be sustained for HW3, where performance returned to that of HW1. Prior heat shock of sufficient temperature has been shown to increase thermal performance in many intertidal organisms, especially when this shock occurs within 48 hours of a subsequent shock (Gunderson et al., 2016, Pasparakis et al., 2016; Zhang et al., 2021), which could explain increased performance between HW1 and HW2. However, it is clear that heat hardening alone is not enough to sustain elevated performance to a subsequent thermal stress, which results in performance reverting to that of HW1 coupled with increased physiological costs, as indicated by elevated Hsp/Hsc70 levels in mussels acclimated to no heat treatments towards the end of the heatwave.  This could indicate that performance to repeated thermal stress events may rely on physiological modifications that are a product of exposure to more long-term thermal complexity in their thermal history.

Regardless of habitat or food availability, thermal performance of unpredictable mussels was the only regime that was consistent across all three heatwave days, suggesting that acclimation to unpredictable thermal stress promotes sufficient resources to handle repeated thermal stress events. During the acclimation period, mussels form the unpredictable treatments were the only mussels to periodically experience temperatures close to the heatwave temperatures, where the highest temperature reached in acclimation was 34°C. It could be that this consistency in thermal performance is a result of mussels tailoring their physiology to maximum temperatures experienced in their previous thermal history, to ensure they are prepared for comparable levels of stress. Recently, Moyen et al. (2020) have shown that *M. californianus* acclimated to 14°C acquire improved heat tolerance when exposed to a single sublethal bout of heat stress (30°C or 35°C for 2hr) up to 3 weeks prior to exposure to an extreme heat stress (40°C for 2hr), that was not observed in mussels exposed to a single sublethal bout of heat stress at 25°C. This suggests that maximum temperature in recent thermal history could be important for initiating physiological adjustments to tolerate future thermal stress. In this study, the mechanism behind this could be related to the high levels of glycogen stores measured in mussels from the unpredictable treatments, which may facilitate thermal performance during warming. Alternatively, high levels of glycogen could expedite recovery and the return of physiological homeostasis in preparation for the next daytime low tide period, allowing consistent performance across multiple thermal stress events.

Finally, the repeated thermal performance of predictable mussels appeared to be also tied to food availability, where low food treatments (SPL and APL) had comparable performance during HW1 and HW2, but experienced reduced performance on HW3. Similarly to mussels acclimated to the no heat treatments, it is clear that at least for mussels from the SPL treatment, this decrease in performance is due to the cumulative physiological cost represented by the high Hsp/Hsc70 levels at H3T and reduced energy stores. In contrast, high food predictable treatments (SPH and APH) were able increase cardiac performance each subsequent day during the heatwave, suggesting that high food availability within a predictable thermal environment allows for increases in performance to try and match elevated physiological demands. However, the rising Hsp/Hsc70 levels exhibited by both SPH and APH treatments suggest that this response may not be able to last indefinitely. The apparent importance of food availability for mussels in predictable environments is not clear in this study, but we speculate it could be associated with the aforementioned potential limits in physiological plasticity and performance breadth exhibited by organisms in predictable thermal environments (Deutsch et al. 2008; Gilchrist 1995; Huey and Kingsolver 1993; Rohr et al. 2018; Stillman 2003; Tewksbury et al. 2008). These restrictions may be further limited in low food scenarios or relieved in high food scenarios. More investigation into the importance of food availability in predictable thermal environments could help elucidate climate induced alterations in thermal performance.

## Conclusion

In conclusion, the responses of mussels to repeated thermal stress events, such as heatwaves, is complex, and influenced by a number of environmental signals. Here we have demonstrated that habitat (tidepool vs tidally-exposed), thermal history predictability (no, predictable, unpredictable) and food availability all have distinctive effects on shaping the physiological performance of mussels to a three day heatwave. Our results indicate that the increases in the number of heatwave events coupled with fluctuations in important determinants of performance, such as food availability and thermal predictability will not have a uniform effect on intertidal organisms residing in tidepools vs. exposed on rocks. Individuals inhabiting shallow aquatic habitats, such as tidepools, will be more at risk of repeated sublethal thermal stress events than tidally-exposed individuals, especially when coupled with low food availability, which may result in drastic shifts in intertidal community composition and functioning. Tidally-exposed individuals exhibited lower physiological costs to the simulated heatwave overall, and this was likely associated with the fact that the sublethal temperature of 35°C is much lower than their upper thermal limit in comparison to tidepool mussels (see Chapter 3). In this study we repeatedly exposed both habitat treatments to 35°C in order to have direct comparisons between habitats, but tidally-exposed organisms typically experience higher aerial temperatures than their tidepool counterparts (Helmuth et al., 2016). While the maximum temperature differences between our tidepool and tidally-exposed robomussels were <4°C, predicted increases in the severity of heatwave events are likely to exacerbate that temperature difference. Further research should look at testing tidally-exposed mussels to heatwave temperatures that are closer to their upper thermal limit in order to advance our understanding of how heatwaves will influence intertidal communities. Finally, in the current study we only measured physiological responses to a three-day heatwave, but as the duration of heatwaves are increasing, intertidal organisms are now experiencing heatwaves consisting of five days or more. Examining physiological responses to longer heatwaves whilst incorporating predicted fluctuations of other environmental variables will allow us to better understand the resilience of the intertidal community to climate change.

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# TABLES

**Table 1.** ANOVA table results of the three GAMM analyses for each heatwave day (HW1, HW2, HW3) displaying the effect to habitat (M), thermal regime (T), food availability (F) (fixed effect predictors) and their interactions as well as the influence of temperature (T) (smooth factor) on mussel heart rate from all twelve treatments (n = 6 for each treatment). Treatments names combine habitat (S: tidepool; A: tidally-exposed) with thermal regime (N: no thermal stress; P: predictable; U: unpredictable) and food availability (L: low; H: high). F and P values are displayed for all factors for each heatwave day. Bold values highlight significant effects.

