RESEARCH ARTICLE

Impact of heating rate on cardiac thermal tolerance in the California mussel, *Mytilus californianus*

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

ABSTRACT

Intertidal communities of wave-swept rocky shores have served as a powerful model system for experiments in ecology, and mussels (the dominant competitor for space in the mid-intertidal zone) play a central role in determining community structure in this physically stressful habitat. Consequently, the ability to account for mussels' physiological responses to thermal stress affects ecologists' capacity to predict the impacts of a warming climate on this ecosystem. Here, we examined the effect of heating rate on cardiac thermal tolerance in the ribbed mussel, *Mytilus californianus*, comparing populations from high and low sites in the intertidal zone where emersion duration leads to different mean daily heating rates. Two temperature-related cardiac variables were examined: (1) the critical temperature (T_{crit}) at which heart rate (HR) precipitously declines, and (2) flatline temperature (FLT) where HR reaches zero. Mussels were heated in air at slow, moderate and fast rates, and HR was measured via an infrared sensor affixed to the shell. Faster heating rates significantly increased T_{crit} in high- but not low-zone mussels, and T_{crit} was higher in high- versus low-zone mussels, especially at the fastest heating rate. By contrast, FLT did not differ between zones, and was minimally affected by heating rate. As heating rate significantly impacted high- but not low-zone mussels' cardiac thermal tolerance, realistic zone-specific heating rates must be used in laboratory tests if those tests are to provide accurate information for ecological models attempting to predict the effects of increasing temperature on intertidal communities.

KEY WORDS: Critical temperature, Flatline temperature, Heart rate, Heat stress, Intertidal zone, Ramping rate

INTRODUCTION

Intertidal communities of wave-swept rocky shores have long served as a model system for experimental ecology, in large part because of the physical extremes the habitat exposes organisms to on a daily basis. When the tide is in, organisms are subjected to the hydrodynamic forces of breaking waves (Carrington et al., 2009; Denny, 1987, 1995; Denny et al., 2003; Paine and Levin, 1981); when the tide is out, they are exposed to the rigors of the terrestrial environment (fluctuations in temperature, desiccation and oxygen stress). On many shores, mussels are the competitive dominant for space in the mid-intertidal zone, thereby controlling much of the community ecology by (on the one hand) excluding other benthic species and (on the other hand) providing 'air conditioned homes'

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for smaller organisms and food for predators (Gaylord et al., 2011). Because mussels are sessile, which limits their ability to behaviorally thermoregulate, they can suffer mass mortalities during heat waves (Harley, 2008; Tsuchiya, 1983), and the threat of such disturbances becomes greater as climate change increases temperature variability, leading to more extreme hot days. Although mussels' physiological responses to heat stress have been previously studied, there are important gaps in our knowledge. In particular, the rate at which a mussel's body temperature increases during low tide varies with a plethora of abiotic factors (e.g. latitude, season, time of low tide, orientation of the substratum), along with their height on the shore and position within the mussel bed. However, it is unknown whether a mussel's ability to tolerate high temperatures depends on the rate at which their body temperature increases and on the acclimatization state of the individual.

A potentially informative approach to examining these issues involves studying the effects of temperature on cardiac function, which can yield data on both sublethal and lethal thermal stress. Laboratory tests of mussels' responses to heat stress have, in fact, focused strongly on cardiac physiology by measuring the effects of acute changes in temperature on heart rate (HR) (Braby and Somero, 2006; Logan et al., 2012). In a typical test, mussels are heated at a certain rate (°C h^{-1}) until their HR begins to precipitously decline; this is the heart's critical temperature (T_{crit}). T_{crit} is not acutely lethal, but does reflect cumulative damage to the cell during the heating process. The types of underlying cellular damage caused by temperatures near or above T_{crit} remain to be fully elucidated. However, in mussels, the expression of stress-related proteins, such as heat-shock proteins, is upregulated at temperatures several degrees below T_{crit} (Gracey et al., 2008; Roberts et al., 1997); and in limpets, T_{crit} is the body temperature at which these protective genes and proteins are maximally upregulated (Han et al., 2013; Zhang et al., 2014). Thus, these data suggest that cardiac dysfunction may be a consequence of a broad suite of underlying thermal effects on the cell, e.g. heat-driven protein unfolding. Consequently, T_{crit} can provide an index of underlying cumulative thermal damage to the cell. Continuing to increase body temperature above T_{crit} will eventually lead to a cessation of HR; this is the flatline temperature (FLT), which typically is lethal to the animal; through a pilot study, we found that mussels died within 1–4 days of achieving their FLT (N.E.M., G.N.S. and M.W.D., unpublished data).

Although the influence of heating rate on cardiac function (or other physiological traits) has received little attention, field studies have documented wide variations of whole-body heating rates in mussels. For example, Denny et al. (2011) found that heating rates of mussel mimics placed in wave-exposed and wave-protected mussel beds at Hopkins Marine Station in Monterey Bay, CA, USA, varied from 1 to 8°C h^{-1}. More recently, Miller and Dowd (2017) measured live mussel (*Mytilus californianus*) body temperature in beds a few meters away from those used in Denny et al.'s (2011) study, and found that heating rate depended

on a mussel's height on shore. High-zone mussels experienced faster mean daily heating rates (6.79°C h^{-1}) than low-zone mussels (1.32°C h^{-1}). Moreover, Miller and Dowd (2017) reported maximum heating rates as fast as 20 and 12°C h^{-1} in high- and low-zone mussel beds, respectively. Here, we built on these data to investigate potential physiological consequences; namely, differences in cardiac thermal responses that might accompany different rates of heating.

Despite these field data showing that mussels experience a wide variation in heating rate, and the fact that heating rate affects thermal tolerance in other taxa (Allen et al., 2012, 2016), we are unaware of any studies directly exploring whether or how heating rate affects thermal tolerance in mussels. Although heating rate was held constant within each previous study of *Mytilus* congeners, heating rate has varied from study to study, ranging from 1.5 to 13°C h^{-1} (Roberts et al., 1997; Braby and Somero, 2006; Logan et al., 2012; Dowd and Somero, 2013). This variation complicates comparisons across *Mytilus* species, and also confounds comparisons within a given *Mytilus* species living at different latitudes and habitats. Therefore, in this study we sought to determine whether heating rate affects cardiac thermal tolerance in the often-studied and ecologically important mussel *M. californianus.* We compared mussels living at high and low intertidal heights to determine whether heating rate differentially affected cardiac thermal tolerance in animals acclimatized to different exposure conditions. We hypothesized that, because high-zone mussels have a higher cardiac thermal tolerance and experience faster mean daily heating rates than low-zone mussels (Compton et al., 2018; Gleason et al., 2018; Miller and Dowd, 2017), high-zone mussels would better tolerate faster heating rates.

MATERIALS AND METHODS
Animal collection and preparation
Specimens of *M. californianus* Conrad 1837 (*n*=70) were collected from high and low zones of a moderately wave-exposed shore at Hopkins Marine Station in Pacific Grove, CA, USA (36.6216°N, 121.9042°W) during the morning low tide of the day testing occurred. To minimize potential seasonal variation in cardiac function, all tests were carried out during a single month: mid-August to mid-September 2018. Intertidal heights were measured by standard surveying methods (GTS-211D Total Station, Topcon, Livermore, CA, USA). Low- and high-zone sites were vertically separated by 0.56 m (0.43 and 0.99 m above mean lower low water, respectively). To minimize other factors that might affect thermal tolerance, only adult mussels with shell lengths within a 20 mm range (i.e. 53–73 mm) were collected.

