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RESEARCH ARTICLE

Mussel acclimatization to high, variable temperatures is lost slowly upon transfer to benign conditions

Nicole E. Moyen*, George N. Somero and Mark W. Denny

ABSTRACT

Climate change is increasing the temperature variability animals face, and thermal acclimatization allows animals to adjust adaptively to this variability. Although the rate of heat acclimatization has received some study, little is known about how long these adaptive changes remain without continuing exposure to heat stress. This study explored the rate at which field acclimatization states are lost when temperature variability is minimized during constant submersion. California mussels (*Mytilus californianus*) with different acclimatization states were collected from high- and low-zone sites (~12 versus ~5°C daily temperature ranges, respectively) and then kept submerged at 15°C for 8 weeks. Each week, the cardiac thermal performance of mussels was measured as a metric of acclimatization state: critical (T_{crit}) and flatline (T_{flat}) temperatures were recorded. Over 8 weeks of constant submersion, the mean T_{crit} of high-zone mussels decreased by 1.07°C from baseline, but low-zone mussels' mean T_{crit} was unchanged. High- and low-zone mussels' mean maximum heart rate (HR) and resting HR decreased ~12 and 35%, respectively. T_{flat} was unchanged in both groups. These data suggest that T_{crit} and HR are more physiologically plastic in response to the narrowing of an animal's daily temperature range than T_{flat} is, and that an animal's prior acclimatization state (high versus low) influences the acclimatory capacity of T_{crit}. Approximately 2 months were required for the cardiac thermal performance of the high-zone mussels to reach that of the low-zone mussels, suggesting that acclimatization to high and variable temperatures may persist long enough to enable these animals to cope with intermittent bouts of heat stress.

KEY WORDS: Common garden, Constant submersion, Critical temperature, Flatline temperature, Heart rate, Heat stress, Intertidal zone, Mollusk, *Mytilus californianus*, Physiological plasticity, Thermal tolerance

INTRODUCTION

Physiological plasticity potentially allows animals to respond quickly to, and mount defenses against, environmental stressors (Burggren, 2018; Gotcha et al., 2018; Gunderson and Stillman, 2015; Rohr et al., 2018; Seebacher et al., 2015). During heat acclimatization – one example of physiological plasticity – several physiological changes occur that protect the organism against elevated temperatures (Somero et al., 2017). Although we have some insights into how quickly ectotherms can acclimatize to thermal stress (e.g. for intertidal mussels, see Braby and Somero, 2006; Gurr et al., 2018; Jimenez et al., 2016; Pickens, 1965; Senius, 1975; Widdows, 1973; Williams and Somero, 1996), much less is known about the rate at which the physiological changes gained with heat acclimatization are lost when high temperatures are absent for a period of time. Understanding the relative rates at which physiological changes are gained and lost becomes pressing in the face of climate change in which both the frequency of heat waves and thermal variability are on the rise (IPCC, 2014). For example, in order to predict an animal's ability to survive large, intermittent temperature swings, it is critically important to first understand whether the animal can retain its heat-acclimatized state for prolonged periods in the absence of high temperatures between heat-stress bouts.

The physiological changes accompanying heat acclimatization are likely to be metabolically costly (e.g. maintaining a higher constitutive level of heat shock proteins; Somero, 2002; Willett, 2010), so it can be advantageous for organisms to re-acclimatize to a lower temperature once heat stress is no longer present (Seebacher et al., 2015). In the few cases where re-acclimatization has been studied in other aquatic organisms (including mollusks), the process was found to take an equal, or longer, amount of time than the initial heat acclimatization (Corey et al., 2017; Cossins et al., 1977; Drake et al., 2017; Healy and Schulte, 2012; Huey and Bennett, 1990; Senius, 1975). However, beyond these limited observations, few of which have involved mussels, very little is understood about the loss of acclimatization to high temperature (its time course and mechanism) when heat stress is removed and/or animals are subjected to constant submersion at cooler temperatures.

Due to their sessile nature, which limits behavioral thermoregulation, intertidal mussels experience large body temperature fluctuations with each tidal cycle (Dowd et al., 2015; Jimenez et al., 2015; Miller and Dowd, 2017). Mussels living at different heights on the shore, and with different wave exposures, experience different daily temperature ranges, which allows one to study members of the same species that live in close proximity, but which have substantially different thermal histories (Denny et al., 2011; Gleason et al., 2018; Jimenez et al., 2015; Miller and Dowd, 2017; Moyen et al., 2019; Zippay and Helmuth, 2012). Furthermore, on many rocky shores, mussels are the dominant competitor for space in the mid-intertidal zone, and therefore play an important role in intertidal community ecology (Bayne et al., 1976; Gaylord et al., 2011; Mislan et al., 2014). Thus, information regarding mussel responses to thermal stress can provide important insights into how the increasing temperature variability expected with climate change (IPCC, 2014) will affect intertidal community ecology. Based on these characteristics, intertidal mussels can serve as a model system in which to study the rates at which heat acclimatization is gained and lost (Pickens, 1965).

In mussels, heat acclimation is typically completed (as measured by improved thermal tolerance or physiological performance) after 2 to 3 weeks of either constant submersion at a higher temperature, or exposure to an expanded range of daily temperature fluctuations (Braby and Somero, 2006; Pickens, 1965; Widdows, 1973, 1976).

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Heat acclimation in mussels induces changes such as a decrease in resting heart rate (HR) (Braby and Somero, 2006), an increase in maximum HR (Pickens, 1965), an increased sensitivity to neurotransmitters (Senius, 1975), an increased heat shock protein pool (Roberts et al., 1997), a restructuring of membrane order (Williams and Somero, 1996), and the re-establishment of routine oxygen consumption and feeding rates after an initial increase with heat stress (Pickens, 1965; Widdows, 1973, 1976). Each of these changes can help confer an increase in physiological performance (Braby and Somero, 2006; Pickens, 1965). Whereas full acclimatization may take 2 to 3 weeks, the rates at which different components of the acclimatization response occur are highly variable. Some can occur within minutes or hours (e.g. increased production of heat shock proteins), but others can take several days to weeks to reach completion (e.g. increased neurotransmitter sensitivity of the cilia on the gills; Newell and Bayne, 1973; Pickens, 1965; Roberts et al., 1997; Senius, 1975).

In mussels, changes in gene regulation, anti-oxidant defense, oxygen consumption and aerobic metabolic machinery are triggered by a tidal cycle and/or temperature variability (Andrade et al., 2018; Gracey et al., 2008; Jimenez et al., 2015). Yet we are aware of only one study that has evaluated the effects of removing this variability. Gleason et al. (2018) found that the thermal performance of both high- and low-zone juvenile mussels' (as assessed by the temperature at which 50% of mussels died, LT_{50}) decreased after 28 days of constant submersion, while the LT_{50} of adult mussels did not change (Gleason et al., 2018). Research on other organisms suggests that adult mussels with different thermal histories might respond differently to constant submersion (Burggren, 2018; Jimenez et al., 2015; Wang et al., 2019; Williams and Somero, 1996) – an avenue that Gleason et al. (2018) explored only in juvenile, but not adult, mussels. Moreover, based on previous literature in other ectotherms like fish (Corey et al., 2017; Healy and Schulte, 2012; Huey and Bennett, 1990), it may take longer than the 28 days of constant submersion used by Gleason et al. (2018) to cause a downward shift in thermal performance, and whereas LT_{50} values may change with constant submersion, it is unclear whether constant submersion affects cardiac thermal performance, a trait which (unlike LT_{50}, i.e. dead versus alive) allows for a more individualized and nuanced experimental understanding of changes in thermal performance through organ-level function.

Cardiac thermal performance experiments on a variety of animals have provided insights into the time course of acclimation and the role of cardiac thermal limits in setting whole-organism thermal performance (Braby and Somero, 2006; Zhang et al., 2014). Critical temperature (T_{crit}, the temperature at which further acute increases in temperature lead to an abrupt decrease in heart rate), and flatline temperatures (T_{flat}, the temperature at which heart rate falls to zero), are the two cardiac traits commonly measured by cardiac thermal performance tests (Braby and Somero, 2006; Drake et al., 2017). Both traits correlate with underlying changes in cellular level stress responses. In *Mytilus californianus*, expression of heat shock proteins commences at temperatures several degrees below T_{crit}, while at temperatures near and above T_{crit} there is a much stronger up-regulation of heat shock genes and expression of genes encoding proteins involved in proteolysis (Gracey et al., 2008). Furthermore, cardiac thermal performance is partially dependent on the animal's vertical position on the shore (Compton et al., 2007; Moyen et al., 2019), which suggests that this performance test is sensitive to each individual's thermal history.

Understanding the intricacies of an organism's acclimatization process is a complex and multi-faceted problem, one that is particularly challenging in intertidal animals because of their exposure to a multitude of stressors that can fluctuate on an hourly basis (e.g. temperature, hypoxia, desiccation). Thus, the goal of this study was to take a step towards better understanding the complex processes surrounding the loss of acclimatization states by exploring (1) if cardiac thermal performance of mussels – as indexed by T_{crit} and T_{flat} – is altered when daily body temperature fluctuations are removed by constantly immersing mussels at local sea surface temperatures for extended periods, and (2) if these responses differ between mussels previously acclimatized to different daily temperature ranges and tidal cycles (i.e. emersion and immersion durations) due to their vertical positions on the shore.