| | **HW1** | | **HW2** | | **HW3** | |
|---|---|---|---|---|---|---|
| **Predictor factor** | ***F*-value** | ***P*-value** | ***F*-value** | ***P*-value** | ***F*-value** | ***P*-value** |
| M | **6.433** | **0.00166** | **5.757** | **0.00324** | **3.993** | **0.0187** |
| T | 1.074 | 0.30034 | 0.032 | 0.85826 | 0.159 | 0.6906 |
| F | **26.712** | **2.72E-07** | **39.877** | **3.66E-10** | **34.863** | **4.47E-09** |
| M * T | 1.17 | 0.31058 | 0.822 | 0.43984 | 0.04 | 0.9605 |
| M * F | 1.741 | 0.17581 | 1.326 | 0.26575 | 0.08 | 0.9231 |
| F * T | **4.552** | **0.0331** | **6.905** | **0.00869** | 0.961 | 0.32703 |
| M * T * F | 2.814 | 0.06032 | 2.278 | 0.10289 | 1.505 | 0.2223 |
| **s(Temperature)** | ***F*-value** | ***P*-value** | ***F*-value** | ***P*-value** | ***F*-value** | ***P*-value** |
| SNL | **59.52** | **<2e-16** | **24.81** | **<2e-16** | **34.64** | **<2e-16** |
| SNH | **38.13** | **<2e-16** | **61.86** | **<2e-16** | **98.47** | **<2e-16** |
| SPL | **64.97** | **<2e-16** | **91.33** | **<2e-16** | **189.29** | **<2e-16** |
| SPH | **33.04** | **<2e-16** | **132.51** | **<2e-16** | **138.94** | **<2e-16** |
| SUL | **125.99** | **<2e-16** | **71.61** | **<2e-16** | **146.75** | **<2e-16** |
| SUH | **313.1** | **<2e-16** | **130.17** | **<2e-16** | **65.67** | **<2e-16** |
| ANL | **20.12** | **<2e-16** | **30.87** | **<2e-16** | **21.94** | **<2e-16** |
| ANH | **30.33** | **<2e-16** | **18.03** | **<2e-16** | **17.08** | **<2e-16** |
| APL | **47.33** | **<2e-16** | **27.07** | **<2e-16** | **17.77** | **<2e-16** |
| APH | **32.04** | **<2e-16** | **17.15** | **<2e-16** | **21.91** | **<2e-16** |
| AUL | **42.88** | **<2e-16** | **47.73** | **<2e-16** | **27.74** | **<2e-16** |
| AUH | **14.7** | **<2e-16** | **19.82** | **<2e-16** | **24.8** | **<2e-16** |

**Table 2.** ANOVA results from the 12 separate GAMM models for the influence of heatwave day (HW1, HW2, HW3) on the cardiac thermal performance for each of the 12 acclimation treatments. Where significant, associated P values from Tukey HSD post hoc analyses are included to highlight differences across the heatwave days. Bold values indicate significant difference in cardiac performance between the days for an individual treatment.

| **Treatment** | **GAMM results** | | **HW1 vs HW2** | **HW1 vs HW3** | **HW2 vs HW3** |
|---|---|---|---|---|---|
| | ***F*-value** | ***P*-value** | Tukey HSD *P* | Tukey HSD *P* | Tukey HSD *P* |
| SNL | **24.77** | **<0.0001** | **0.0027** | 0.9535 | **0.0434** |
| SNH | **17.65** | **<0.0001** | **<0.0001** | 0.9359 | **<0.0001** |
| SPL | **21.73** | **<0.0001** | 0.7343 | **0.0002** | **0.0001** |
| SPH | **31.1** | **<0.0001** | **<0.0001** | **0.0001** | **0.0241** |
| SUL | 1.29 | 0.276 | | | |
| SUH | 1.57 | 0.209 | | | |
| ANL | **14.82** | **<0.0001** | **0.013** | 0.0545 | **<0.0001** |
| ANH | **8.33** | **<0.0001** | **0.045** | 0.9570 | **0.0489** |
| APL | **21.46** | **<0.0001** | 0.506 | **0.0293** | **<0.0001** |
| APH | **23.35** | **<0.0001** | **0.0260** | **<0.0001** | **0.0052** |
| AUL | 2.73 | 0.067 | | | |
| AUH | 2.14 | 0.119 | | | |

**Table 3.** Breakpoint temperatures and maximum heart rates recorded for each of the 12 treatments for day 1 (HW1), day 2 (HW2), and day 3 (HW3) of the heatwave. Tidally-exposed treatments (ANL – AUH) did not experience a break in cardiac performance during warming and thus do not have a breakpoint temperature. Treatments names combine habitat (S: tidepool; A: tidally-exposed) with thermal regime (N: no thermal stress; P: predictable; U: unpredictable) and food availability (L: low; H: high). Values are mean ± SEM of 6 individuals for each treatment.

| | **Breakpoint temperature** | | | **Maximum heart rate** | | |
|---|---|---|---|---|---|---|
| | **HW1** | **HW2** | **HW3** | **HW1** | **HW2** | **HW3** |
| SNL | 24.41 ± 0.79 | 25.74 ± 0.68 | 24.16 ± 0.54 | 46.92 ± 4.56 | 51.51 ± 4.19 | 46.91 ± 3.82 |
| SNH | 23.92 ±0.46 | 25.55 ± 1.08 | 23.89 ± 0.54 | 33.02 ± 6.43 | 37.16 ± 5.24 | 35.15 ± 6.21 |
| SPL | 27.58 ± 0.55 | 28.07 ± 0.73 | 25.92 ± 1.61 | 47.03 ± 3.11 | 55.93 ± 3.35 | 47.58 ± 2.07 |
| SPH | 24.26 ± 0.27 | 25.54 ± 0.47 | 25.57 ± 0.66 | 31.09 ± 3.10 | 38.48 ± 5.98 | 39.45 ± 5.31 |
| SUL | 26.60 ± 0.18 | 26.8 ± 0.68 | 27.14 ± 0.49 | 41.38 ± 3.39 | 45.59 ± 3.23 | 44.18 ± 4.77 |
| SUH | 27.88 ± 0.61 | 27.02 ± 0.74 | 28.07 ± 0.53 | 40.67 ± 2.88 | 41.24 ± 2.97 | 41 ± 1.79 |
| ANL | NA | NA | NA | 17.99 ± 0.63 | 19.02 ± 1.27 | 17.7 ± 0.26 |
| ANH | NA | NA | NA | 15.82 ± 0.77 | 17.85 ± 1.15 | 16.49 ± 0.94 |
| APL | NA | NA | NA | 21.15 ± 0.72 | 21.29 ± 1.03 | 20.52 ± 0.91 |
| APH | NA | NA | NA | 20.98 ± 0.74 | 21.39 ± 0.92 | 21.67 ± 0.87 |
| AUL | NA | NA | NA | 20.93 ± 0.89 | 21.28 ± 0.35 | 23.46 ± 0.95 |
| AUH | NA | NA | NA | 24.13 ± 1.35 | 23.74 ± 0.98 | 24.25 ± 1.29 |

**Table 4.** T and P values from the 12 separate LME models assessing post warming recovery of heart rate on day 1 (HW1), day 2 (HW2), day 3 (HW3) of the heatwave in comparison to the baseline day for each acclimation treatment. Bold values indicate significant deviation from baseline day.