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. Water was wiped from the external surface of the mussels to avoid evaporative cooling, and each individual's baseline body mass was measured using a digital scale (accurate to 0.0001 g). Next, in order to record individual mussel body temperature, a thermocouple (Type K, 26-gauge wire, Omega Engineering, Santa Ana, CA, USA) was carefully inserted into the drilled hole and secured with cyanoacrylate glue. To record HR during heating, an infrared sensor (model IR-AMP03-EX, Newshift, Leira, Portugal) was attached to each mussel's shell on its dorsal side, directly over the pericardial sac 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 (AD Instruments, LabChart 6 software, Colorado Springs,

CO, USA). HR was sampled at 4 Hz with a low-pass filter of 10 Hz (Burnett et al., 2013).

Heating rate experiments
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 specific rates using a temperature control box (Newport Electronics, iSeries Temperature Controller, 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, baseline HR, mussel body temperature and air temperature (via thermocouple) were recorded. Six mussels from the same zone were tested during each trial.

After baseline measurements were taken, mussels were subjected to increasing air temperature at slow, moderate and fast heating rates that were selected to mimic the typical range of mussel body heating rates experienced throughout the year at Hopkins Marine Station at low and high intertidal heights (Denny et al., 2011; Miller and Dowd, 2017). The planned mussel heating rates were 3.0, 5.5 and 8.0°C h^{-1} for the slow, moderate and fast heating rates, respectively. As mussels were emersed during heating, the chamber (air) heating rates needed to be slightly faster than the planned mussel body heating rates because of a temporal lag between air and mussel temperatures, especially at the fastest heating rates (see Discussion for more details). Individual overall heating rates were calculated for each trial. To standardize these calculations, a mussel's baseline body temperature was subtracted 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. This was at the end of hours 6, 3 and 2 for the slow, moderate and fast heating rates, respectively.

HR, body temperature and air temperature were recorded every 15 min throughout heating, as well as when T_{crit} (Braby and Somero, 2006) and FLT (Stenseng et al., 2005) occurred. FLT was determined by pinpointing the mussel's body temperature at which their last heartbeat occurred (defined by a HR of zero for at least 3 min). Because of the large variability in HR signature, heartbeats were manually counted for 30 s at each recording time and then multiplied by 2 to yield HR in beats min^{-1} (see Fig. 1 for sample HR signatures). Baseline HR, maximum HR, the total change in HR (maximum minus minimum HR) and the Q_{10} of HR were used to evaluate the mussels' physiological responses to heat stress.

Once mussels reached their FLT, they were removed from the chamber and the thermocouple and infrared sensor were detached. Mussels were patted dry and a post-trial body mass was recorded. Absolute water loss (in grams) throughout heating was calculated by subtracting each individual's post-trial body mass from their pre-trial body mass. Lastly, morphometric measurements of shell height (the longest distance from the dorsal to ventral surface), shell width (the widest part of the mussel across both closed valves) and shell length (the longest distance from anterior to posterior) were made using digital calipers (Beggel et al., 2015). To determine the mussels' reproductive status, within 24 h of completion of each trial, the gonads and somatic tissues were dissected and placed into separate aluminium weigh boats; samples
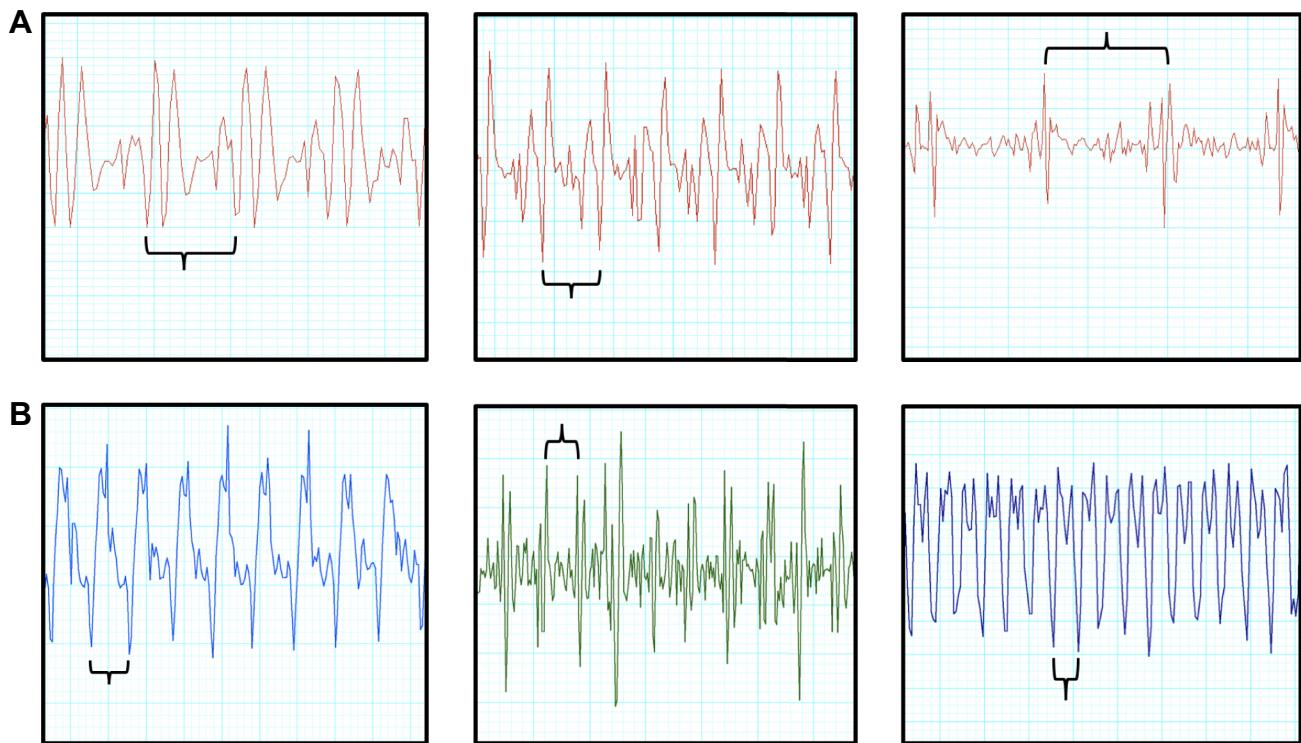
Fig. 1. Sample heart rate (HR) recordings for *Mytilus californianus*. Note: the time scale of each image is slightly different for best visualization of HR; furthermore, the graph units (i.e. one square) do not correspond to the same amount of time as LabChart adjusts the actual time between units based on the scaling. (A) One low-zone mussel's HR at three different time points during a moderate heating rate trial, reflecting different HR and body temperatures at 2 h (left: HR=16 beats min^{-1}, body temperature=30.4°C), 3 h (middle: HR=11 beats min^{-1}, body temperature=34.5°C) and 3.75 h (right: HR=4 beats min^{-1}, body temperature=37.5°C) into the trial. (B) HR of three different low-zone mussels at the same time point in one of the slow heating rate trials (1 h into the trial): blue, HR=11 beats min^{-1}, body temperature=23.7°C; green, HR=9 beats min^{-1}, body temperature=23.9°C; purple, HR=22 beats min^{-1}, body temperature=24.3°C. The black bracket indicates one heartbeat for each specific snapshot. HR was measured based on previous work in mussels as described by Burnett et al. (2013), where one heartbeat is denoted by the time between two large peaks. It is expected that often there will be one to two smaller peaks following the largest peak, and together these two to three peaks comprise one heartbeat (Burnett et al., 2013). Note the large variability within and between mussels' HR signatures at each time point.

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 mass plus gonadal mass (Logan et al., 2012).