MATERIALS AND METHODS

To evaluate the effects of constant submersion and reduced thermal variation on cardiac thermal performance, high- (N=125) and low-zone (N=126) *Mytilus californianus* Conrad 1837 were collected from a moderately wave-exposed shore at Hopkins Marine Station in Pacific Grove, CA, USA (36.6216°N, 121.9042°W). High- and low-zone groups were collected from the same site, but were vertically separated by 0.56 m (0.43 and 0.99 m above mean lower low water, respectively, as measured with a GTS-211D Total Station, Topcon, Livermore, CA, USA). To minimize any effects that size might have on thermal performance, only adult mussels with shell lengths within a 30 mm range (i.e. 50–80 mm) were used.

Constant submersion experiments

Experimental design and animal preparation

To determine if cardiac thermal performance changed over 8 weeks of constant submersion, different sets of 10–12 mussels each from both the high- and low-zone sites were tested at baseline (Week 0), and then one set each Week thereafter over the course of 8 weeks. Because cardiac thermal performance tests are lethal if animals are taken to their flatline temperatures (see Results), each set could only be tested once.

Because of equipment constraints, we could test only six mussels at any given time, and each trial took ∼4 h to complete. Therefore, for practical purposes, we completed the required 38 trials across three Rounds, where each Round represented an entire 8 weeks of constant submersion. For Rounds 1, 2 and 3, mussels were collected from the same high- and low-zone sites on 12 September 2018, 23 November 2018 and 14 August 2019, respectively. At the beginning of each Round, within the first 7 days of collection, 10–15 high- and low-zone mussels underwent cardiac thermal performance tests to establish a baseline measurement (i.e. Week 0) for mussels that had not yet been subjected to constant submersion. This served as the baseline group for the rest of the mussels being tested during that Round. For each Round, different numbers of mussels were tested each Week, with the over-arching goal that across all three Rounds, 10–12 mussels from each of the high- and low-zone sites were tested for each Week (Table S1 outlines how many mussels were tested each Week for each Round of experiments).

After collection, all mussels were kept together in the same flow-through aquarium system. This tank holds approximately 60 liters and has a flow rate of ∼0.05 liters s^{-1}; the tank's water was thus turned over approximately every 20 min. Sea water in the flow-through system came directly from Monterey Bay, but was sand filtered before entering the tanks; thus, the water temperature was not controlled and matched that of the Bay. As such, for each of the Rounds the water temperatures were (mean±s.d.): Round 1, 15.2±1.1°C, range 13.2–17.9°C; Round 2, 14.8±1.4°C, range 11.7–17.2°C; Round 3, 14.9±0.8°C, range 13.3–17.4°C. The overall water

temperature throughout the 24 weeks of testing was 15.0±1.1°C (range 11.7–17.9°C). Although we did not obtain mussel temperatures from the high- and low-zone sites prior to collecting the mussels, recent field work at Hopkins Marine Station recorded live mussel temperatures, indicating that during the months of July and August, the mean individual maximum temperatures for high- and low-zone mussels were ∼25.8 and 19.8°C, respectively, while the mean individual minimum temperatures for the high- and low-zone mussels were ∼13.9 and 15.0°C, respectively. The mean and maximum sea surface temperatures during this same period were 17.1 and 21.0°C, respectively (Miller and Dowd, 2017).

Mussels were fed a commercial shellfish mix three to four times per week (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA). To avoid confounding effects of feeding on heart rate (Pickens, 1965), mussels were starved for 24 h before cardiac thermal performance tests.

Cardiac thermal performance tests

Before testing commenced, each individual's body mass was obtained (digital scale accurate to 0.0001 g). HR and internal mussel temperatures were recorded for each individual during cardiac thermal performance tests. For measurements of internal mussel temperature, a 1.5 mm diameter hole was drilled through the anterior end of each mussel's shell using a diamond bit. Next, in order to record individual mussel body temperatures, a thermocouple (Type K, 26-gauge wire, Omega Engineering, Santa Ana, CA, USA) was carefully inserted into the drilled hole, secured with cyanoacrylate glue, and then connected to a thermocouple amplifier (MAX31856 Universal Thermocouple Amplifier; Adafruit, New York, NY, USA) controlled by an Arduino microcontroller (Uno R3; Arduino, Scarmagno, Italy). To record HR during heating, an infrared sensor (model IR-AMP03-EX; Newshift, Leira, Portugal) was positioned on each mussel's shell on its dorsal side directly over the pericardial sac, and held in place using mounting putty (Fun-Tak Mounting Putty; Loctite, Henkel Corporation, Rocky Hill, CT, USA). The infrared sensor was connected to an amplifier (model AMP03-EX; Newshift) and interfaced with a PowerLab data logger (LabChart 6 software; AD Instruments, Colorado Springs, CO, USA). Heart rate was sampled at 4 Hz with a low-pass filter of 10 Hz (Burnett et al., 2013).

Once thermocouples and HR sensors were attached, each mussel was placed on a wire rack inside an insulated chamber where air temperature could be tightly regulated. Mussels were heated in air (i.e. emersed), as they would be during low tide. Air temperature inside the chamber was increased at a specific air heating rate using a temperature control box (iSeries Temperature Controller; Newport Electronics, Omega Engineering, Santa Ana, CA, USA) that received feedback from a resistance temperature detector, which regulated a heating element inside the chamber. A small fan circulated air inside the chamber to provide uniform heating. After all mussels were placed inside the chamber and the lid was secured, there was a 20 min baseline equilibration period during which air temperature inside the chamber was steady at 22°C. At the end of the equilibration period, resting HR (the lowest HR during this equilibration period) and body temperatures were recorded. Six mussels from the same zone were tested during each trial.

As mussels were emersed during heating, the chamber (air) heating rate needed to be slightly faster than the planned mussel body heating rates due to a temporal lag between air and mussel temperatures (for more details about the temporal temperature lag, see Moyen et al., 2019). Mussels were tested at only a single air heating rate of 9.0°C h^{-1}, which elicited a mean±s.d. mussel heating rate of 7.8±0.6°C h^{-1} (range 6.0–9.0°C h^{-1}). In our previous study (Moyen et al., 2019) we found that high-zone mussels had the highest T_{crit} at this heating rate, and that this rate elicited the biggest difference in T_{crit} between high- and low-zone mussels. Additionally, this rate is similar to typical heating rates that high-zone mussels experience on a daily basis at our site (Miller and Dowd, 2017). The mean individual mussel heating rates presented above were calculated for each trial by subtracting each mussel's baseline body temperature from its body temperature at the end of the last complete hour of the experiment (when all mussels were still alive), and then divided by the total amount of time between the two temperatures (in this case, at the end of 2 h).

Mussel HR and body temperatures were recorded every 15 min throughout heating, as well as when the T_{crit} (Braby and Somero, 2006) and T_{flat} (Stenseng et al., 2005) occurred. Along with resting HR, maximum HR (defined as the highest HR during the cardiac thermal performance test) and the total HR range (maximum minus minimum HR) were also used to evaluate each mussel's physiological responses to heat stress. T_{flat} was determined by pinpointing the mussel's body temperature when their last heartbeat occurred (defined by a heart rate of zero for at least 3 min). Because of the large variability in HR signatures, heartbeats were manually counted for 30 s at each recording time and then multiplied by two to yield HR in beats per minute (for example HR signatures, see Moyen et al., 2019).

Once mussels reached their T_{flat}, they were removed from the chamber and the thermocouple and infrared sensor were detached. Morphometric measurements of shell height (the longest distance from dorsal to ventral surfaces), shell width (the widest part of the mussel across both closed valves) and shell length (the longest distance from the anterior to posterior) were made using digital calipers (Beggel et al., 2015). To determine a mussel's reproductive status, immediately after morphometric measurements were collected, mussel gonads and somatic tissues were dissected and placed into separate aluminium weigh boats; samples were dried in a drying oven at 60°C for 48 h or until brittle. Relative gonad mass (as a percentage of total dry tissue mass) was calculated as gonadal mass divided by the sum of somatic plus gonadal masses (Logan et al., 2012).

T_{crit} and T_{flat} lethality experiments

Although exposure to temperatures near T_{crit} is not immediately lethal, such exposure activates many components of the cellular stress response (Somero, 2020), which indicates that cellular damage has occurred. However, it is currently unknown whether exposure to T_{crit} is eventually lethal over longer periods, e.g. several days or weeks. It has been observed that T_{flat} can be lethal (Moyen et al., 2019); however, this has not yet been methodically tested. To clarify the extent to which exposures to T_{crit} and T_{flat} are lethal, and thus develop a better ecological context for our findings, we conducted additional cardiac thermal performance tests. For this purpose, we used adult mussels (N=36) that were freshly collected from a moderately wave-exposed site at Hopkins Marine Station, whose elevation ranged from 0.95 to 1.22 m above mean lower low water. Pooled means±s.d. for morphometric data were as follows: body mass, 29.9±6.5 g; shell height, 28.9±2.2 mm; shell width, 26.4±2.0 mm; shell length, 60.5±6.3 mm.

Cardiac thermal performance tests were conducted using the same methods as detailed above, and mussels were heated at the same rate (air heating rate of 9°C h^{-1}). HR was continuously measured so that T_{crit} and T_{flat} could be identified. Mussel internal body temperatures were not measured during the tests for two reasons: (1) the goal of the tests was to determine if T_{crit} and T_{flat} are

lethal to the organism rather than to determine the animal's actual T_{crit} or T_{flat}; (2) to measure internal temperature, thermocouples must be glued onto the mussel's shell, making it difficult to quickly remove each mussel from the chamber at its specific T_{crit} or T_{flat}, without altering the temperature inside the chamber or disturbing the HR measurements of other mussels.