| **Treatment** | **HW1** | | **HW2** | | **HW3** | |
|---|---|---|---|---|---|---|
| | ***T-value*** | ***P*-value** | ***T-value*** | ***P*-value** | ***T-value*** | ***P*-value** |
| SNL | **17.326** | **<0.0001** | **4.143** | **<0.0001** | **2.436** | **0.015** |
| SNH | 0.947 | 0.344 | 0.813 | 0.417 | 1.442 | 0.150 |
| SPL | 1.792 | 0.0734 | **11.531** | **<0.0001** | **3.993** | **<0.0001** |
| SPH | 0.928 | 0.354 | 1.483 | 0.138 | 1.430 | 0.153 |
| SUL | 0.425 | 0.671 | **4.886** | **<0.0001** | **6.114** | **<0.0001** |
| SUH | 1.240 | 0.215 | 0.45 | 0.653 | 1.021 | 0.308 |
| ANL | **3.52** | **0.00047** | **2.965** | **0.003121** | 1.249 | 0.2118 |
| ANH | **3.546** | **0.000415** | 0.405 | 0.685 | 0.684 | 0.494 |
| APL | **6.910** | **<0.0001** | **7.001** | **<0.0001** | **7.904** | **<0.0001** |
| APH | **3.717** | **<0.0001** | **2.262** | **0.002** | **5.133** | **<0.0001** |
| AUL | **3.711** | **<0.0001** | **4.921** | **<0.0001** | 0.788 | 0.433 |
| AUH | **7.384** | **<0.0001** | **5.727** | **<0.0001** | 1.302 | 0.194 |

# FIGURES

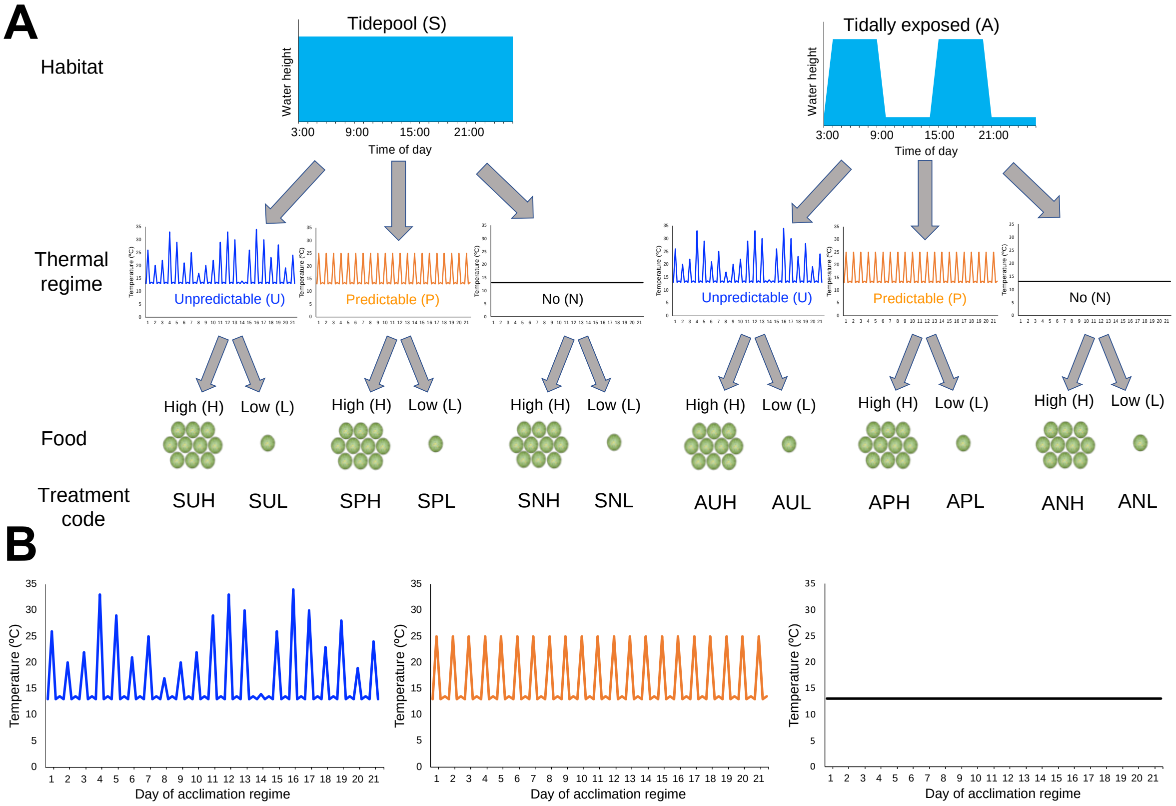

**Figure 1.** A: Schematic of the 12 treatments for acclimation. *Mytilus californianus* were acclimated for 3 weeks to either tidepool (S) or tidally-exposed (A) treatments combined with either unpredictable (U), predictable (P) or no (N) thermal stress and high (H) or low (L) food availability for a total of twelve 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 three thermal regimes incorporated during acclimation. Unpredictable (blue)

mussels were warmed to a different maximum temperature during daytime low tide, and this profile mimicked a 3-week period of maximum temperatures from the ‘master’ logger profile. Predictable (orange) mussels 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. Finally, no (black) heat stress, where mussels were not warmed during daytime low tide and experienced ambient temperatures (13°C).