Statistical analyses

R 3.5.2 (https://cran.r-project.org/) and R studio (https://www.rstudio.com/) were used for all statistical analyses and models. Given the large variability in heating rate (as a result of temporal lag between air and body temperature; see Discussion), we used two methods to separate the data for analyses. In method 1, we included all samples in the dataset while using regression models to represent the data as a continuous variable (e.g. Fig. 3). For this method, 33 low-zone and 37 high-zone mussels (*n*=70) were included. In method 2, we separated the data into bins based on heating rate (i.e. slow, moderate and fast) to determine whether there were statistical differences among rates. However, to ensure that the actual individual heating rates did not overlap between bins, we excluded individuals from each heating rate that did not fall within a 2°C range of each of the planned mussel heating rates (i.e. 3.0, 5.5 and 8.0°C h^{-1}). For the slow, moderate and fast heating rates, individuals with heating rates within the following ranges were included: 1.6–3.6, 4.0–6.0 and 6.5–8.5°C h^{-1}, respectively. For these analyses, four high-zone mussels were excluded that fell outside of these 2°C ranges, resulting in 33 mussels in each of the high and low zones (*n*=66).

For method 1, model fits (i.e. linear versus quadratic versus cubic) were compared by calculating the Akaike information criterion (AIC) for each model, where the lowest AIC determined the best model fit (Angilletta, 2006). For method 2, independent *t*-tests were used to determine whether high- versus low-zone mussels were statistically different for any variables within a given heating rate (e.g. baseline HR in high- versus low-zone mussels for the slow heating rate), and α<0.05 defined significance. When evaluating differences across heating rates for each of the high- and low-zone mussels, a one-way ANOVA (1 zone×3 heating rates) was used. For statistically significant *F*-scores, independent *t*-tests with a Bonferroni correction (*P*<0.017) were used to determine whether significant differences existed between heating rates.

RESULTS

Table 1 presents some key morphometric data for specimens from different zones and exposed to different heating rates. As planned, within each zone, mussel heating rates were significantly different from each other across heating rates (e.g. slow versus moderate versus fast heating rates in high-zone mussels; all *P*<0.05), but for a given heating rate, no differences existed between zones (all *P*>0.05). High-zone mussels had a significantly higher pre-trial body mass (shell included) than low-zone mussels by ~6 g (*P*<0.05); this difference in body mass was not a result of differences in total dry-tissue mass (gonadal plus somatic body mass; overall mean±s.d. for both zones: 1.18±0.27 g; *P*>0.05),

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Table 1. Morphometric and individual heating rate data for low- and high-zone mussels

	Slow		Moderate		Fast		All heating rates	
	Low	High	Low	High	Low	High	Low	High
Sample size	12	11	10	11	11	11	33	33
Overall heating rate (°C h^{-1})	2.9±0.3‡	2.8±0.2‡	4.7±0.6‡	4.9±0.4‡	7.5±0.6‡	7.2±0.6‡	5.0±2.0	5.0±1.9
Body mass (g)	20.29±4.20	26.89±6.48*	21.04±5.83	25.95±4.89*	20.99±4.29	26.93±4.14*	20.75±4.64	26.59±5.11*
Absolute water loss (g)	−3.98±2.03	−4.22±1.78	−3.70±2.48	−3.34±1.55	−3.28±1.85	−4.05±2.09	−3.66±2.07	−3.89±1.81
Gonad mass (% total dry body mass)	16.19±2.51	14.51±3.12*	13.95±1.76	15.38±2.96*	17.39±6.01	15.37±2.91*	15.91±4.04	15.09±2.93

Data are means±s.d. *Significant difference between zones for that specific heating rate ($P<0.05$); ‡significant difference between all heating rates within that specific zone ($P<0.01$).

suggesting that the internal organs in high- and low-zone mussels were likely of similar sizes. Shell length and height were similar between zones (overall means±s.d. for both zones: 61.55±4.14 mm and 26.98±2.09 mm, respectively; $P>0.05$), but high-zone shells were ~3 mm wider than low-zone shells (overall mean±s.d. for high- versus low-zone mussels: 26.36±2.33 versus 23.81±1.88 mm; $P<0.05$). Water loss also did not differ across heating rates or between zones (all $P>0.05$). Thus, the body mass disparity between zones was likely due to thicker shells in high- versus low-zone individuals; however, more research is needed to confirm this hypothesis. Body mass played a negligible role in how quickly mussels heated up at the slow and moderate heating rates; however, at the fast heating rate, heavier mussels tended to heat up more slowly (Pearson r correlation coefficients for each heating rate: slow=−0.08, $P=0.70$; moderate=−0.17, $P=0.45$; fast=−0.37, $P=0.06$).

HR variables

Five HR variables were examined to lay a foundation for the comparisons of heating rate effects: baseline HR, total change in HR during heating (i.e. maximum minus minimum HR), maximum HR, the slope of HR as a function of body temperature and the Q_{10} of HR. When combining data from all trials, total change in HR and maximum HR were significantly higher in high- versus low-zone mussels (mean±s.d. for high versus low zone: total change in HR=13.1±4.2 versus 11.0±3.9 beats min^{-1}, maximum HR=24.6±4.0 versus 22.4±4.6 beats min^{-1}; both $P<0.05$), but this statistical difference was largely the result of higher maximum HR in high-zone mussels at the fastest heating rate. By contrast, baseline HR

was similar between zones (baseline HR in high versus low zone: 12.4±4.8 versus 12.3±5.2 beats min^{-1}, respectively; $P=0.79$) and across heating rates for the high- and low-zone mussels (all $P>0.05$). These data indicate that all mussels started the trials in a similar cardiovascular state (in terms of baseline HR), and that the recent tidal conditions or time of day the mussels were collected did not independently affect T_{crit} results. Total change in HR was also not significantly different across heating rates within each zone (high zone: $P=0.60$; low zone: $P=0.33$) or between zones for any given heating rate (all $P>0.05$).

Maximum HR was not significantly different across heating rates for low-zone mussels ($P=0.67$). However, maximum HR in high-zone mussels at the fast heating rate (27.0±3.0 beats min^{-1}) was significantly higher than that during heating at the slow (22.3±3.7 beats min^{-1}, $P<0.01$) but not the moderate (24.7±4.0 beats min^{-1}) rate ($P>0.05$). Maximum HR was only higher in the high- versus low-zone mussels at the fastest heating rate (low zone HR=22.4±3.7 beats min^{-1}, $P<0.05$). There were no other significant differences in maximum HR between zones at the slow or moderate heating rates (all $P>0.05$).

Fig. 2 depicts the averaged body temperature versus HR data, classified by heating rate and intertidal zone. There were no differences across heating rates or between zones in the slope of the HR versus body temperature response up until T_{crit} (overall mean±s.d. for high versus low zone: 0.65±0.32 versus 0.59±0.40 beats min^{-1} °C^{-1}; all $P>0.05$). The HR slope was also unrelated to T_{crit} or FLT in both high- and low-zone mussels ($P>0.05$). These data indicate that mussel HR likely responds to the animal's absolute body temperature, and not necessarily to the rate of change in body
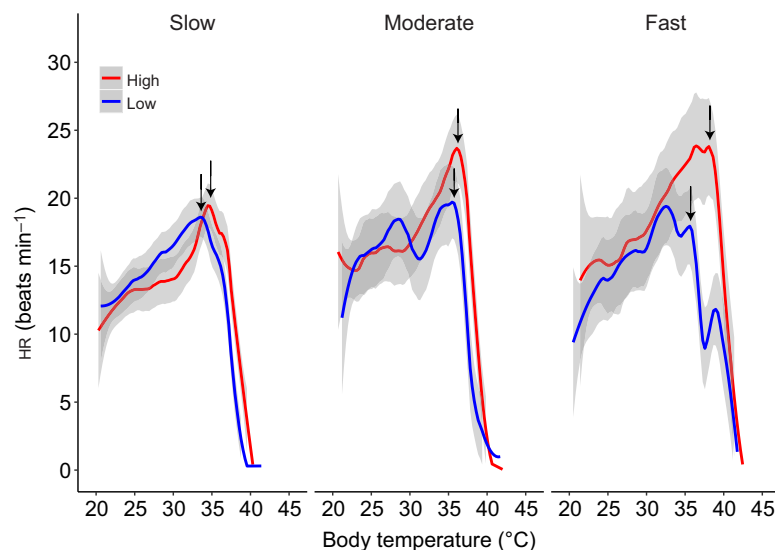