For the T_{crit} group (N=12), each mussel was removed from the heat chamber when it reached its T_{crit} and placed immediately into ∼15°C seawater. Mussels in the T_{flat} group (N=12) were heated until they reached their T_{flat}, and then immediately placed into ∼15°C seawater. Lastly, the control mussel group (N=12) was emersed in the chamber at 22°C (room temperature) for the same amount of time that it took (on average) for the experimental mussels to reach T_{flat} (4 h), before then being placed back into ∼15°C seawater.

After testing, mussels were placed in a flow-through aquarium at 15.14±0.95°C and fed three to four times per week so that mussel mortality could be monitored for 3 weeks (Dowd and Somero, 2013).

Statistical analyses

R 3.5.2 (https://cran.r-project.org/) and R studio (https://www.rstudio.com/) were used for all statistical analyses. Because testing occurred in three different Rounds at different dates over the course of one year, we first determined if there were any differences among Rounds in T_{crit} and T_{flat} at Week 0 (i.e. baseline, no constant submersion) potentially resulting from seasonal changes in thermal performance. To do so, we conducted separate one-way ANOVAs (across Rounds) for the high- and low-zone mussels at Week 0. An α value of <0.05 defined significance for all statistical tests. We found no significant differences among Rounds at Week 0 for either the high- or low-zone mussels' T_{crit} or T_{flat} (d.f.=2, all $F \leq 2.7$, all $P>0.05$). Consequently, we took all of the Week 0 mussels from the three Rounds of testing, and randomly selected 12 mussels to represent each of the high- and low-zone groups at Week 0, thus providing a sample size equal to that in subsequent weeks for the remainder of the statistical analyses.

As the cardiac thermal performance tests are lethal, we had to use new mussels each Week for testing, which made this study a between-subjects design. We therefore used two-way between-between ANOVAs (2 Zone×9 Week) to evaluate whether there was a Zone by Week interaction effect, a main effect of Zone, or a main effect of Week for each of the heart rate, morphometric, reproductive and thermal performance variables. For any tests with statistically significant F scores, pairwise comparisons with a Bonferroni correction were used to evaluate whether significant differences existed among Weeks, or between Zones for a specific Week. See Table S4 in the Supplemental Information for the ANOVA statistics for each variable. Pearson r correlations were used to assess whether any relationships existed between T_{crit} or T_{flat} with the following variables: individual heating rate, resting HR, maximum HR, total HR range, and/or any morphometric and reproductive variables.

Lastly, to determine the effects of constant submersion on the changes in T_{crit}, T_{flat}, resting HR, maximum HR and total HR range from Week 0, we calculated each individual's change score from the Week 0 mean value that was specific to that Round and Zone. For example, to evaluate the mean change in T_{crit} for the Round 1 high-zone mussels at Week 8, we subtracted each high-zone mussel's T_{crit} at Week 8 from the mean of the high-zone mussels' T_{crit} at Week 0 for Round 1. For each variable, we first fitted a linear model that included the predictors of Week and Zone, plus a Week×Zone interaction effect to see if the effects of constant submersion differed based on Zone (i.e. whether the slopes of the lines for each Zone were statistically different based on Week). If there was no interaction effect, then we fitted a linear model with just Week as the predictor (e.g. change in T_{crit}=slope×Week). The intercepts in these regressions were all forced through zero (i.e. regression through the origin), as the change from Week 0 is by definition zero. The fraction of overall variance explained by the linear model (r^2) was calculated according to Eisenhauer (2003), where $r^2 = \sum \hat{Y}_i^2 / \sum Y_i^2$. Here, $\hat{Y}_i$ is the predicted mean change value for a given Week, and Y_i is the actual mean change value for that Week. An α value of <0.05 defined significance.

RESULTS

Heating rates and morphometric variables with constant submersion

Individual heating rates did not differ between Zones for any specific Week, between Zones, or across Weeks (all $P>0.05$). As we used a between-subject experimental design, we wanted to confirm that basic morphometrics were similar among groups (across all 8 Weeks), and also that Week 0 mussels were of similar size and reproductive status compared with all other Weeks. Therefore, we conducted two-way ANOVAs (Zone by Week) for each of the morphometric variables (see Table S4 for ANOVA statistics). The pooled means±s.d. for all 251 mussels' morphometric and reproductive status data were: body mass, 25.41±7.82 g; shell height, 26.83±2.60 mm; shell width, 24.93±2.94 mm; shell length, 61.05±6.18 mm; gonad mass (as a percentage of total dry weight), 12.8±4.1% (see Table S2 for detailed morphological parameters separated by Week and Zone).

There were no Zone by Week interaction effects for any of the variables (all $P>0.05$), except for shell height (P=0.02). However, *post-hoc* tests with a Bonferroni correction (significant at $P<0.006$ based on eight tests for Weeks 1–8) found no significant differences between Zones based on Week for shell height (all $P>0.006$). Similar to our previous study (Moyen et al., 2019), the high-zone mussels were slightly larger on average, with a higher body mass (by ∼5 g), and greater shell height and width (both by ∼1.5 mm; all $P<0.001$). Mussels from Weeks 4 and 5 were slightly larger than mussels in the other Weeks (main effect of Week; $P<0.05$; see Tables S1 and S4 for details). However, these slight morphometric differences likely did not impact the study results as the only morphometric variables that significantly, but weakly, correlated with T_{crit} were body mass and shell width (r=0.19 and 0.16, respectively; both $P<0.02$). These correlations were probably due to the high-zone mussels being slightly larger and having a higher T_{crit} overall compared with the low-zone mussels. None of the morphometric variables correlated with T_{flat} (all $P>0.05$). It is unlikely that these slight differences in morphometric variables caused any changes in cardiac thermal performance, because if that were the case we would expect the mussels from Weeks 4 and 5 to have higher cardiac thermal performance values than the other Weeks, but this did not occur (see below). Thus, these slight differences in morphometric data across the 8 weeks of constant submersion did not appear to affect thermal tolerance, and the differences in cardiac thermal performance with constant submersion are likely to be the result of differences in acclimatization status between high- versus low-zone mussels.

HR indices with constant submersion

There were no Week by Zone interaction effects for any of the HR variables when assessing the change values from baseline (all $P>0.05$), indicating that high- and low-zone mussel heart rates were similarly affected by 8 weeks of constant submersion. Both high- and low-zone mussels' mean maximum HR decreased by 2.83±0.91 beats min^{-1} [mean±95% confidence interval (CI)] over the

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course of 8 weeks of constant submersion, a 0.35±0.06 beats min^{-1} (mean±s.e. of the slope) decrease per Week (r^2=0.16; P<0.001; Fig. 1). Mussel mean resting HR decreased by ∼5.52± 1.29 beats min^{-1} (mean±95% CI) over the 8 weeks, a 0.69± 0.08 beats min^{-1} (mean±s.e. of the slope) decrease per Week (r^2=0.26; P<0.001; Fig. 2). These decreases are large when taken as a percent change of the mussels' Week 0 mean maximum HR (∼12% decrease overall) and mean resting HR (∼35% decrease overall). Although both maximum and resting HR decreased, the total HR range (maximum–minimum HR) remained unchanged with constant submersion (P=0.01, r^2=0.04). Lastly, these mean changes in HR did not account for any changes that occurred in mean T_{crit} (as assessed by regression analyses; all P>0.05).

As these data demonstrate, constant submersion altered the mean change values of resting and maximum HR from baseline. However, when comparing the actual values at each time point (rather than the changes relative to baseline), constant submersion did not have any discernible effect on resting HR, maximum HR, or the total HR range for either zone: there were no significant Zone by Week interaction effects, main effects of Zone, or main effects of Week (all P>0.05; see Table S4 for ANOVA statistics). The pooled means± s.d. for resting HR, maximum HR and the total HR range were 12.9±5.6, 22.5±4.0, and 11.9±4.1 beats min^{-1}, respectively. Resting HR (both zones included) was significantly, but weakly, correlated with T_{flat} (r=0.15, P=0.03).

T_{crit} and T_{flat} with constant submersion

Eight weeks of constant submersion significantly decreased the mean T_{crit} in high-zone mussels only, by ∼1.07±0.48°C (mean± 95% CI) from Week 0 to Week 8, or 0.13±0.03°C (mean±s.e. of the slope) per week (r^2=0.17; P<0.001; Fig. 3). This is an approximately 2.7% change from high-zone mussels' baseline T_{crit}. Low-zone mussels' mean T_{crit} did not change with constant submersion (P=0.23). Despite the fact that high-zone mussels' mean T_{crit} decreased over the 8 weeks and low-zone mussels' T_{crit} did not, there was not a significant interaction effect for Zone and Week for the mean change in T_{crit} (P=0.15).

High-zone mussels had a significantly higher T_{crit} overall (i.e. main effect of Zone) versus low-zone mussels (pooled means±s.d., 38.5±1.6 versus 37.1±2.3°C, respectively; P<0.001). Constant submersion did not differentially affect the high- versus low-zone mussels' absolute T_{crit} (i.e. no Zone by Week interaction effect; P=0.59), and the only significant difference across Weeks (i.e. main effect of Week) was that the T_{crit} for Week 1 was significantly higher than that of Week 3 (P<0.05; see Table S3 in the Supplementary Information for absolute T_{crit} and T_{flat} data separated by Zone and Week, and Table S4 for ANOVA statistics).