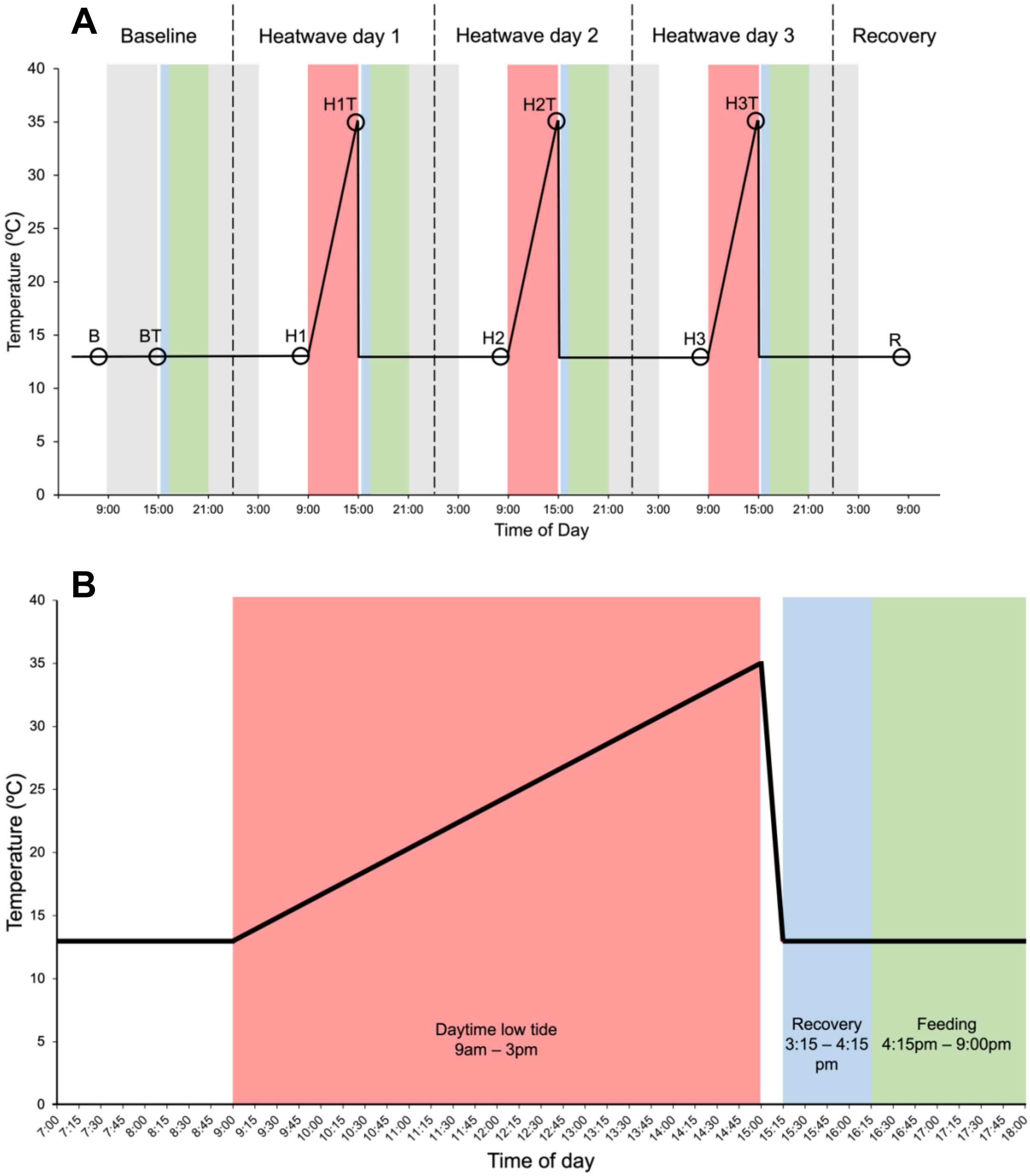

**Figure 2.** (A) Schematic of the simulated heatwave event. Black circles represent when tissue sampling occurred, which was just before, and at the end of each daytime low tide period, with the exception of the Recovery day where a sample was only taken just before daytime low tide. The black line indicates the temporal change in temperature throughout the heatwave event. No

warming occurred on the baseline day, then mussels were warmed to 35°C from ambient (13°C) during the daytime low tide period (9 am-3pm) at a rate of 3.67°C $h^{-1}$, immediately followed by a fast (within 15 minutes) cooling to ambient temperature (13°C), which was maintained until the next warming period. Red periods indicated daytime low tides (9am-3pm) when warming occurred. Grey periods indicate low tides where no warming occurred (daytime low tide on baseline day and night-time low tides [9pm-3am] on all days). Blue periods indicated when heart rate was assessed for post warming recovery each day (3:15- 4:15pm). Green periods indicate when mussels were fed (4:15-9pm), which immediately followed the post warming recovery period and coincided with daytime high tide. White periods indicated night-time high tide. Acclimation conditions with regards to habitat and food availability were maintained throughout the heatwave event. Tidepool mussels remained permanently submerged throughout the course of the heatwave event and experienced temperature change during immersion. Tidally-exposed individuals experienced a tidal cycle and were emersed during low tide periods and submerged during high tide periods, and thus experienced temperature change during emersion. High food treatments were fed 100,000 cells ml during feeding periods and low food treatments were fed 10,000 cells ml throughout the heatwave event (B) Close up of the warming and post warming period on a typical heatwave day.

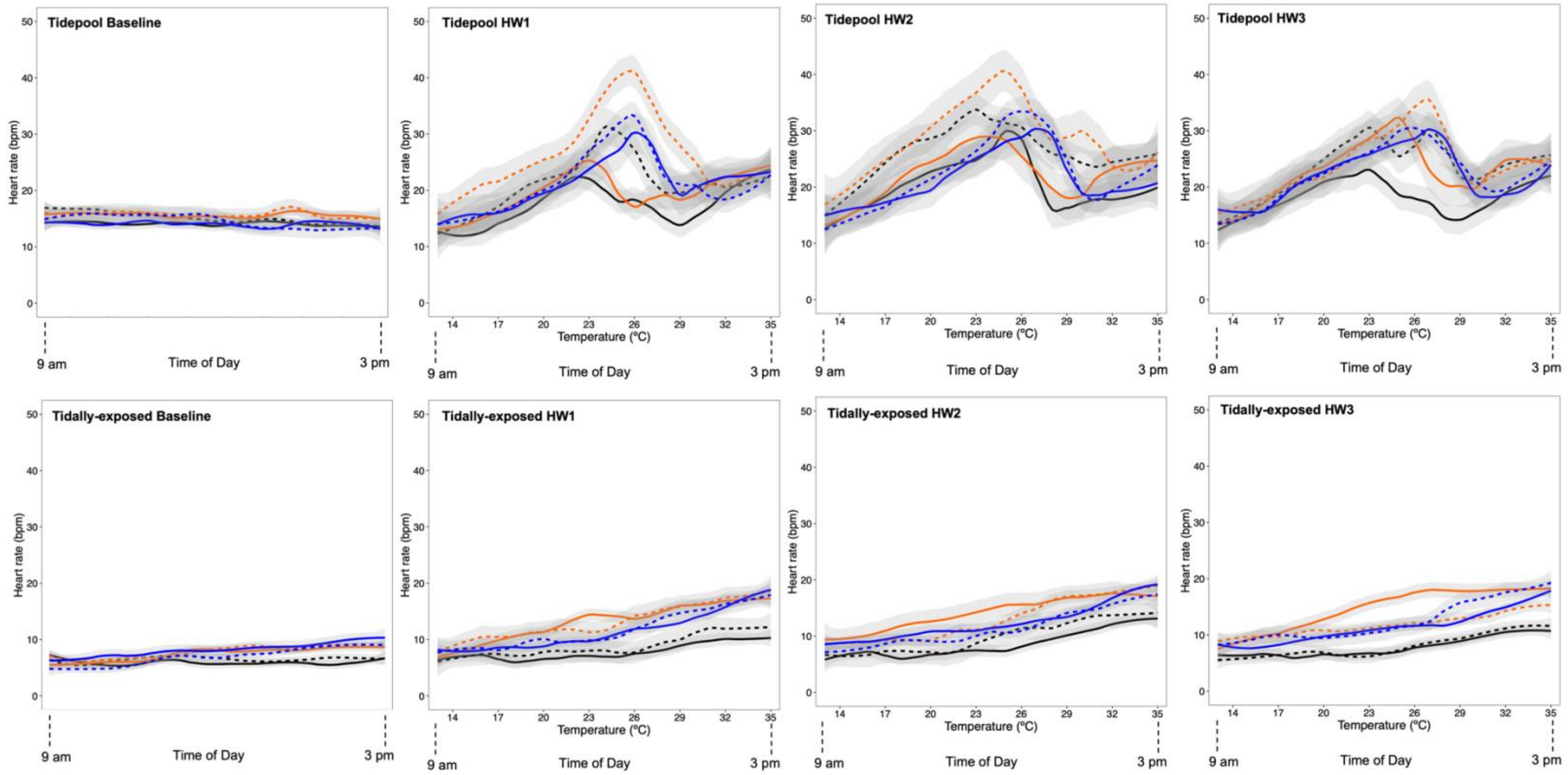

**Figure 3.** Baseline cardiac responses (left) and thermal performance curves on day 1 (centre left) day 2 (centre right) and day 3 (right) of the heatwave event for *Mytilus californianus* acclimated to tidepool (top) and tidally-exposed (bottom) habitats. Line colour represents the different thermal regimes where unpredictable is blue, predictable is orange and acclimation to no heat stress in black. Solid lines reflect mussels from high food treatments and dashed lines reflect mussels from low food treatments. Mussels from tidepool treatments were warmed in water, whereas mussels from tidally-exposed treatments were warmed in air. Lines are mean of 6 individuals and the grey shaded area represents 95% confidence interval for each line.