Fig. 2. Averaged time series data for mussel heart rate versus body temperature. Data were sampled every 15 min throughout the trials, and separated by zone (high/low). Gray shading indicates 95% confidence intervals. The black arrows indicate estimates of the mean T_{crit} for that heating rate and zone (see Results for actual values); the critical temperature (T_{crit}) and flatline temperature (FLT) were not determined from this plot, but instead each individual's T_{crit} and FLT were analyzed individually, and then the group mean was taken. Thus, these plots are simply pictorial representations of the HR data during heating.
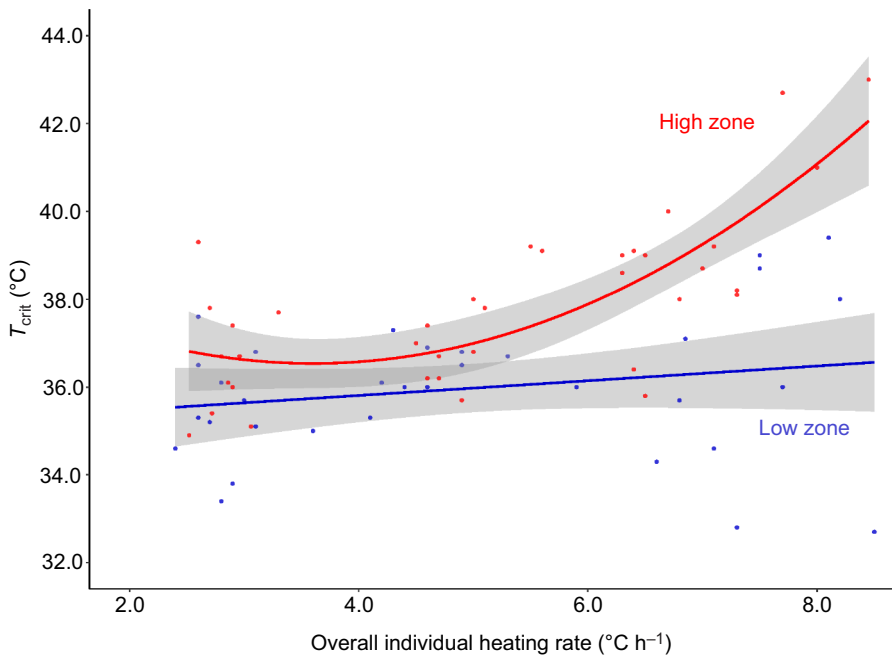
Fig. 3. T_{crit} versus individually measured heating (ramping) rate. Data are separated by intertidal zone. Each point represents a separate mussel [n=70 total: n=37 high zone (red), n=33 low zone (blue)]; gray shading around plots represents 95% confidence intervals. Heating rate significantly affected T_{crit} in high-zone (r^2=0.58, P<0.01) but not low-zone mussels (r^2=0.05, P=0.43).

temperature. Moreover, HR sensitivity to temperature did not appear to differ between zones. The Q_{10} of HR was also calculated for each individual and, as would be expected from the lack of difference in the HR slopes, there were no significant differences in the Q_{10} of HR between zones for any heating rate, or across heating rates for either zone (high-zone means±s.d. for slow, moderate and fast heating rates: 1.42±0.50, 1.44±0.63 and 1.53±0.49; low zone means±s.d. for slow, moderate and fast heating rates: 1.51±0.36, 1.49±0.68 and 1.72±0.52; all P>0.05).

Heating rate affects T_{crit} and FLT

Fig. 3 shows the effect of heating rate on T_{crit} in high- and low-zone mussels. For high-zone mussels, individual heating rate accounted for 58% of the variance in T_{crit}; the faster an individual's body temperature increased, the higher the T_{crit}. A second-order polynomial best modeled the data compared with linear and cubic fits: T_{crit}=39.6−1.7x+0.2x^2, where T_{crit} is expressed in °C and x is

the individual mussel's heating rate in °C h^{-1} (F=23.31, P<0.001). A forward regression was used to evaluate whether adding other physiologically related variables (e.g. baseline HR, shell height or width) to the model better explained the variance in T_{crit}. However, adding these variables did not significantly improve the model fit (i.e. AIC values were higher than those with heating rate alone), indicating that in high-zone mussels, an individual's heating rate is the best available predictor of T_{crit}. By contrast, heating rate did not discernibly affect T_{crit} of low-zone mussels (r^2=0.05, P=0.43). The best predictor of low-zone mussels' T_{crit} was the total change in HR (r^2=0.12, P=0.04). However, as total change in HR is only a weak to moderate predictor of T_{crit}, and it is also not a feasible measure to obtain before heat stress occurs, it is probably not a suitable variable for modeling low-zone mussels' thermal responses in an ecological context.

High-zone mussels had a significantly higher T_{crit} than low-zone mussels at the slow and moderate heating rates (by ~1°C) and at the fast heating rate (by ~3°C, all P<0.05; see Fig. 3 and Fig. 5A).
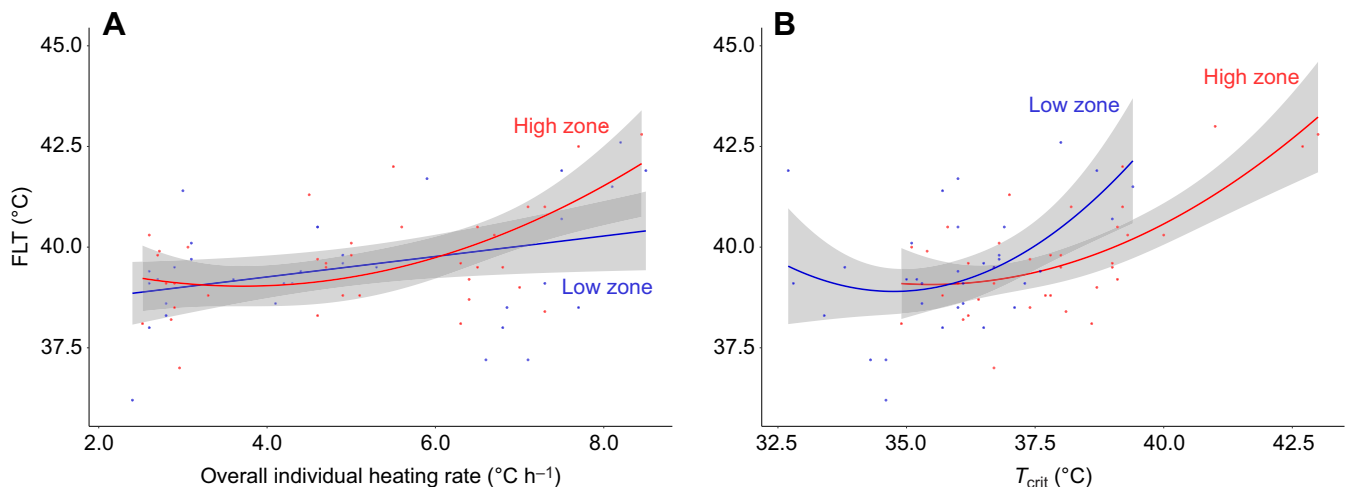

Fig. 4. Relationship of FLT with individual heating rate and T_{crit}. Each point represents a separate mussel [n=70 total: n=37 high zone (red), n=33 low zone (blue)]; gray shading around plots represents 95% confidence intervals. Heating rate significantly affected FLT in high-zone (r^2=0.33) and low-zone mussels (r^2=0.12; A). However, T_{crit} was a better predictor of FLT for both high-zone (r^2=0.49) and low-zone mussels (r^2=0.29; all P<0.01; B).