T_{flat} did not change over the course of the 8 Weeks of constant submersion (either as a mean change value from baseline or as the actual values at each time point), did not differ between Zones for any specific Week, and did not differ between Zones overall (pooled

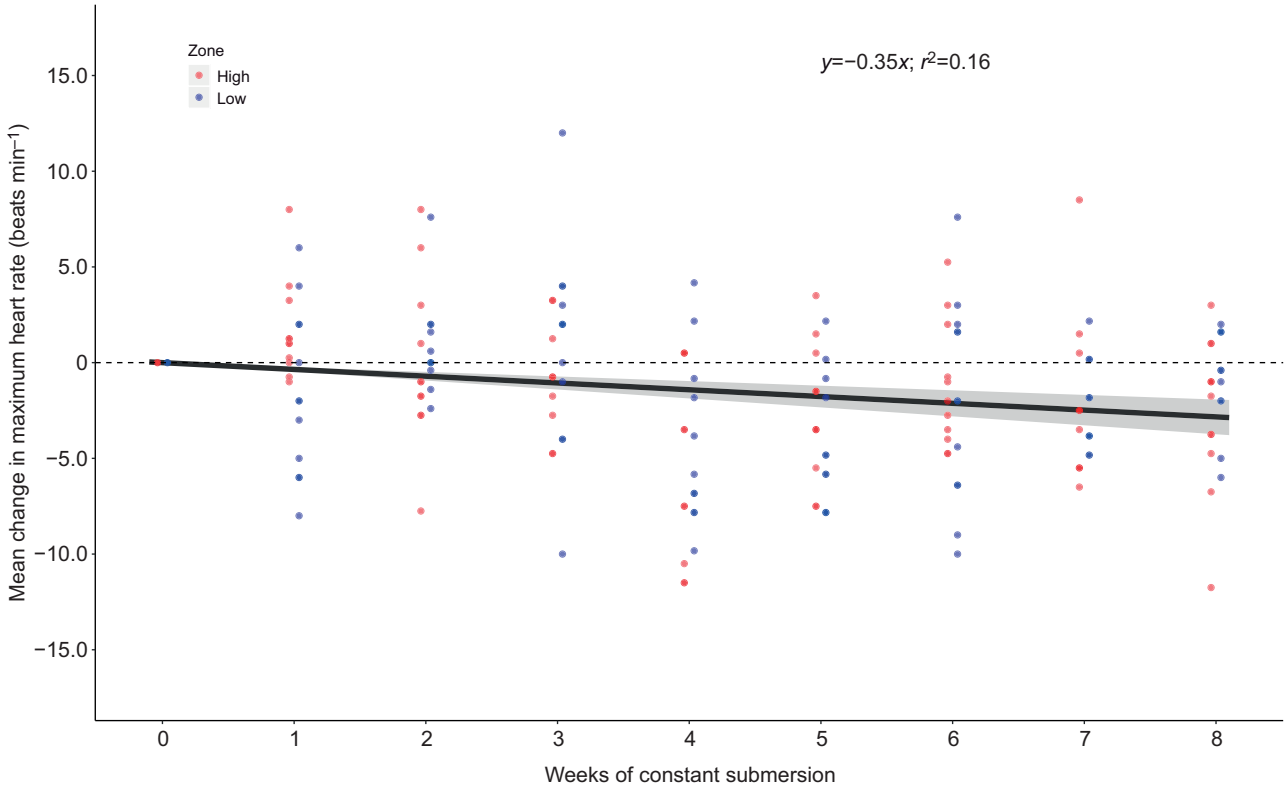

Fig. 1. Changes in *Mytilus californianus* maximum HR from Week 0 over 8 weeks of constant submersion. Each point represents an individual's change value, which is calculated by subtracting each individual's maximum HR for that Week from the Week 0 mean maximum HR, specific to their Round and Zone. Individuals from the high Zone are in red, and those from the low Zone are in blue (note that the points are slightly offset for better visualization). Mussels' maximum HR significantly decreased from Week 0 (r^2=0.16; P<0.001) by 0.35±0.06 beats min^{-1} (mean±s.e. of the slope) per Week, and by ∼2.83± 0.91 beats min^{-1} overall (mean±95% CI). In the regression equation, y is the mean change in maximum HR from Week 0 (beats min^{-1}), and x is the number of Weeks of constant submersion. Constant submersion did not differentially affect high- versus low-zone mussels' maximum HR response (P=0.57). The dashed horizontal line at zero represents no change in mean maximum HR from baseline (i.e. Week 0, no constant submersion), while the gray shading around the black best fit line indicates the 95% CI.

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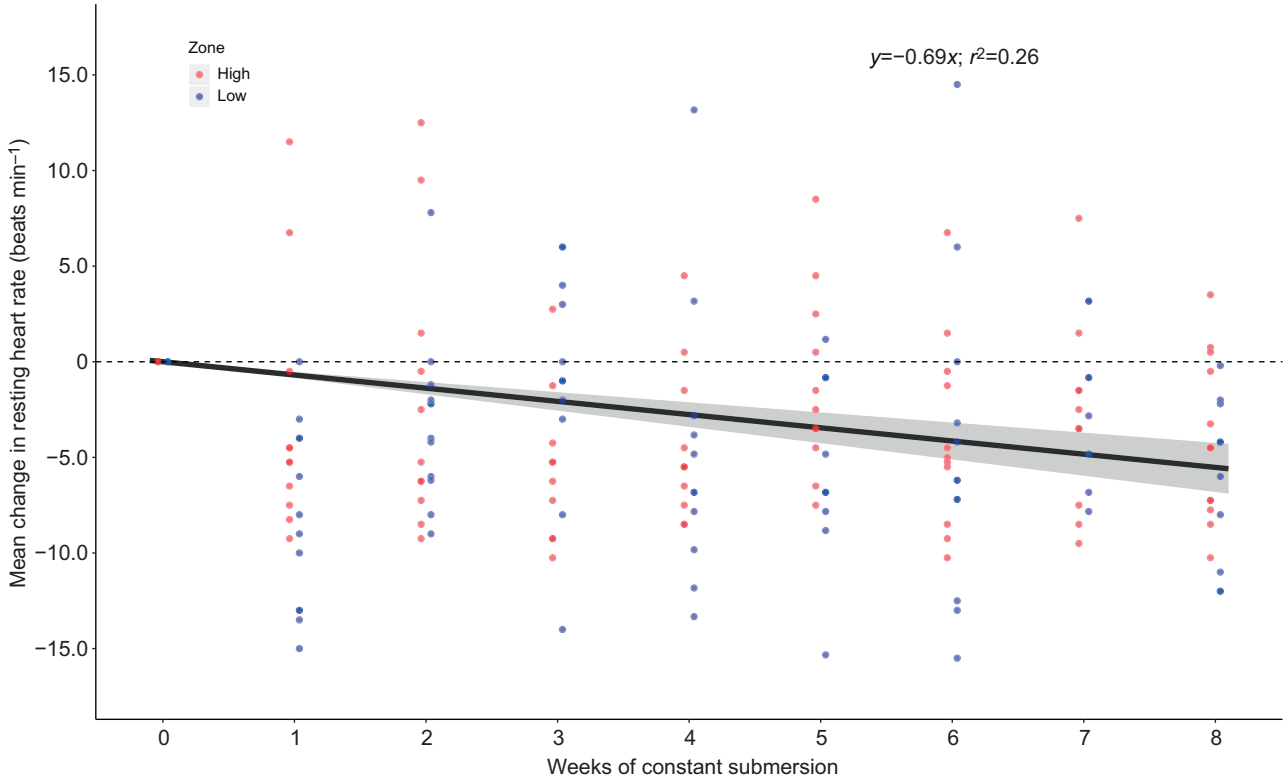

Fig. 2. Changes in mussel resting HR from Week 0 over 8 weeks of constant submersion. Each point represents an individual's change value, which is calculated by subtracting each individual's resting HR for that Week from the Week 0 mean resting HR, specific to their Round and Zone. Individuals from the high Zone are in red, and those from the low Zone are in blue (note that the points are slightly offset for better visualization). Mussels' resting HR significantly decreased from Week 0 (r^2=0.26; P<0.001) by 0.69±0.08 beats min^{-1} (mean±s.e. of the slope) per Week, and by ∼5.52±1.29 beats min^{-1} overall (mean±95% CI). In the regression equation, y is the mean change in resting HR from Week 0 (beats min^{-1}), and x is the number of Weeks of constant submersion. Constant submersion did not differentially affect high- versus low-zone mussels' resting HR response (P=0.96). The dashed horizontal line at zero represents no change in mean resting HR from baseline (i.e. Week 0, no constant submersion), while the gray shading around the black best fit line indicates the 95% CI.

mean±s.d., 40.4±1.3°C; all P>0.05). The absolute temperature difference between T_{flat} and T_{crit} was wider in low- versus high-zone mussels overall (pooled means±s.d. for T_{flat}–T_{crit}, 3.31±2.19 versus 1.93±1.52°C, respectively; P<0.001); however, constant submersion had no discernible effect on this difference (all P>0.05; see Table S4 for ANOVA statistics).

T_{flat} significantly correlated with T_{crit} (r=0.40; P<0.001), T_{crit} significantly correlated with the absolute temperature difference between T_{flat} and T_{crit} (r=−0.80, P<0.001), and individual heating rate and T_{flat} were positively correlated (r=0.33; P<0.001).

T_{crit} and T_{flat} lethality

Three out of 12 mussels (25%) died after reaching their T_{crit}; two of these mussels died 12 days after the test, and the other died 15 days after the test. All 12 mussels (100%) died within 4 days after reaching their T_{flat}. As expected, none of the control mussels (0%) died from 4 h of benign emersion at 22°C.