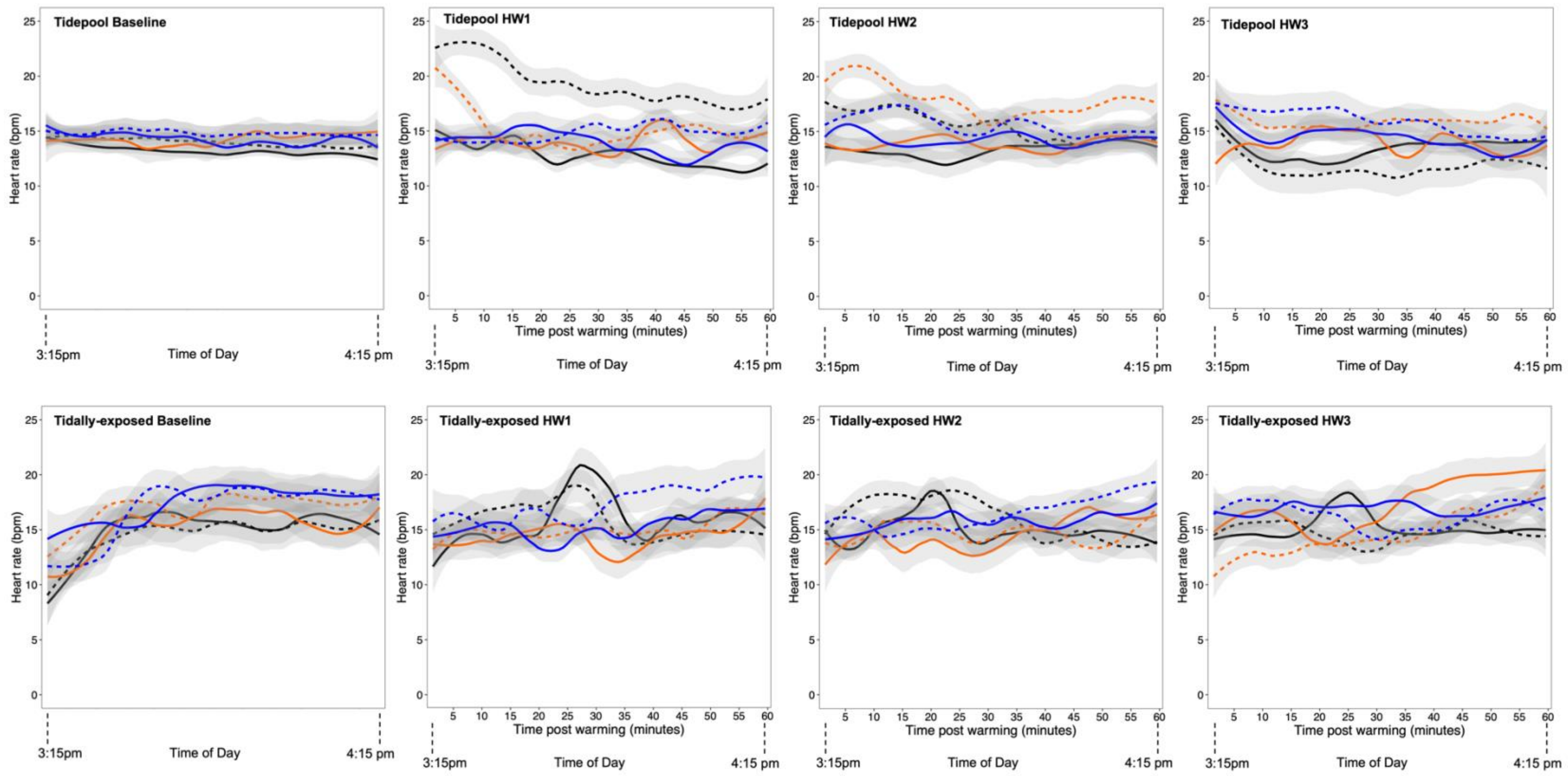

**Figure 4.** Baseline cardiac responses after daytime low tide (left) and post warming recovery heart rates on day 1 (centre left) day 2 (centre right) and day 3 (right) of the heatwave event for *Mytilus californianus* acclimated to tidepool (top) and tidally-exposed (bottom) habitats. Line colour represents the different thermal regimes where unpredictable is blue, predictable is orange and acclimation to no heat stress in black. Solid lines reflect mussels from high food treatments and dashed lines reflect mussels from low food treatments. Mussels from tidepool treatments were warmed in water, whereas mussels from tidally-exposed treatments were warmed in air. Lines are mean of 6 individuals and the grey shaded area represents 95% confidence interval for each line.