There were no differences across heating rates in T_{crit} for the low-zone mussels (means±s.d. for slow, moderate and fast heating rates: 35.4±1.2, 36.4±0.6 and 36.2±2.4°C, respectively; $P>0.05$). In high-zone mussels, T_{crit} was significantly higher in the fast versus the slow and moderate heating rates (all $P<0.01$), but was not different ($P>0.05$) between the slow and moderate heating rates (means±s.d. for slow, moderate and fast heating rates: 36.6±1.3, 37.3±1.2 and 39.4±2.1°C, respectively). Despite differences in T_{crit} between zones, because of the large variability in T_{crit} and individual heating rates for each trial, the amount of time to reach T_{crit} was statistically indistinguishable between zones for any given heating rate ($P>0.05$); however, as expected, the total time to reach T_{crit} significantly decreased ($P<0.01$) as heating rate increased (overall means±s.d. for slow, moderate and fast heating rates: 4.96±0.79, 3.12±0.28 and 2.22±0.42 h, respectively). In summary, high-zone mussels' cardiac thermal tolerance is significantly dependent on heating rate, while low-zone mussels' cardiac thermal tolerance is unaffected by heating rate. T_{crit} of high-zone mussels was higher than that of low-zone mussels at all heating rates, but the disparity between zones was largest at the fastest heating rate (see Fig 5A).

Heating rate was weakly related to FLT in low-zone mussels ($r^2=0.12$, $P<0.05$) and moderately related to FLT in high-zone mussels ($r^2=0.33$, $P<0.01$). Based on AIC values, a linear fit best modeled low-zone data, while a quadratic fit best modeled high-zone data (see Fig. 4A). In both high- and low-zone mussels, T_{crit} was a stronger predictor of FLT than was heating rate. A curvilinear relationship best explained the relationships between T_{crit} and FLT (low zone: $r^2=0.29$; high zone: $r^2=0.49$; both $P<0.01$; Fig. 4B). FLT was not statistically different between zones for any heating rate (all $P>0.05$; see Fig. 5B). In low-zone mussels, FLT was not significantly different across heating rates (means±s.d. for slow, moderate and fast heating rates: 39.1±1.3, 39.8±0.9 and 39.7±2.0°C, respectively; $P=0.31$). In high-zone mussels, FLT was only significantly higher ($P<0.01$) at the fast versus slow heating rate (means±s.d. for slow, moderate and fast heating rates: 39.0±1.0, 39.9±1.1 and 40.7±1.6°C, respectively). For each heating rate, high- and low-zone mussels reached FLT in the same amount of time

($P>0.05$); and, as expected, as heating rate increased, the total time to reach FLT significantly decreased (overall means±s.d. for slow, moderate and fast heating rates: 6.35±0.47, 3.98±0.37 and 2.86±0.50 h, respectively; $P<0.01$). Independent of intertidal height, T_{crit} was negatively correlated with (1) the absolute temperature difference ($r=-0.72$) between FLT and T_{crit} (both $P<0.001$; see Fig. 6), and (2) the time ($r=-0.58$) between FLT and T_{crit}. In other words, mussels with a lower T_{crit} took more time to reach their FLT, and, consequently, there was a larger temperature difference between their T_{crit} and FLT; those with higher T_{crit} had a smaller amount of time and difference in temperature between their T_{crit} and FLT.

DISCUSSION

Each day, as the ocean recedes at low tide, a mussel's body temperature changes at a rate that depends on a variety of abiotic factors: the time of day that the low tide occurs, air temperature, wind speed, solar irradiance, the height on shore where the mussel occurs and its orientation to the sun. We sought to determine whether heating rate affected cardiac thermal tolerance in California mussels and, if this was the case, whether this relationship differed between high- and low-zone mussel beds. Three main findings emerged: (1) faster heating rates increased T_{crit} of high- but not low-zone mussels, (2) T_{crit} of high-zone mussels was higher than that of low-zone mussels at all heating rates, with differences being largest at the fastest heating rate, and (3) FLT was minimally affected by heating rate and did not differ between zones.

T_{crit}

Heating rate greatly affected T_{crit} of high-zone mussels, accounting for 58% of the variance in this trait, while T_{crit} of low-zone mussels was unaffected by heating rate. This difference between sites could be related to the different mean daily heating rates experienced at each zone, as well as the absolute temperatures reached during emersion; both thermal variables might lead to different acclimatization states. Low-zone mussels at Hopkins Marine Station typically experience mean daily maximum heating rates of
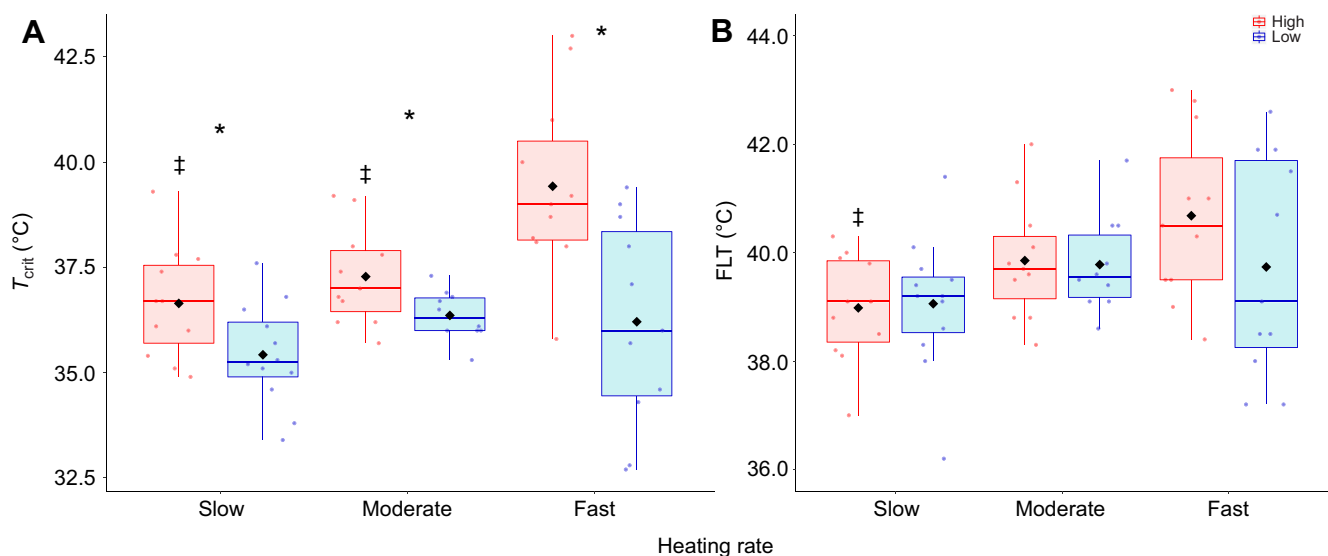