DISCUSSION

The goal of this study was to take a first step in exploring the role of temperature variability and emersion duration in maintaining an animal's acclimatization state. We did this by evaluating how quickly cardiac thermal performance changes when a mussel's typical (field) temperature range is replaced by stable temperatures under constant submersion, and whether these changes were dependent on an animal's prior intertidal location on shore (i.e. their previous acclimatization state). Our studies demonstrate the utility of using cardiac variables, notably HR and T_{crit}, to monitor the acclimation status of mussels over time. Phenotypic plasticity in these two traits was evident over the 8 week acclimation period under constant submersion at local sea surface temperatures, and while the patterns of change in HR were similar for both zones, only high-zone mussels' T_{crit} decreased.

Changes in heart rate with constant submersion

Eight weeks of constant submersion similarly affected HR in high- and low-zone mussels: mean maximum and resting HR both decreased, while the total HR range remained unchanged. Previous research has shown that some HR indices are physiologically plastic. Resting and maximum HR can change with heat and cold acclimation (Braby and Somero, 2006; Pickens, 1965), while the total HR range (i.e. maximum–minimum HR) remains fixed. Similar to previous studies, we found that the total HR range did not change with constant submersion because maximum and resting HR both shifted downwards with constant submersion. Our mussels' downward shift in mean maximum HR is a similar percent change from pre-acclimation HR (∼12% decrease) to that found by Pickens (1965) after mussels were constantly submerged at 16°C for 41 days (Pickens, 1965). Aside from the Pickens (1965) study, however, we are unaware of any other studies in *Mytilus* that have evaluated changes in HR with cold acclimation or re-acclimation to cooler temperatures. Thus, our data are some of the first to investigate changes in resting and maximum HR with prolonged constant submersion, where influences of temperature variability and tidal cycles are minimized or eliminated.

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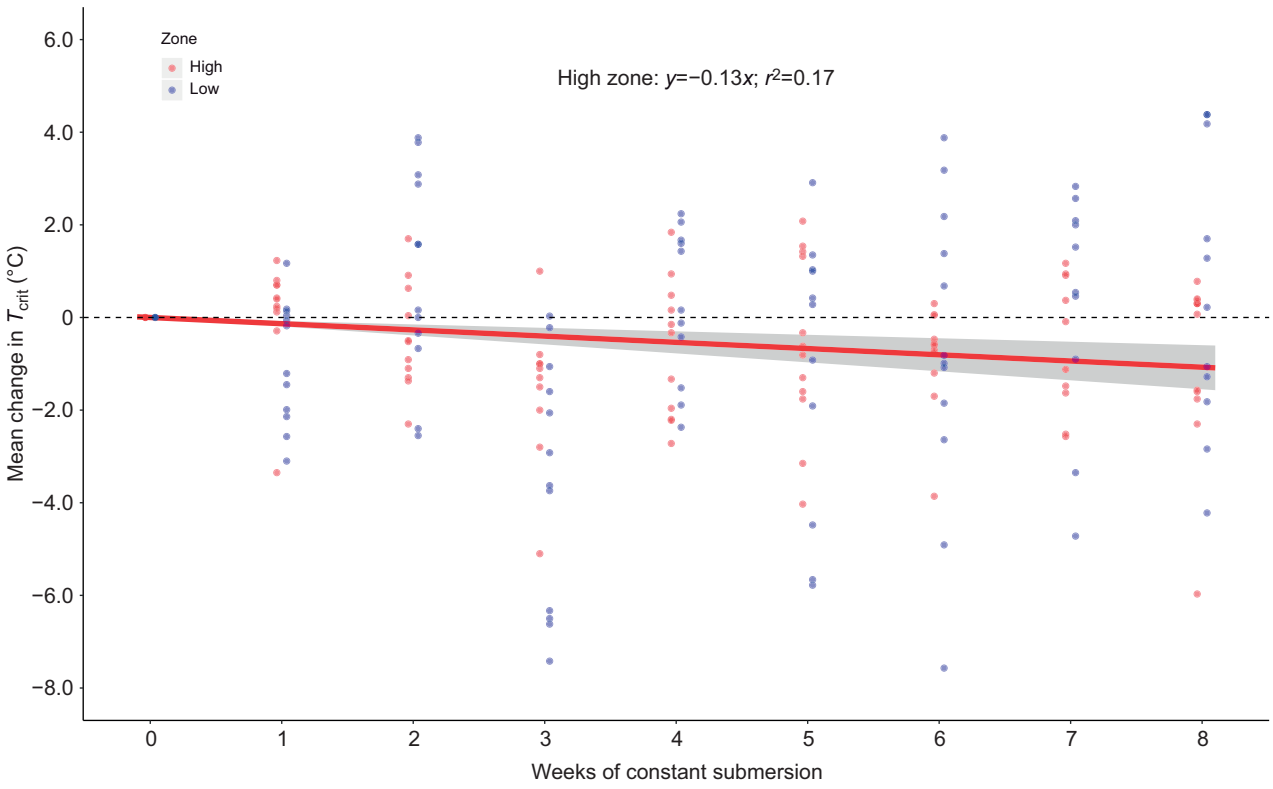

Fig. 3. Change in mussel T_{crit} from Week 0 over 8 weeks of constant submersion. Each point represents an individual's change value, which is calculated by subtracting each individual's T_{crit} for that Week from the Week 0 mean T_{crit}, specific to their Round and Zone. The red line of best fit is for the high-zone mussels only. Constant submersion decreased high-zone mussels' mean T_{crit} (r^2=0.17; P<0.001) by 0.13±0.03°C (mean±s.e. of the slope) per Week, leading to an overall decrease in mean T_{crit} of 1.07±0.48°C from Week 0 (mean±95% CI). Low-zone mussels' mean T_{crit} did not change with constant submersion (P=0.23), which is why there is no line of best fit for the low-zone mussels in this figure. In the regression equation, y is the mean change in T_{crit} (°C) from Week 0, and x is the number of Weeks of constant submersion. The dashed horizontal line at zero represents no change in mean T_{crit} from Week 0, while the gray shading around the red best fit line indicates the 95% CI.

As our mussels were constantly submerged for 2 months, it may be that, along with the lack of temperature variability, the lack of the tidal cycle (or emersion) also affected their hearts. Collins et al. (2020) compared heart rate responses during heating of subtidal (constantly submerged) versus intertidal (normal tidal cycle) blue mussels (*Mytilus galloprovincialis*; Collins et al., 2020). The authors found that when subtidal mussels were emersed (in air), their HR decreased by ∼25% when heated from 16 to 24°C, whereas the intertidal animals' heart rates remained unchanged when heated to a similar temperature range (Collins et al., 2020). It could be that after 8 weeks of constant submersion at ∼15°C, our mussels' hearts developed a phenotype similar to that of subtidal animals' hearts, which exhibit a higher heart rate when submerged versus emersed (Collins et al., 2020). This may partially explain why our mussels' mean percent change in resting HR decreased by ∼35% from baseline (as resting HR was taken when mussels were emersed at ∼22°C). It is unclear at this point what would cause mussels' maximum and resting heart rates to decrease with constant submersion, but there is evidence suggesting that it could be related to changes in neurotransmitter sensitivity, thermal effector sensitivity, oxygen consumption, metabolic machinery, or possibly shifts in gene expression (Andrade et al., 2018; Bakhmet, 2017; Braby and Somero, 2006; Domnik et al., 2016; Gracey et al., 2008; Jayasundara and Somero, 2013; Logan and Somero, 2011; Pickens, 1965; Seebacher et al., 2015; Stenseng et al., 2005; Widdows, 1973, 1976; Williams and Somero, 1996; Wu et al., 2016).

It is unclear exactly how these changes in resting and maximum HR with constant submersion would affect the organism within an ecological context, as we are unaware of any field studies exploring how changes in HR are linked to the organism's metabolic rate and long-term survival. Therefore, we can only speculate that these downward shifts in maximum and resting HR (during emersion) may be indicative of reduced respiratory rates characteristic of subtidal animals (Tagliarolo et al., 2012). However, as changes in HR did not account for any changes in T_{crit} or T_{flat}, and T_{flat} did not change with constant submersion, it appears that these changes in HR may be secondary to mussel survival, and that other mechanisms probably exert greater control in determining mussels' thermal tolerance. Further research is required to determine the stimuli, or lack thereof, required to alter and/or maintain changes in these HR variables, along with the ecological implications of these changes in HR for mussel survival in the field.

Changes in cardiac thermal performance responses with constant submersion

Taking our data together with previous research, it appears that in the absence of high temperatures, T_{crit} is lost at one-tenth of the rate that it is gained with heat acclimation (i.e. lost at ∼0.13°C per week versus increased by ∼1.3–1.5°C per week; Braby and Somero, 2006; Pickens, 1965). This finding in mussels is similar to findings in other marine organisms (Corey et al., 2017; Drake et al., 2017; Healy and Schulte, 2012; Huey and Bennett, 1990; Palumbi, 1984), where animals acclimatize to elevated temperatures much faster (i.e. within 1 day to 3 weeks) than they lose those changes when placed at lower temperatures (i.e. ≥3–8 weeks). In our study, the slow

decline in high-zone mussels' T_{crit} might be attributable to the fact that the stimulus (i.e. simply the absence of the warmer temperatures typically experienced by our mussels in the field) was not sufficient to rapidly or completely reverse the changes that led to the animals' initial acclimatization state (Palumbi, 1984). However, it could also be that the decrease in high-zone mussels' T_{crit} resulted from molecular and biochemical changes due to lack of a tidal cycle (emersion), rather than an absence of heat stress (Andrade et al., 2018; Gleason et al., 2017; Gracey et al., 2008; Jimenez et al., 2015). Future research is required to tease apart the effects of temperature variability versus emersion on thermal performance, and how thermal history influences the rates at which temperature acclimatization is gained versus lost, i.e. the rates at which re-acclimatization to new thermal conditions occurs.