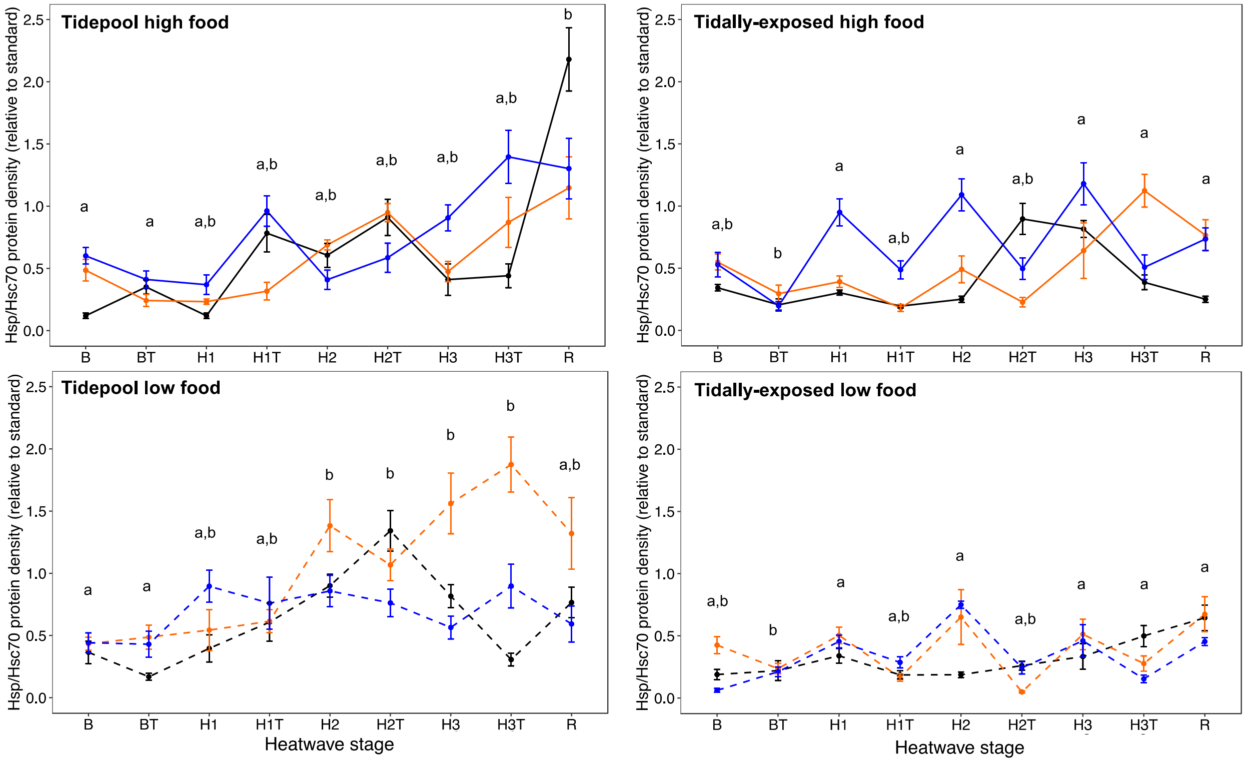

**Figure 5.** Hsp/Hsc70 of *Mytilus californianus* gill tissue taken at the 9 different sampling stages during the heatwave, just before (B, H1, H2, H3, R) and at the end (BT, H1T, H2T, H3T) of the daytime low tide period. B=baseline, H1= heatwave day 1, H2= heatwave day 2, H3 = heatwave day 3, R = recovery. Due to a statistically significant interactive effect between food availability heatwave stage exhibited by tidepool mussels, plots have been separated out into tidepool high food (top left) and low food (bottom left), and for continuity so has tidally-exposed high food (top right) and low food (bottom right) treatments. In all cases, mussels acclimated to unpredictable treatments are in blue, predictable in orange and no heat stress in black. Solid lines represent high food treatments and dashed lines represent low food treatments. Mussels from tidepool treatments were warmed in water, whereas mussels from tidally-exposed treatments were warmed in air. Values are mean ± SEM. Different lower case levels letters represent a significant difference in levels at different heatwave stages (Tukey HSD, p<0.05). For tidally-

exposed treatments, mussels from the high food treatments overall had significantly different Hsp/Hsc70 levels to low food treatments. There was no difference between thermal regimes in either tidepool or tidally-exposed mussels.

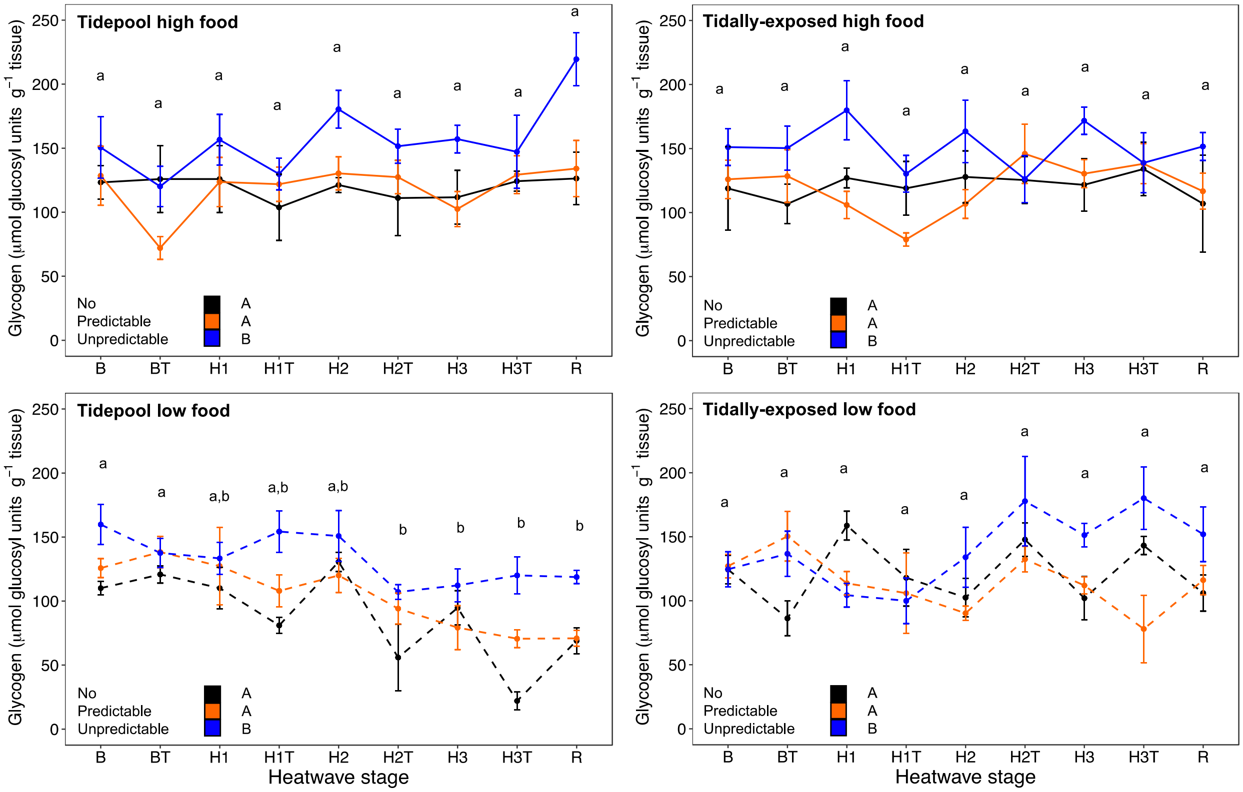

**Figure 6.** Glycogen content of *Mytilus californianus* mantle tissue taken at the 9 different sampling stages during the heatwave, taken just before (B, H1, H2, H3, R) and at the end (BT, H1T, H2T, H3T) of the daytime low tide period. B=baseline, H1= heatwave day 1, H2= heatwave day 2, H3 = heatwave day 3, R = recovery. Due to a statistically significant interactive effect between habitat, food availability and heatwave stage, plots have been separated out into tidepool high food (top left) and low food (bottom left) treatments, as well as and tidally-exposed high food (top right) and low food (bottom right) treatments. In all cases, mussels acclimated to unpredictable treatments are in blue, predictable in orange and no heat stress in black. For continuity with other plots, solid lines represent high food treatments and dashed lines represent low food treatments. Mussels from tidepool treatments were warmed in water, whereas mussels from tidally-exposed treatments were warmed in air. Values are mean ± SEM. Different lower case levels letters represent a significant difference in levels at different heatwave stages (Tukey

HSD, $p<0.05$). Different upper-case letters represent a significant difference between thermal regimes.