Fig. 5. T_{crit} and FLT based on heating rates in high-zone versus low-zone mussels. Boxplots outline the 25th and 75th percentiles, and the midline indicates the median. Black diamonds inside each boxplot indicate the mean for each heating rate and zone. Each data point represents an individual mussel [$n=33$ high-zone mussels (red), $n=33$ low-zone mussels (blue)]. T_{crit} was significantly higher for high-zone mussels than for low-zones mussels at all heating rates (*$P<0.05$; A). FLT did not differ between zones for any heating rate (B). ‡Significant difference from high-zone mussel T_{crit} or FLT at the fast heating rate ($P<0.05$).
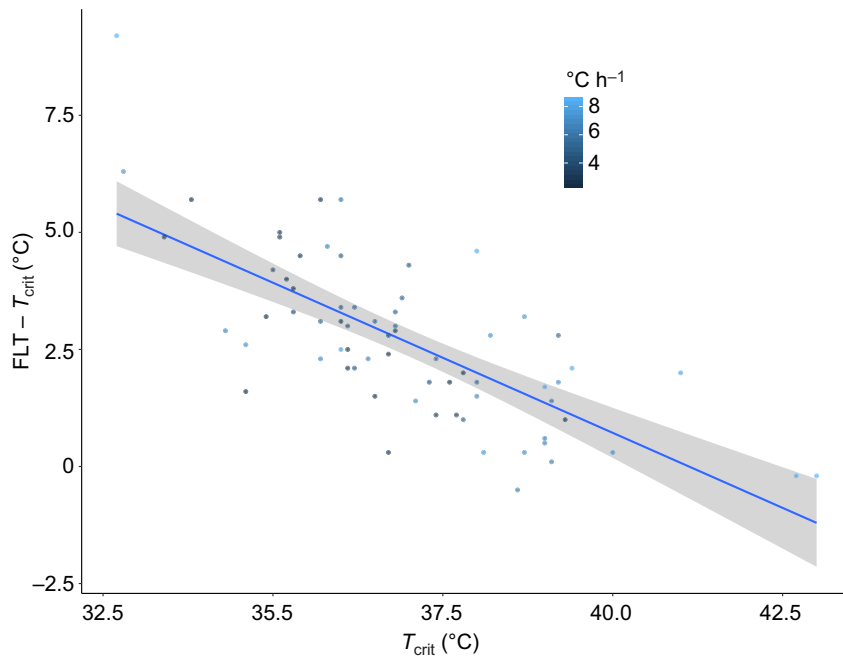
Fig. 6. Absolute temperature difference between FLT and T_{crit} versus T_{crit}. Each point represents an individual mussel (*n*=70, data include both high- and low-zone specimens); points are colored on a scale based on each individual's internal heating rate (°C h^{-1}) during the trial. Gray shading around the blue line represents the 95% confidence intervals. T_{crit} negatively correlated with the absolute temperature difference between FLT and T_{crit} (*r*=−0.72, *P*<0.001), and this relationship appeared to be independent of internal heating rate or intertidal height.

1.3°C h^{-1} (Miller and Dowd, 2017), which is slower than our slowest experimental heating rate (2.9°C h^{-1}). In contrast, our fastest heating rate (7.2°C h^{-1}) was slightly faster than the mean daily maximum heating rates that high-zone mussels reportedly experience at our site (6.8°C h^{-1}; Miller and Dowd, 2017). Although the high- and low-zone mussels in our study were tested across the same absolute range of heating rates (i.e. 2.9–7.2°C h^{-1}), the midpoint of this range differs with regard to the mean daily

heating rates an individual experiences in the field. In our study, the range in heating rates we utilized included the mean daily heating rate experienced by high-zone mussels, but was ~1.5°C h^{-1} faster than the mean daily heating rate experienced by low-zone animals. As such, perhaps T_{crit} of low-zone mussels would be affected by heating rate (similar to that of high-zone mussels) if we had utilized heating rates spanning their mean daily heating rate. However, the slow rates required to achieve this range would have little meaning
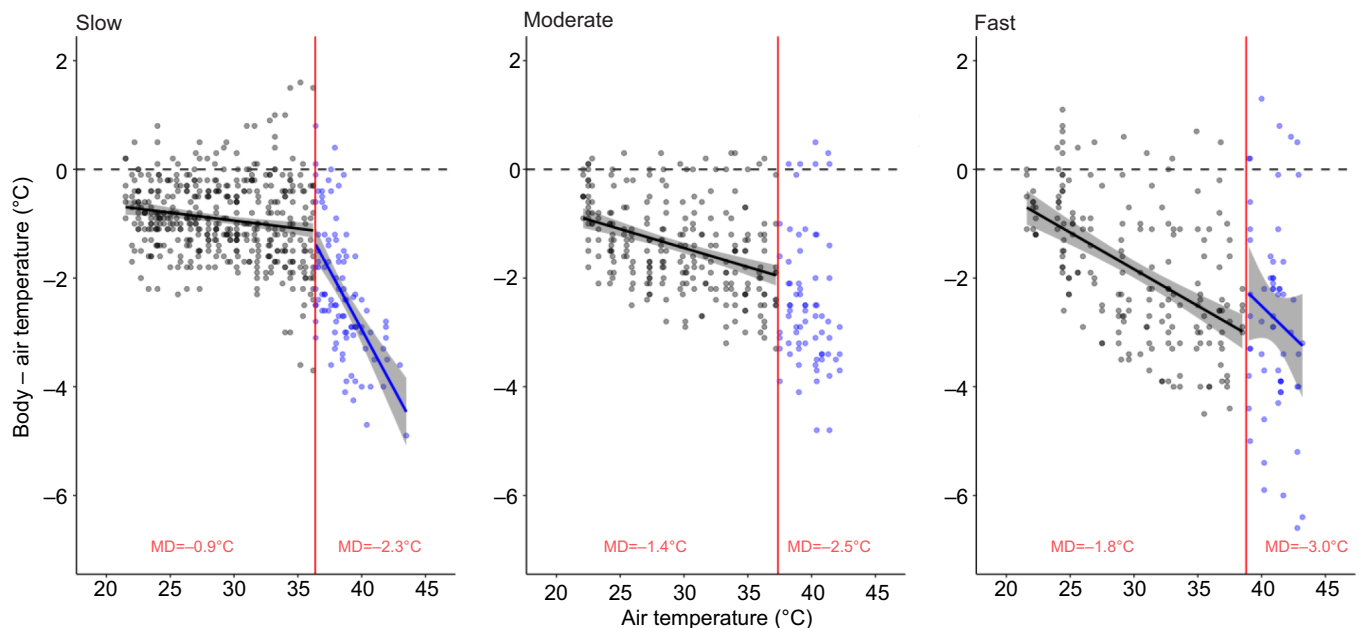

Fig. 7. Modified Bland–Altman plot (residuals versus actual air temperature) representing the temporal lag between body versus air temperature for each heating rate. Each individual data point represents an individual's temperature difference for a given air temperature; low- and high-zone mussels are combined for each heating rate (*n*=66 total). Gray shading around the lines represents the 95% confidence intervals. The horizontal dashed line at zero represents the point of no temporal lag. The red line in each plot demarcates the air temperature at which the mean T_{crit} occurred for that specific heating rate. Lines were fitted to the data points prior to and after the T_{crit} for each heating rate. MD is the mean difference in body versus air temperature for that group of data. For all heating rates, the mean difference increased by ~0.5–1.2°C after reaching T_{crit} (red line). It is also important to note that the temporal lag (i.e. air versus body temperature difference) widened as the heating rate became faster (Pearson *r* for slow, moderate and fast heating rates below T_{crit}: −0.17, −0.39 and −0.52, respectively; all *P*<0.01); the fastest heating rate led to the greatest difference between air and body temperatures. Note that no line was fitted to the moderate heating rate data after T_{crit} as this relationship was not statistically significant (*P*=0.26).

from an ecological perspective, as rates below 3.0°C h^{-1} are so slow that it is unlikely low-zone mussels would ever be emersed for periods long enough to reach their T_{crit}.