Low-zone mussels' T_{crit} did not change with constant submersion, probably because the constant submersion treatment was not substantially different from these mussels' typical daily temperature fluctuations and emersion durations in the field. Our low-zone mussels at Hopkins Marine Station typically experience a narrower temperature range on a daily basis than high-zone mussels (i.e. ∼5 versus ∼12°C daily temperature range, respectively), and also undergo much slower mean daily heating rates (∼1.3 versus ∼6.8°C h^{-1}, respectively; Miller and Dowd, 2017). Moreover, our low-zone mussels are immersed for a much larger percentage of their day (around two-thirds of the day) compared with our high-zone mussels, which are immersed for approximately one-third of each day (Tide Predictions - NOAA Tides and Currents, 2019). In general, animals that experience less temperature variability (like our low-zone mussels) appear to have less physiological plasticity (Denny and Dowd, 2012; Seebacher et al., 2015), and as a result, are often less able to acclimatize to temperatures or conditions outside their normal temperature range (Wang et al., 2019). For example, Wang et al. (2019) heat-acclimated limpets (*Lottia limatula*) from three locations with very different thermal profiles, and then conducted thermal tolerance tests. Limpets from the site with the highest daily temperature fluctuations had the largest changes in T_{crit} with heat acclimation (i.e. the greatest physiological plasticity), while limpets from the most thermally stable environment actually had a decrease in thermal tolerance with heat acclimation (Wang et al., 2019). The idea that physiological plasticity is dependent on an animal's thermal history is in line with findings in mussels showing that living in different intertidal locations (e.g. high- versus low-zone sites) leads to distinct thermal histories (i.e. acclimatization states; Miller and Dowd, 2017), which can result in very different physiological responses to heat stress (Compton et al., 2007; Denny et al., 2011; Gleason et al., 2017; Logan et al., 2012; Moyen et al., 2019; Pickens, 1965; Somero, 2002; Tanner and Dowd, 2019; Williams and Somero, 1996).

However, other factors (e.g. the temperature difference between T_{crit} and T_{flat}) could also govern an individual's capacity for thermal plasticity. For example, gastropods whose T_{crit} is close to their T_{flat} have smaller increases in T_{crit} with heat acclimation than those with a larger difference between their T_{crit} and T_{flat} (Armstrong et al., 2019; Stenseng et al., 2005). If we apply these findings to our data, we would expect that our low-zone mussels would have experienced the largest change in T_{crit} with constant submersion as their T_{flat}–T_{crit} difference is significantly wider than that of high-zone mussels. However, this was not the case. As the aforementioned studies (Armstrong et al., 2019; Stenseng et al., 2005) examined plasticity in the context of heat acclimation, it may be that the opposite is true with cold acclimation (or re-acclimatization), where animals with a wider T_{flat}–T_{crit} gradient experience minimal decreases in their T_{crit} because it is already near the minimum of the possible T_{crit} range. Further research is needed to confirm this hypothesis.

Changes in cardiac thermal performance within an ecological context

Previous studies on limpets (Dong et al., 2017; Han et al., 2013) show that some mollusks can recover from reaching T_{crit} but that T_{flat} is invariably lethal. This is in contrast to crabs, which die if they reach T_{crit} (Tepolt and Somero, 2014). In the absence of similar data on mussels, we conducted experiments to determine whether T_{crit} and T_{flat} are lethal. We found that the majority of mussels recovered after reaching T_{crit} when followed by an immediate return to cool seawater (∼15°C); however, reaching T_{flat} was invariably lethal (see Results).

These findings are important to consider in the context of our constant-submersion results, where mean T_{crit} decreased (by ∼1.1°C or 2.7% from baseline) in high-zone mussels, but there was no change in T_{flat} for either zone. Although merely reaching T_{crit} is generally not lethal to mussels, it is probable that prolonged time at T_{crit} would be lethal, as this temperature is linked to maximal expression levels of stress-related genes and proteins in mollusks (Gracey et al., 2008; Han et al., 2013; Zhang et al., 2014). Thus, it may be presumed that having a higher T_{crit} would be beneficial to the organism. However, similar to our previous study (Moyen et al., 2019), we found that animals with a higher T_{crit} had the same T_{flat} as those with a lower T_{crit} (e.g. T_{crit} in two animals differed by 6°C, but their T_{flat} was the same at 41°C). This finding begs the question as to the importance of T_{crit} plasticity if T_{flat} remains unchanged. Although contrary to the predominant beliefs in the field, it may be that if T_{flat} remains fixed, having a lower (versus higher) T_{crit} is beneficial to the organism. For example, suppose that two mussels with different critical temperatures (e.g. 33 versus 39°C) but the same T_{flat} (41°C), reach a sublethal temperature of 38°C during an afternoon low tide. The mussel with a T_{crit} of 33°C will spend considerably more time with a lower HR (and presumably lower oxygen consumption) while their temperature is between 33 and 38°C, while the mussel with a T_{crit} of 39°C will be at near-maximum HR and oxygen consumption for a longer duration. As such, the animal with the lower T_{crit} would have saved a considerable amount of energy that can then be used for recovery and repair post-heat stress. Therefore, assuming that reaching temperatures between T_{crit} and T_{flat} are not acutely lethal, it may be that it is more beneficial to have a lower T_{crit} (if T_{flat} does not change); however, this energy trade-off is likely to depend on the frequency with which the animal experiences heat stress.

Although we are unaware of any studies evaluating changes in T_{flat} with temperature acclimation in mussels, based on limpet (Drake et al., 2017) and snail (Stenseng et al., 2005) studies, T_{flat} may (Drake et al., 2017) or may not change (Stenseng et al., 2005) with temperature acclimation. Gleason et al. (2018) found that mussel lethal temperature (assessed by LT_{50}) was decreased after 1 month of constant submersion in juveniles, but not in adults. Thus it may be that the animal's lethal temperature (in this particular case assessed by heart rate reaching zero) is set early in life based on the temperatures each individual experiences during a critical window of development (Burggren, 2018; Gleason et al., 2018; Karunanithi and Brown, 2015). It could also be that high- or low-zone mussels' T_{flat} would change if a larger stimulus (outside of their normal temperature range) was provided (Huey and Bennett, 1990; Palumbi, 1984), and that simply narrowing a mussel's daily temperature range does not change its T_{flat}. However, we cannot rule out the possibility that T_{flat} is predominantly determined by genetics (Kelly, 2019; Morley et al., 2017; Sorby et al., 2018).

It is clear that our findings highlight the need for additional research that might pinpoint the cellular and molecular mechanisms, along with the life stage, that determine T_{flat} in mussels. Moreover, studies should aim to better understand the energy trade-offs associated with making changes to T_{crit} (and the rate at which that occurs), if and when T_{flat} remains fixed. Additionally, it is important to explore how reaching temperatures at or above T_{crit} (but before T_{flat}) affects mussel cardiac function and survival, during single and repeated bouts of heat stress.

Considerations

Laboratory research on thermal acclimation of mussels has often utilized common garden (typically, constant submersion) treatments as the first step in its protocols. Mussels are held in aquaria, constantly submerged without a tidal cycle, for a period of time ranging from as short as 2 weeks to as long as 6 months (Andrade et al., 2018; Braby and Somero, 2006; Dowd and Somero, 2013; Logan et al., 2012; Yao and Somero, 2013). Common garden exposure of this type is intended to establish similar thermal responses in all specimens, yet we are unaware of any studies that have validated this assumption. Notably, it took 2 months for our high-zone mussels to reach a similar T_{crit} to that of low-zone mussels, which calls into question the assumptions of some prior work using constant submersion for less than 2 months to start animals at the same baseline thermal tolerance.

Moreover, we note that 2 months of constant submersion did not alter the variability in critical or flatline temperatures among individuals: the standard deviations of T_{crit} and T_{flat} remained unchanged across 8 weeks of constant submersion. Therefore, 'acclimation' to a constant (sea surface) temperature does not result in all individuals reaching a similar T_{crit} (i.e. regression to the mean). Instead, it may be that, during acclimation, each individual undergoes a similar change in T_{crit} relative to its initial T_{flat}–T_{crit} range (e.g. heat acclimation leads to a 10% increase in all mussels' T_{crit}, relative to their overall T_{flat}–T_{crit} range). As merely reaching T_{crit} is generally not lethal in mussels, future studies could employ T_{crit} tests on the same individuals before and after temperature acclimation to quantify each individual's change in T_{crit} (Healy and Schulte, 2012). Measuring these individual changes in thermal tolerance will help elucidate the cellular and molecular changes underpinning temperature acclimatization (Tanner and Dowd, 2019).