# APPENDIX

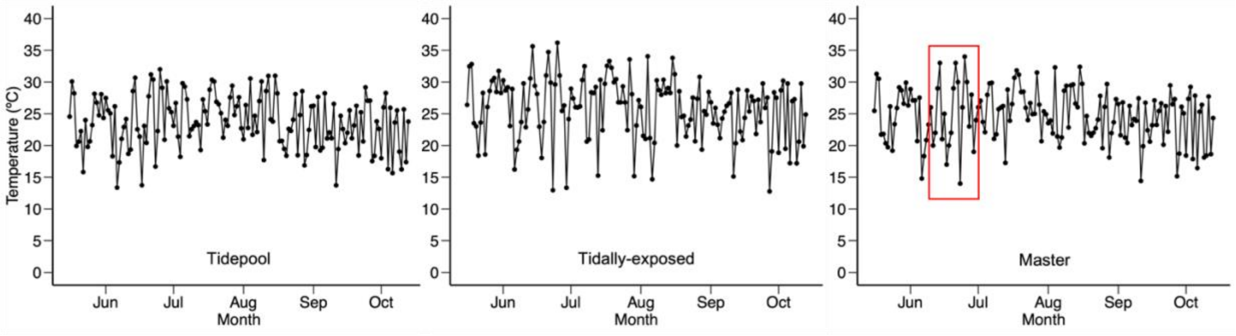

**Figure A1.** Maximum temperatures for each day from the robomussels situated in a tidepool (left) and attached to tidally-exposed rock (centre). Temperature data from both the tidepool and tidally-exposed logger were combined to create the master temperature profile (right). The red outline on the master profile represents the section on logger data that was use to inform the unpredictable thermal regime.

# CHAPTER 5

## General Discussion

The overall goal of this research was to understand how key aspects of the intertidal environment interact to influence thermal performance in the California mussel (*Mytilus californianus*). Historically, the majority of previous studies investigating temperature stress in the intertidal have acclimated organisms to constant, submerged conditions and have focused on determining upper thermal limits and the physiological mechanisms regulating those limits. In reality, intertidal organisms do not live in a constant, submerged environment but rather a dynamic environment, which experiences fluctuation of several environmental variables with the movement of the tide. Furthermore, it is important to understand sublethal impacts of thermal stress, as these animals must recover daily from low tide and carry out important biological processes associated with fitness (i.e. feeding, growth, reproduction) during high tide periods. With my PhD research I found that habitat, thermal predictability, food availability and degree of thermal stress (lethal or sublethal; single or repeated thermal stress events) all interacted in nuanced ways to shape the thermal performance of *M. californianus*. It also became apparent that mussels tailor their physiological responses to predictable signals in their environment. In the intertidal zone, the most predictable environmental signal is the transition between immersion and emersion due to the movement of the tide. Here, I found that acclimation to a tidal cycle of immersion and emersion plays a fundamental role in shaping physiological responses in mussels and was the predominant driver for regulating thermal performance to both lethal and sublethal thermal stress. So, despite the attention on thermal stress and thermal history by intertidal physiologists for decades (e.g Barry et al., 1995; Somero, 2005; Stillman, 2002), exposure to air is the strongest factor driving the physiology of a sessile intertidal organism.

The importance of experiencing thermal stress within a tidal cycle framework became apparent with Chapter 2, where acclimation to a tidal cycle was the main driver for determining upper thermal limits of cardiac function. This became more apparent in Chapter 3, where mussels from tidally-exposed habitats exhibited elevated final breakpoint temperatures and flatline temperatures in comparison to tidepool mussels. In Chapter 3 it became clear that the habitat medium in which thermal stress occurs (air or water) was the predominant regulator for determining the metabolic strategy utilised to tolerate thermal stress. Tidepool mussels that experienced temperature increases in water exhibited a ramp in cardiac performance in order to match the increases in metabolic demand as a result of rising temperatures, but were faced with cardiac capacity limitations and exhibited multiple breaks in cardiac function during the acute thermal ramp. In contrast, experiencing temperature increases in air resulted in metabolic depression, with tidally exposed mussels experiencing a single break in cardiac function close to their flat line temperature. These differences in cardiac performance and metabolic strategies were maintained in Chapter 4 in response to sublethal, repeated thermal stress events, highlighting that exposure to air during low tide is the largest overall driver in shaping the thermal responses in the California mussel. What became noticeable over Chapters 3 and 4 was that tidepool mussels exhibit cardiac capacity limitations at temperatures they would frequently experience in their natural environment. In both Chapter 3 and 4, tidepool mussels reached their maximum cardiac capacity limit between 24-28°C, and while cardiac function was able to be sustained through the help of Hsp/Hsc70 and glycogen stores, tidepool mussels were unable to reach similar maximal levels again in the same warming period. This could indicate that sublethal temperatures of 24-28°C represents a critical physiological setpoint in cardiac function when mussels experience warming in an aquatic environment that is not observed when mussels experience warming in air. Typically, temperature fluctuations in water (i.e. tidepools) are dampened compared to neighbouring rock in air by a few degrees, which could explain why tidepool mussels can persist through heatwaves.

While habitat played a major role in shaping physiological responses, thermal unpredictability fine-tuned the response, indicating that the natural cycle of temperature fluctuation that mussels experience *in situ* plays and important role in shaping physiological responses to temperature change. Across Chapters 2, 3 and 4 I saw that mussels acclimated to unpredictable thermal regimes exhibited increases in cardiac performance and upper thermal limits above all other groups. These results could suggest that the increase in thermal unpredictability and variability predicted by climate change models may serve to increases upper thermal tolerance in intertidal organisms, although it would be important to test the degree of thermal unpredictability which is beneficial. A recurrent theme throughout Chapter 2, 3, and 4 was that thermal unpredictability increased glycogen stores, which suggests that having sufficient energy stores is a high priority when dealing with an unpredictable thermal environment. In tidepool mussels it was clear that high energy stores facilitated thermal tolerance during warming, and was able to fuel the increased metabolic demand and sustain cardiac function, which likely contributed towards increasing upper thermal limits. In contrast, as tidally-exposed mussels exhibited metabolic depression during the warming periods, glycogen did not play a direct role in tolerating increasing temperatures, so the prioritisation of maintaining high glycogen stores in tidally-exposed mussels in unpredictable thermal environments remains unclear. Energy stores can be used to fuel a number of physiological responses to changing conditions (Sokolova et al., 2012) and thus does appear to be a suitable mechanism for tolerating unpredictable environments. As tidally-exposed mussels metabolically depress during warming, it is possible that glycogen stores aid in restoring physiological homeostasis during re-immersion. Another possible explanation is that acclimation to unpredictable thermal regime results in a more efficient metabolism, which allows mussels allocate more energy into storage. We saw some evidence of this in Chapter 2, where tidally-exposed mussels acclimated to an unpredictable regime had lower malate dehydrogenase activity (MDH) and succinate content going into the thermal ramp, which could indicate that unpredictable mussels have lower baseline costs and can invest more in energy storage. A more formal, comprehensive analysis of metabolic pathways and use of different fuel sources (i.e. carbohydrates vs. lipids) during fluctuating thermal conditions is needed to better understand mechanisms of increased performance of mussels acclimated to unpredictable thermal regimes.