In other ectothermic invertebrates (e.g. insects), heating rates have been shown to have a large effect on thermal tolerance, specifically with regard to the maximum critical temperature an organism can tolerate (i.e. CT$_{max}$, the point typically where an animal loses coordinated locomotor abilities). However, previous research does not agree on the directionality of the relationship between CT$_{max}$ and heating rate. When evaluating the relationship between heating rate and CT$_{max}$, some have found a positive relationship (Casta; da et al., 2015; Santos et al., 2012), while others have found a negative relationship (Chidawanyika and Terblanche, 2010), and some have found no relationship (Terblanche et al., 2011). This might be because the directionality of the relationship can be species dependent, as well as habitat dependent (Allen et al., 2012). Our findings are similar to previous research in that they demonstrate a habitat-dependent relationship between heating rate and thermal tolerance. Altogether, our data, combined with previous findings, highlight the complexity of thermal acclimatization; heating rate appears to be a strong component of thermal acclimatization, but its influence is apparently species and habitat dependent.

The effects of habitat thermal conditions on the heating rate versus thermal tolerance relationship merit analysis in the context of latitude as well as vertical position at a single location, especially for a species like *M. californianus* that has a wide latitudinal range. To examine this issue, Logan et al. (2012) compared *M. californianus* populations living at different latitudes along the eastern Pacific coastline, ranging from Punta Morro, Baja California, Mexico, up to Port Townsend, WA, USA, using an 8°C h^{-1} heating rate for their cardiac thermal tolerance tests. Interestingly, they found no differences in mussel T_{crit} among these sites. This result may seem surprising given the fact that mussels at southern latitudes would be expected to experience hotter temperatures than those at more northern latitudes; however, it could be that the mussels at northern latitudes experienced heating rates similar to or faster than those of mussels at southern latitudes, because low tides at the higher latitudes occur closer to midday when temperatures are usually hottest. Thus, the lack of difference in T_{crit} among mussels living at different latitudes could be a result of their acclimatization state to different heating rates, and it could be that if these animals were tested at heating rates they commonly experience in the field, the results may differ.

It is also interesting to note that the range of T_{crit} we observed was largest at the fastest heating rate, and that this T_{crit} range was similar between high- and low-zone mussels (T_{crit} ranges of 7.2 and 6.7°C, respectively). Miller and Dowd (2017) reported a large range in the heating rates experienced within both high- and low-zone mussel beds over a 21 day period, encompassing a heating rate range of up to 14.7 and 10.8°C h^{-1}, respectively. It is likely that the mussels we tested from high- and low-zone sites experienced ranges of heating rates in the field comparable to those reported by Miller and Dowd (2017). Thus, the variability in T_{crit} at the fastest heating rate in both high- and low-zone mussels may reflect a large variability in acclimatization to heating rates (and, therefore, absolute temperatures) within the same mussel bed.

The observed variability in cardiac thermal tolerance responses raises two important questions. First, how do we best obtain biologically realistic values of T_{crit} within an ecological context, in light of its dependence on the rate of heating and the acclimatization state of the individual? Second, what is the physiological

significance of T_{crit}, in terms of reflecting underlying (cellular level) effects of thermal stress? Previous research – which has not taken heating rate into account – has suggested that mussels' T_{crit} values are, on average, ∼5–7°C higher than the average maximum habitat temperature they experience (Compton et al., 2018). Taking this difference at face value, it might seem that cardiac function is not threatened by high temperatures in the field, and that in this context mussels may be 'over-engineered' for their habitats. However, prior studies of cellular-level damage from heat at temperatures below T_{crit} (see below) and our observation that, in high-zone mussels, T_{crit} depends on the rate at which body temperature rises during periods of emersion indicate that a more detailed reanalysis of this question of vulnerability to heat stress is required.

There are two components to this reanalysis of T_{crit} and habitat temperatures. First, when zone-specific heating rate is taken into account, do mussels' hearts still appear to be 'over-engineered' (and lack vulnerability) to heat stress relative to average maximum temperatures experienced? Second, is average maximum temperature experienced within that mussel bed the appropriate measure of potential stress? To examine the first question, we compared T_{crit} of our high- and low-zone mussels with the individual average maximum temperatures that Miller and Dowd's (2017) mussel beds experienced. Using the fast heating rates in our study (comparable to those seen in the field), our high-zone mussels had an average T_{crit} ∼13°C higher than the average individual maximum temperature in the high-zone mussel bed. Similarly, using a heating rate appropriate for the low-zone mussels in the field (comparable to our slow heating rate), we found our low-zone mussels had a T_{crit}∼15°C higher than the average individual maximum temperature in the low-zone mussel bed. Thus, when average heating rate is taken into account, mussels appear even less vulnerable and more 'over-engineered' than earlier studies suggest. However, a comparison of T_{crit} with average individual maximum temperature may not be the appropriate metric by which to judge mussels' thermal tolerance. Instead, using Miller and Dowd's (2017) field data, it appears that for both our high- and low-zone mussel beds, the absolute maximum temperature experienced in that bed (and not the average individual maximum temperature) is the best predictor of critical temperature at a given zone-specific heating rate. Our T_{crit} values are only 1–2°C higher than the overall maximum temperature experienced in the high- and low-zone mussel beds in the limited duration of Miller and Dowd's (2017) study, and even higher temperatures are likely to occur during heat waves. In this context, mussels appear to be barely adequately engineered. This suggests that mussels' cardiac thermal tolerance has evolved or undergone acclimatization in response to extreme (absolute) rather than average individual maximum temperatures, a suggestion in line with the evolution of thermal tolerance in limpets (Denny and Dowd, 2012).

The fact that high-zone mussels experience faster heating rates and a wider range of body temperatures on a daily basis than low-zone mussels (Miller and Dowd, 2017) explains, at least from an organismal perspective, why it is advantageous that their cardiac thermal tolerance improves at faster heating rates. However, it remains unclear what morphological or physiological mechanisms would allow for this adaptive adjustment in T_{crit}. Differences in gross morphological characteristics seem unlikely to account for the adjustments in T_{crit}. For example, the fact that there was no difference between zones in absolute (or percentage) water loss during heating indicates that differences between high- and low-zone animals are unlikely due to phenomena related to osmotic

stress. Instead, we hypothesize that there are likely cellular and molecular changes (via acclimatization) allowing for the high-zone mussels' improved cardiac thermal tolerance at faster heating rates.

In the context of what mechanisms might underlie adaptive changes in T_{crit}, it is relevant to emphasize that T_{crit} is not an index of a 'threshold' temperature at which thermal damage to cells commences. Rather, there is considerable evidence that heat-induced damage to cells in mussels and other intertidal mollusks occurs at temperatures several degrees below T_{crit}. For example, the expression of stress-related genes such as those encoding heat shock proteins is upregulated at temperatures between 23 and 25°C in *M. californianus*, temperatures that lie anywhere from 9 to 16°C below mussels' T_{crit} (Gracey et al., 2008; Buckley et al., 2001; Halpin et al., 2004; Roberts et al., 1997). Thus T_{crit} likely reflects cumulative heat damage to cells that is initiated during earlier stages of heating and gradually builds up to a level that causes heart dysfunction at the critical temperature. The T_{crit} of cardiac function, while an index of organ-level dysfunction, thus can also serve as an indicator that sufficient thermal damage of cellular structures has occurred to render the heart suboptimal in its performance. Furthermore, the plasticity found in T_{crit} values from mussels in high versus low sites indicates that cells have the capacity to control the amount of heat-induced damage that occurs, possibly by up-regulating constitutive levels of heat shock proteins and other stress-related proteins during acclimatization to hotter temperatures. Roberts et al. (1997) found significantly higher amounts of heat shock protein 70 in high-zone *M. californianus* specimens relative to conspecifics from a lower tidal height. At body temperatures above T_{crit}, more severe heat-induced damage to cellular constituents occurs. As temperatures approach or exceed FLT, some of this damage is irreversible, as indicated by the upregulation of genes that encode proteins responsible for removing irreversibly damaged proteins from the cell (Gracey et al., 2008).