Conclusions

Lack of substantial temperature variability and daily tidal cycles from 8 weeks of constant submersion led to a decrease in maximum and resting HR in both low- and high-zone mussels. Moreover, high-zone mussels' mean T_{crit} decreased by 1.07±0.48°C (~2.7% change) from baseline, but low-zone mussels' mean T_{crit} remained unchanged. Constant submersion did not change T_{flat} in either high- or low-zone animals. It thus appears that T_{crit} is more sensitive (physiologically plastic) to the narrowing of an animal's daily temperature range than T_{flat}. This study is in line with previous literature showing that physiological changes with heat acclimation (e.g. an increase in T_{crit}) occur much more quickly than they are lost when the stimulus (i.e. heat) is removed. In ecological models predicting animal survival with climate change, it is important to consider not only how increases in temperature variability might impact the organism, but also how the narrowing of an animal's temperature range (e.g. during winter) might result in a loss of any previously acquired improvements in cardiac thermal performance. Moreover, it is important to consider our findings when designing laboratory-based experiments utilizing a constant submersion (or common garden) protocol at the start of an experiment, as we found that the group's mean cardiac thermal performance is lost at a slow rate and individual variability remains. These data lay the foundation for future work exploring the role of T_{crit} plasticity in organism survival during single and repeated bouts of heat stress, especially when T_{flat} might remain unchanged.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.E.M., G.N.S., M.W.D.; Methodology: N.E.M., G.N.S., M.W.D.; Validation: N.E.M., G.N.S., M.W.D.; Formal analysis: N.E.M., M.W.D.; Investigation: N.E.M.; Resources: M.W.D.; Data curation: N.E.M.; Writing - original draft: N.E.M.; Writing - review & editing: N.E.M., G.N.S., M.W.D.; Visualization: N.E.M.; Supervision: N.E.M., G.N.S., M.W.D.; Project administration: N.E.M.; Funding acquisition: M.W.D.

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Data availability

Data are available from Mendeley: http://dx.doi.org/10.17632/k3msdp67cn.1.

Supplementary information

Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.222893.supplemental

References

Andrade, M., Soares, A., Figueira, E. and Freitas, R. (2018). Biochemical changes in mussels submitted to different time periods of air exposure. *Environ. Sci. Pollut. Res.* **25**, 8903-8913. doi:10.1007/s11356-017-1123-7

Armstrong, E. J., Tanner, R. L. and Stillman, J. H. (2019). High heat tolerance is negatively correlated with heat tolerance plasticity in nudibranch mollusks. *Physiol. Biochem. Zool.* **92**, 430-444. doi:10.1086/704519

Bakhmet, I. N. (2017). Cardiac activity and oxygen consumption of blue mussels (*Mytilus edulis*) from the White Sea in relation to body mass, ambient temperature and food availability. *Polar Biol.* **40**, 1959-1964. doi:10.1007/s00300-017-2111-6

Bayne, B. L., Bayne, C. J., Carefoot, T. C. and Thompson, R. J. (1976). The physiological ecology of *Mytilus californianus* Conrad. *Oecologia* **22**, 229-250. doi:10.1007/BF00344794

Beggel, S., Cerwenka, A., Brandner, J. and Geist, J. (2015). Shell morphological versus genetic identification of quagga mussel (*Dreissena bugensis*) and zebra mussel (*Dreissena polymorpha*). *Aquat. Invasions* **10**, 93-99. doi:10.3391/ai.2015.10.1.09

Braby, C. E. and Somero, G. N. (2006). Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*). *J. Exp. Biol.* **209**, 2554-2566. doi:10.1242/jeb.02259

Burggren, W. (2018). Developmental phenotypic plasticity helps bridge stochastic weather events associated with climate change. *J. Exp. Biol.* **221**, jeb161984. doi:10.1242/jeb.161984

Burnett, N. P., Seabra, R., De Pirro, M., Wethey, D. S., Woodin, S. A., Helmuth, B., Zippay, M. L., Sarà, G., Monaco, C. and Lima, F. P. (2013). An improved noninvasive method for measuring heartbeat of intertidal animals. *Limnol. Oceanogr. Methods* **11**, 91-100. doi:10.4319/lom.2013.11.91

Collins, C. L., Burnett, N. P., Ramsey, M. J., Wagner, K. and Zippay, M. L. (2020). Physiological responses to heat stress in an invasive mussel *Mytilus galloprovincialis* depend on tidal habitat. *Mar. Environ. Res.* **154**, 104849. doi:10.1016/j.marenvres.2019.104849

Compton, T. J., Rijkenberg, M. J. A., Drent, J. and Piersma, T. (2007). Thermal tolerance ranges and climate variability: a comparison between bivalves from differing climates. *J. Exp. Mar. Biol. Ecol.* **352**, 200-211. doi:10.1016/j.jembe.2007.07.010

Corey, E., Linnansaari, T., Cunjak, R. A. and Currie, S. (2017). Physiological effects of environmentally relevant, multi-day thermal stress on wild juvenile Atlantic salmon (*Salmo salar*). *Conserv. Physiol.* **5**, cox014. doi:10.1093/conphys/cox014

Cossins, A. R., Friedlander, M. J. and Prosser, C. L. (1977). Correlations between behavioral temperature adaptations of goldfish and the viscosity and fatty acid

composition of their synaptic membranes. *J. Comp. Physiol.* **120**, 109-121. doi:10.1007/BF00619309

Denny, M. W. and Dowd, W. W. (2012). Biophysics, environmental stochasticity, and the evolution of thermal safety margins in intertidal limpets. *J. Exp. Biol.* **215**, 934-947. doi:10.1242/jeb.058958

Denny, M. W., Dowd, W. W., Bilir, L. and Mach, K. J. (2011). Spreading the risk: small-scale body temperature variation among intertidal organisms and its implications for species persistence. *J. Exp. Mar. Biol. Ecol.* **400**, 175-190. doi:10.1016/j.jembe.2011.02.006

Domnik, N. J., Polymeropoulos, E. T., Elliott, N. G., Frappell, P. B. and Fisher, J. T. (2016). Automated non-invasive video-microscopy of oyster spat heart rate during acute temperature change: impact of acclimation temperature. *Front. Physiol.* **7**, 236. doi:10.3389/fphys.2016.00236

Dong, Y.-W., Li, X.-X., Choi, F. M. P., Williams, G. A., Somero, G. N. and Helmuth, B. (2017). Untangling the roles of microclimate, behaviour and physiological polymorphism in governing vulnerability of intertidal snails to heat stress. *Proc. R. Soc. B* **284**, 20162367. doi:10.1098/rspb.2016.2367

Dowd, W. W. and Somero, G. N. (2013). Behavior and survival of *Mytilus* congeners following episodes of elevated body temperature in air and seawater. *J. Exp. Biol.* **216**, 502-514. doi:10.1242/jeb.076620

Dowd, W. W., King, F. A. and Denny, M. W. (2015). Thermal variation, thermal extremes and the physiological performance of individuals. **218**, 1956-1967. doi:10.1242/jeb.114926

Drake, M. J., Miller, N. A. and Todgham, A. E. (2017). The role of stochastic thermal environments in modulating the thermal physiology of an intertidal limpet, *Lottia digitalis*. *J. Exp. Biol.* **220**, 3072-3083. doi:10.1242/jeb.159020

Eisenhauer, J. G. (2003). Regression through the origin. *Teach. Stat.* **25**, 76-80. doi:10.1111/1467-9639.00136

Gaylord, B., Hill, T. M., Sanford, E., Lenz, E. A., Jacobs, L. A., Sato, K. N., Russell, A. D. and Hettinger, A. (2011). Functional impacts of ocean acidification in an ecologically critical foundation species. *J. Exp. Biol.* **214**, 2586-2594. doi:10.1242/jeb.055939

Gleason, L. U., Miller, L. P., Winnikoff, J. R., Somero, G. N., Yancey, P. H., Bratz, D. and Dowd, W. W. (2017). Thermal history and gape of individual *Mytilus californianus* correlate with oxidative damage and thermoprotective osmolytes. *J. Exp. Biol.* **220**, 4292-4304. doi:10.1242/jeb.168450

Gleason, L. U., Strand, E. L., Hizon, B. J. and Dowd, W. W. (2018). Plasticity of thermal tolerance and its relationship with growth rate in juvenile mussels (*Mytilus californianus*). *Proc. R. Soc. Biol.* **285**, 20172617. doi:10.1098/rspb.2017.2617

Gotcha, N., Terblanche, J. S. and Nyamukondiwa, C. (2018). Plasticity and cross-tolerance to heterogeneous environments: divergent stress responses co-evolved in an African fruit fly. *J. Evol. Biol.* **31**, 98-110. doi:10.1111/jeb.13201

Gracey, A. Y., Chaney, M. L., Boomhower, J. P., Tyburczy, W. R., Connor, K. and Somero, G. N. (2008). Rhythms of gene expression in a fluctuating intertidal environment. *Curr. Biol.* **18**, 1501-1507. doi:10.1016/j.cub.2008.08.049

Gunderson, A. R. and Stillman, J. H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proc. R. Soc. Biol.* **282**, 20150401. doi:10.1098/rspb.2015.0401

Gurr, S. J., Goleski, J., Lima, F. P., Seabra, R., Gobler, C. J. and Volkenborn, N. (2018). Cardiac responses of the bay scallop *Argopecten irradians* to diel-cycling hypoxia. *J. Exp. Mar. Bio. Ecol.* **500**, 18-29. doi:10.1016/j.jembe.2017.12.011

Han, G.-D., Zhang, S., Marshall, D. J., Ke, C.-H. and Dong, Y.-W. (2013). Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion. *J. Exp. Biol.* **216**, 3273-3282. doi:10.1242/jeb.084269

Healy, T. M. and Schulte, P. M. (2012). Factors affecting plasticity in whole-organism thermal tolerance in common killifish (*Fundulus heteroclitus*). *J. Comp. Physiol. B* **182**, 49-62. doi:10.1007/s00360-011-0595-x

Huey, R. and Bennett, A. (1990). Physiological adjustments to fluctuating thermal environments: an ecological and evolutionary perspective. In *Stress Proteins in Biology and Medicine* (ed. R. I. Morimoto, A. Tissières and C Georgopoulos), pp. 37-59: Cold Spring Harbor Laboratory Press.