With the importance of glycogen stores made apparent in the unpredictable treatment in Chapter 2, for Chapters 3 and 4 I explored the role of food availability in influencing the thermal performance in mussels. In Chapter 3, I had anticipated that reduced food availability would result in reduced thermal tolerance. Ultimately, I found that food availability had little influence on the upper thermal tolerance of mussels exposed to an acute, single thermal stress event, regardless of habitat type or thermal regime. This indicates that potential future reductions in food availability may still be sufficient for mussels to maintain performance to a single, acute thermal stress event. However, intertidal animals do not see single heat stress events in isolation. When investigating physiological responses to sublethal, repeated thermal stress events in Chapter 4, I found that food availability played a much larger role in shaping physiological performance, but only in tidepool mussels. As tidally-exposed mussels tolerated warming periods by metabolic depression, it was perhaps unsurprising that food availability had minimal effect in shaping thermal performance. In the tidepool treatments, mussels acclimated to low food availability exhibited increased thermal sensitivity to sublethal warming rates in comparison to high food treatments. This increased sensitivity was coupled with a progressive increase in Hsp/Hsc70 and a progressive decline in glycogen stores indicating that mussels with low food availability were unable to restore physiological homeostasis after each thermal stress event, which resulted in cumulative physiological costs as the heatwave progressed. Interestingly these trends in physiological responses were the most notable in mussels acclimated to a regime with no heating and predictable heating, which suggests that mussels residing in predictable aquatic habitats may struggle to respond to repeated thermal stress events, such as heatwaves, if there is low food availability. In contrast, this trend in physiological responses was not as apparent in mussels acclimated to unpredictable thermal regimes, which suggests that mussels in unpredictable thermal environments may be better prepared to tolerate repeated thermal stress events, and are not as reliant on food availability for structuring thermal performance. The heatwave used in Chapter 4 was only 3 days and with the predicted increase in heatwave duration (Stillman, 2019), it would be important to continue this type of research with longer heatwave periods.

## Future directions

While we now have a clearer understanding of how aspects of the intertidal environment interact to shape the physiological performance of mussels to thermal stress, my PhD research has produced more questions. Firstly, it was clear from the results of Chapter 3 and Chapter 4 that the habitat medium (air or water) in which thermal stress occurs was a strong determinant for informing metabolic performance during both lethal and sublethal ramp. A more comprehensive investigation into the physiological adjustments that are a product of long-term periodic exposure to air (i.e. acclimation to a tidal regime) as well how various physiological mechanisms change during air exposure will help identify important mechanisms utilised by tidally-exposed mussels for tolerating environmental change during low tide periods. Here, I focused on heat shock proteins and energy stores due to the emphasis of their importance for tolerating periods of thermal stress in previous studies (Feder and Hofmann, 1999; Sokolova et al., 2012). As the majority of previous studies have either acclimated intertidal organisms to submerged conditions or exposed them to an acute heat shock in water, it made sense that Hsp/Hsc70 and glycogen played an important role in structuring thermal performance for tidepool mussels. However, when under more ecologically realistic conditions of experiencing thermal stress in conjunction with air exposure, I found that heat shock proteins and glycogen stores played a minimal role during the warming period in tidally-exposed mussels due to metabolic depression. Assessment of metrics more focused on metabolism and energy dynamics as well as oxidative stress may provide more useful insights for understanding the mechanisms regulating the thermal stress response in tidally-exposed mussels. Similarly, comparisons of the metabolic strategy utilised by tidepool and tidally-exposed mussels indicate that metabolic depression appears to be a more effective mechanism to tolerate thermal stress as exhibited by tidally-exposed mussels, which allowed mussels to ultimately reduce physiological demands during low tide and increase upper thermal tolerance. This raises the question, why isn’t this strategy also employed by tidepool mussels to tolerate thermal stress? Even when mussels were subjected to repeated thermal stress events, tidepool mussels still maintained the same metabolic strategy, even when experiencing high levels of Hsp/Hsc70 and depleted glycogen stores as exhibited in some of the low food treatments. This suggests that there is some component of tidally-exposed environment that drives this ability to metabolically depress (i.e. air is the environmental trigger that initiates declines in aerobic metabolism). Here we have postulated that because tidally-exposed treatments always experience air (predictable signal) prior to warming (unpredictable signal) mussels can initiate anticipatory metabolic adjustments prior to temperatures reaching suboptimal levels. Investigation into the role of air exposure as a signal that modulates metabolism could be a fruitful avenue for understanding how air exposure may modulate physiological responses in mussels.

Similarly, a consistent trend throughout Chapter 2, 3, and 4 was that acclimation to an unpredictable thermal regime increased glycogen stores, suggesting that having elevated energy stores is important for tolerating unpredictable thermal stress. In Chapter 2 we hypothesised that the high glycogen levels was to provide more energy to tolerate warming during the daytime low tide period. While this was the case with mussels acclimated to tidepool conditions in Chapter 3, we saw no change in glycogen levels in tidally-exposed mussels during warming, suggesting perhaps high energy stores may have played a role during re-immersion and restoring homeostasis more quickly to resume normal biological processes at high tide. Assessment of how energy dynamics change over the course of the tidal cycle, and how thermal unpredictability alters those dynamics could provide more insights on the importance of energy stores. Similarly, with increased investment into energy stores exhibited by mussels in unpredictable acclimation treatments, it will be important to understand if this results in a trade-off with important life history traits such as reproduction. Understanding how exposure to unpredictable temperature fluctuations will alter fitness dynamics will be important for understanding populations changes in the future.

Here, we worked with adult mussels, but evidence suggests that earlier life stages are more vulnerable environmental changes (Byrne et al., 2012; Przeslawski et al., 2015) and may exhibit different responses to the interaction of environmental variables than adult individuals. Indeed, while my research has shown that experiencing thermal stress in combination with air exposure serves to increase upper thermal limits, evidence indicates that this may have the opposite effect in young juvenile mussels (Jurgens and Gaylord, 2016). Incorporating more realistic environmental complexity into experimental designs across multiple life stages will be paramount for understanding how mussels and other intertidal species will respond to climate change.

## Conclusion

The key takeaway from my research is that thermal performance in *Mytilus californianus* is complex and can be regulated by a number of environmental variables. Habitat, thermal predictability, food availability and degree of thermal stress (lethal or sublethal; single or repeated thermal stress events) all interacted in nuanced ways to shape the thermal performance of the California mussel. Moving forwards, my research indicates that it is essential to incorporate ecologically relevant fluctuations in environmental variables in order to truly understand how intertidal organisms may respond to predicted climate change scenarios. Similarly, evolution in the way we apply thermal stress in physiological experiments needs to develop beyond single, lethal heat ramps for understanding thermal tolerance and move towards assessing tolerance to sublethal repeated thermal stress events. Furthermore, assessment of how thermal stress events influence physiological performance should advance beyond just assessing what is happening during thermal stress, by also investigating the physiological responses post warming at ecologically relevant timescales in order to understand the physiological ramifications of climate change. By incorporating more realistic approaches across all aspects of our experimental design, we can further our understanding of how climate-induced changes in the intertidal environment may shape the population dynamics of mussels in the decades to come.

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