As HR was the main physiological variable we measured during heating, we can only speak to the potential acclimatization of this organ and the ganglia that innervate it and modulate the rate of beating. Notably, the slope of the HR versus temperature response did not differ across heating rates or between zones (see Fig. 2). This indicates that HR is responsive to absolute body temperatures and not to the rate of change in body temperature. We also found that maximum HR was higher in high- versus low-zone mussels only at the fastest heating rate. And in high-zone mussels, maximum HR was only higher at the fast versus slow heating rates (∼27 versus 22 beats min^{-1}). By contrast, there were no significant differences in the total change in HR (i.e. maximum minus minimum HR) between zones or across heating rates. These data suggest the total HR range is somewhat fixed in mussels, but that this HR range can be shifted higher or lower depending on heating rate and acclimatization (e.g. an upward shift would lead to higher baseline and maximum HR). This finding is corroborated by other studies showing an upward shift in the HR range, leading to a higher baseline and maximum HR after heat acclimation (Bakhmet, 2017; Xing et al., 2016). However, it remains undetermined whether these changes in baseline and maximal HR are beneficial to the organism.

FLT

FLT was less affected by heating rate than T_{crit} in both high- and low-zone mussels. Unlike T_{crit}, FLT was not different between zones for any heating rate; and only high-zone mussels' FLT was significantly higher at the fastest versus slowest heating rate (but only by ∼1.7°C; see Fig. 5B). Given that only T_{crit} was different between zones and across heating rates, it appears that T_{crit} is a more

physiologically plastic trait than FLT. This finding is similar to results of a study of congeneric snails of the genus *Tegula*: after undergoing heat acclimation, snails had larger increases in cardiac T_{crit} than in FLT (Stenseng et al., 2005). Moreover, our data show that FLT is similar between individuals with low versus high T_{crit} (see Fig. 6). For example, in our study, two mussels had similar individual heating rates of 8.5°C h^{-1}, but one had one of the lowest T_{crit} (32.7°C), while the other had one of the highest (43.0°C); however, the two mussels had a similar FLT (41.9 and 42.8°C, respectively). These data suggest that acute factors, such as heating rate, do not substantially affect FLT, but leave open the possibility that more chronic (long-term) factors, like heat acclimatization, are necessary to alter FLT. T_{crit}, in contrast, appears to be a more acutely plastic trait that can be influenced by transient abiotic factors, like heating rate. Overall, these results highlight the complexities of pinpointing markers that best define thermal tolerance in mussels. Clearly, further research is required to determine the abiotic and biotic factors that drive acute versus long-term changes in mussels' thermal tolerance (i.e. T_{crit} versus FLT) and therefore survival.

Thermal inertia

In the context of experimental design, it is important to note that during our experiments, as air temperature increased during heating, the internal temperature of the mussels was on average ∼1–3°C lower than the air temperature; this temporal lag increased as air temperature became hotter and heating rate was increased (Pearson r-values for the slow, moderate and fast heating rates until reaching T_{crit}: −0.19, −0.39 and −0.52, respectively; all $P<0.01$; see Fig. 7). Fig. 7 shows that the mean difference between body and air temperature increased by ∼1°C after mussels achieved their T_{crit}. At the slow heating rate, the temporal lag was greater after T_{crit} than before (Pearson $r=−0.59$, $P<0.01$). However, at the fast heating rate, the temporal lag decreased after achieving T_{crit} (Pearson $r=−0.35$, $P<0.01$). Although we did not directly measure it, observationally we found that many mussels started to gape upon achieving their T_{crit}. This may have allowed for evaporative cooling, thus slowing individual heating rates and leading to an even larger discrepancy between air and body temperature. More research is needed to determine whether achieving T_{crit} leads to gaping. In addition, there was large variability among mussel body temperatures for any given air temperature, ranging from 1.5°C at lower air temperatures to >6°C for higher air temperatures. Most notably, these data highlight the importance of measuring individual mussel body temperatures to obtain accurate T_{crit} and FLT when conducting laboratory experiments where mussels are emersed, rather than relying on air temperature as a proxy for mussel body temperature.

Considerations

There are several points that are important to highlight about our findings to provide steps for future research. At Hopkins Marine Station, our high-zone mussels experience considerably faster mean daily heating rates than the low-zone mussels, which likely explains why our high-zone mussels tolerated faster heating rates. However, it is possible that there are sites at different latitudes or locations in the world where low-zone mussels experience faster mean daily heating rates than high-zone mussels. In this case, it could be that the relationship between heating rate and thermal tolerance at those sites would be different from (or opposite to) our findings. That being said, future studies should explore site-specific relationships between heating rate and thermal tolerance for high- versus low-zone mussels, as this relationship is likely dependent on the mussels' mean daily heating rates in the field (i.e. acclimatization state). Moreover, we did not monitor mussels' gaping behavior during the study because

previous work at our site has found that gaping was not related to mussel body temperature, and therefore mussels did not use gaping for evaporative cooling (Fitzhenry et al., 2004; Miller and Dowd, 2017). However, it could be that at other locations, behavioral gaping (and thus evaporative cooling) occurs on hot days, and therefore would modify the relationship between thermal tolerance and heating rate. Moreover, if mussels at these other locations indeed utilize gaping for evaporative cooling, then it is likely that relative humidity would also modify the heating rate versus thermal tolerance relationship by limiting evaporative cooling (therefore leading to faster heating rates) at high humidity. This is another potential area for future work. Lastly, the comparative plasticity between T_{crit} and FLT should be considered under a more controlled laboratory study. We have begun experiments exploring the plasticity in these variables across weeks of constant submersion in the hope of providing a more concrete idea as to how long-term alterations to the daily tidal cycle (and therefore mean daily heating rates) will impact mussels' thermal tolerance (i.e. T_{crit} and FLT).

Conclusions

Faster heating rates increased high- but not low-zone mussels' cardiac thermal tolerance, as indexed by T_{crit}. For all heating rates, high-zone mussels had a higher T_{crit} compared with low-zone mussels, an effect that was most apparent at the fastest heating rate. However, there were no differences between zones in FLT, and FLT was minimally affected by heating rate. These findings can be applied to both laboratory and ecological settings in several key ways. When conducting thermal tolerance tests in the laboratory, we suggest selecting heating rates for the animal that are appropriate for that mussel bed's mean daily heating rate. This will allow for the most ecologically valid predictions of a mussel's T_{crit} and survivability within their mussel bed. Testing animals at their location-specific mean daily heating rates will also help to make more ecologically valid comparisons between animals from differing intertidal heights. In an ecological context, as climate change continues to result in hotter air temperatures and more frequent heat waves, animals will likely experience faster heating rates, which will consequently affect mussels' site-specific thermal survival. Moving forward, it is important that the effects of heating rate and acclimatization are taken into consideration in ecological models predicting mussel survivability, and hence intertidal community ecology. As with all predictions, these ecological forecasts will benefit from increased understanding of the mechanisms by which mussels physiologically adapt to their thermal environment.

Data availability
The full dataset from this study is available from Mendeley (doi:10.17632/6gcx3ckxh8.1).

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