IPCC (2014). Climate Change 2014: synthesis report. Contribution of Working Groups I, II and II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (ed. R. Pachauri and L. Meyer). IPCC, Geneva.

Jayasundara, N. and Somero, G. N. (2013). Physiological plasticity of cardiorespiratory function in a eurythermal marine teleost, the longjaw mudsucker, *Gillichthys mirabilis*. *J. Exp. Biol.* **216**, 2111-2121. doi:10.1242/jeb.083873

Jimenez, A. G., Alves, S., Dallmer, J., Njoo, E., Roa, S. and Dowd, W. W. (2016). Acclimation to elevated emersion temperature has no effect on susceptibility to acute, heat-induced lipid peroxidation in an intertidal mussel (*Mytilus californianus*). *Mar. Biol.* **163**, 55. doi:10.1007/s00227-016-2828-8

Jimenez, A. G., Jayawardene, S., Alves, S., Dallmer, J. and Dowd, W. W. (2015). Micro-scale environmental variation amplifies physiological variation among individual mussels. *Proc. R. Soc. Biol.* **282**, 20152273. doi:10.1098/rspb.2015.2273

Karunanithi, S. and Brown, I. R. (2015). Heat shock response and homeostatic plasticity. *Front. Cell. Neurosci.* **9**, 68. doi:10.3389/fncel.2015.00068

Kelly, M. (2019). Adaptation to climate change through genetic accommodation and assimilation of plastic phenotypes. *Phil. Trans. R. Soc. B* **374**, 20180176. doi:10.1098/rstb.2018.0176

Logan, C. A. and Somero, G. N. (2011). Effects of thermal acclimation on transcriptional responses to acute heat stress in the eurythermal fish *Gillichthys mirabilis* (Cooper). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R1373-R1383. doi:10.1152/ajpregu.00689.2010

Logan, C. A., Kost, L. E. and Somero, G. N. (2012). Latitudinal differences in *Mytilus californianus* thermal physiology. *Mar. Ecol. Prog. Ser.* **450**, 93-105. doi:10.3354/meps09491

Miller, L. P. and Dowd, W. W. (2017). Multimodal in situ datalogging quantifies inter-individual variation in thermal experience and persistent origin effects on gaping behavior among intertidal mussels (*Mytilus californianus*). *J. Exp. Biol.* **220**, 4305-4319. doi:10.1242/jeb.164020

Mislan, K. A. S., Helmuth, B. and Wethey, D. S. (2014). Geographical variation in climatic sensitivity of intertidal mussel zonation. *Glob. Ecol. Biogeogr.* **23**, 744-756. doi:10.1111/geb.12160

Morley, S. A., Nguyen, K. D., Peck, L. S., Lai, C.-H. and Tan, K. S. (2017). Can acclimation of thermal tolerance, in adults and across generations, act as a buffer against climate change in tropical marine ectotherms? *J. Therm. Biol.* **68**, 195-199. doi:10.1016/j.jtherbio.2016.09.007

Moyen, N. E., Somero, G. N. and Denny, M. W. (2019). Impact of heating rate on cardiac thermal tolerance in the California mussel, *Mytilus californianus*. *J. Exp. Biol.* **222**, jeb203166. doi:10.1242/jeb.203166

Newell, R. C. and Bayne, B. L. (1973). A review on temperature and metabolic acclimation in intertidal marine invertebrates. *Neth. J. Sea Res. Eur. Symp. Mar. Biol.* **7**, 421-433. doi:10.1016/0077-7579(73)90063-X

Palumbi, S. R. (1984). Tactics of acclimation: morphological changes of sponges in an unpredictable environment. *Science* **225**, 1478-1480. doi:10.1126/science.225.4669.1478

Pickens, P. E. (1965). Heart rate of mussels as a function of latitude, intertidal height, and acclimation temperature. *Physiol. Zool.* **38**, 390-405. doi:10.1086/physzool.38.4.30152416

Roberts, D. A., Hofmann, G. E. and Somero, G. N. (1997). Heat-shock protein expression in *Mytilus californianus*: acclimatization (seasonal and tidal-height comparisons) and acclimation effects. *Biol. Bull.* **192**, 309-320. doi:10.2307/1542724

Rohr, J. R., Civitello, D. J., Cohen, J. M., Roznik, E. A., Sinervo, B. and Dell, A. I. (2018). The complex drivers of thermal acclimation and breadth in ectotherms. *Ecol. Lett.* **21**, 1425-1439. doi:10.1111/ele.13107

Seebacher, F., White, C. R. and Franklin, C. E. (2015). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nat. Clim. Chang.* **5**, 61. doi:10.1038/nclimate2457

Senius, K. E. O. (1975). The thermal resistance and thermal resistance acclimation of ciliary activity in the *Mytilus* gills. *Comp. Biochem. Physiol.* **51A**, 957-961. doi:10.1016/0300-9629(75)90080-8

Somero, G. N. (2002). Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. *Integr. Comp. Biol.* **42**, 780-789. doi:10.1093/icb/42.4.780

Somero, G. N. (2020). The cellular stress response and temperature: function, regulation, and evolution. *J. Exp. Zool. A*. doi:10.1002/jez.2344

Somero, G. N., Lockwood, B. and Tomanek, L. (2017). *Biochemical Adaptation: Response to Environmental Challenges, from Life's Origins to the Anthropocene*: Sinauer Associates, Incorporated Publishers.

Sorby, K. L., Green, M. P., Dempster, T. D. and Jessop, T. S. (2018). Can physiological engineering/programming increase multi-generational thermal tolerance to extreme temperature events? *J. Exp. Biol.* **221**, jeb174672. doi:10.1242/jeb.174672

Stenseng, E., Braby, C. E. and Somero, G. N. (2005). Evolutionary and acclimation-induced variation in the thermal limits of heart function in congeneric marine snails (Genus *Tegula*): implications for vertical zonation. *Biol. Bull.* **208**, 138-144. doi:10.2307/3593122

Tagliarolo, M., Clavier, J., Chauvaud, L., Koken, M. and Grall, J. (2012). Metabolism in blue mussel: intertidal and subtidal beds compared. *Aquat. Biol.* **17**, 167-180. doi:10.3354/ab00464

Tanner, R. L. and Dowd, W. W. (2019). Inter-individual physiological variation in responses to environmental variation and environmental change: integrating across traits and time. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **238**, 110577. doi:10.1016/j.cbpa.2019.110577

Tepolt, C. K. and Somero, G. N. (2014). Master of all trades: thermal acclimation and adaptation of cardiac function in a broadly distributed marine invasive species, the European green crab, *Carcinus maenas*. *J. Exp. Biol.* **217**, 1129-1138. doi:10.1242/jeb.093849

Tide Predictions - NOAA Tides & Currents (2019). National Oceanographic and Atmospheric Administration, center for operational oceanographic products and services, Retrieved from www.tidesandcurrents.noaa.gov/noaatidepredictions.html. Station ID: 9413450.

Wang, T., Tanner, R. L., Armstrong, E. J., Lindberg, D. R. and Stillman, J. H. (2019). Plasticity of foot muscle and cardiac thermal limits in the limpet *Lottia*

limatula from locations with differing temperatures. *Aquat. Biol.* **28**, 113-125. doi:10.3354/ab00714

Widdows, J. (1973). Effect of temperature and food on the heartbeat, ventilation rate and oxygen uptake of *Mytilus edulis*. *Mar. Biol.* **20**, 269-276. doi:10.1007/BF00354270

Widdows, J. (1976). Physiological adaptation of *Mytilus edulis* to cyclic temperatures. *J. Comp. Physiol. B* **105**, 115-128. doi:10.1007/BF00691115

Willett, C. S. (2010). Potential fitness trade-offs for thermal tolerance in the intertidal copepod *Tigriopus californicus*. *Evolution* **64**, 2521-2534. doi:10.1111/j.1558-5646.2010.01008.x

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

Wu, F., Lu, W., Shang, Y., Kong, H., Li, L., Sui, Y., Hu, M. and Wang, Y. (2016). Combined effects of seawater acidification and high temperature on hemocyte parameters in the thick shell mussel *Mytilus coruscus*. *Fish Shellfish Immunol.* **56**, 554-562. doi:10.1016/j.fsi.2016.08.012

Yao, C. L. and Somero, G. N. (2013). Thermal stress and cellular signaling processes in hemocytes of native (*Mytilus californianus*) and invasive (*M. galloprovincialis*) mussels: cell cycle regulation and DNA repair. *Comp. Biochem. Physiol. A* **165**, 159-168. doi:10.1016/j.cbpa.2013.02.024

Zhang, S., Han, G.-D. and Dong, Y.-W. (2014). Temporal patterns of cardiac performance and genes encoding heat shock proteins and metabolic sensors of an intertidal limpet *Cellana toreuma* during sublethal heat stress. *J. Therm. Biol.* **41**, 31-37. doi:10.1016/j.jtherbio.2014.02.003

Zippay, M. L. and Helmuth, B. (2012). Effects of temperature change on mussel, *Mytilus*. *Integr. Zool.* **7**, 312-327. doi:10.1111/j.1749-4877.2012.00310.x