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Physiological responses to heat stress in an invasive mussel *Mytilus galloprovincialis* depend on tidal habitat

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ABSTRACT

Mussels are ecologically important organisms that can survive in subtidal and intertidal zones where they experience thermal stress. We know little about how mussels from different tidal habitats respond to thermal stress. We used the mussel *Mytilus galloprovincialis* from separate subtidal and intertidal populations to test whether heart rate and indicators of potential aerobic (citrate synthase activity) and anaerobic (cytosolic malate dehydrogenase activity) metabolic capacity are affected by increased temperatures while exposed to air or submerged in water. Subtidal mussels were affected by warming when submerged in water (decreased heart rate) but showed no effect in air. In contrast, intertidal mussels were affected by exposure to air (increased anaerobic capacity) but not by warming. Overall, physiological responses of mussels to thermal stress were dependent on their tidal habitat. These results highlight the importance of considering the natural habitat of mussels when assessing their responses to environmental challenges.

1. Introduction

Many marine species can survive in both subtidal habitats, where they experience constant submersion, and in intertidal habitats, where they experience regular periods of aerial exposure (Newell, 1979; De Pirro et al., 1999a). Across these habitats, organisms can experience a variety of abiotic stressors, including thermal stress, osmotic stress, and hypoxic stress, that are known to affect organismal performance and survival (e.g., Menge and Olson, 1990; Helmuth and Hofmann, 2001; Zacherl et al., 2003; Zippay and Hofmann, 2010; Logan et al., 2012). Habitat temperature is an especially prominent stressor that can influence the performance, survival, and geographic distribution of marine organisms (e.g., Schneider and Helmuth, 2007; Tagliorolo and McQuaid, 2015; Monaco and McQuaid, 2019). The temperature of a subtidal organism is directly tied to the temperature of sea water, which generally remains constant over short (i.e., daily) time scales (Helmuth and Hofmann, 2001). In contrast, the temperature of an intertidal organism is determined by the sea water temperature during high tide (i.e., submersion) but can be drastically increased during low tide (i.e., aerial exposure) based on the ambient air temperature, solar radiation, relative humidity, and wind speed (Helmuth and Hofmann, 2001). Thus, organisms living in intertidal habitats experience different magnitudes and temporal patterns of temperatures than organisms living in subtidal habitats. Additionally, these temperatures can often covary with other stresses (e.g., salinity) that may alter how organisms respond to temperature (Dahlhoff et al., 2001, 2002). Water and air temperatures in coastal areas have been warming in recent years and are expected to continue warming (Harley et al., 2006, IPCC, 2013), so it is important to know how warming affects the physiological performance and survival of marine organisms across the sub- and inter-tidal zones. Many studies of thermal physiology have compared organisms living in different regions of the intertidal zone (e.g., Jimenez et al., 2015; Gleason et al., 2017; Ober et al., 2019), as well as organisms at different points within a tidal cycle (e.g., Gracey and Connor, 2016). However, we still know little about how physiological responses to temperature are affected by an animal's tidal habitat (i.e., subtidal *versus* intertidal) (but see Tagliorolo et al., 2012), especially at organismal and subcellular levels.

Environmental temperature dictates the body temperature of marine ectotherms and can therefore influence their physiology (Sandison, 1967; Bannister, 1974; Stillman and Somero, 1996; Kern et al., 2015). It is widely accepted that there is an optimal thermal range associated with physiological performance for most organisms and that this largely

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corresponds to the thermal range of their native habitat (Pörtner et al., 2000; Pörtner, 2010; Somero, 2010). As temperature has pervasive effects on the rate of metabolic processes of ectotherms (Widdows and Bayne, 1971; Hochachka and Somero, 2002; Hofmann and Todgham, 2010), these organisms have evolved compensatory responses that help to buffer an organism's performance when the optimal thermal range is exceeded. For instance, organisms can compensate for thermal stress through defense mechanisms such as the cellular stress response, which controls metabolism, the cell cycle, and apoptosis (Kültz 2003, 2005; Sokolova et al., 2012), or by upregulating physiological processes, such as ventilation (Sokolova et al., 2012) and cardiac activity (Braby and Somero, 2006; Lockwood and Somero, 2011). Understanding these coping mechanisms is critical to understanding how an organism's physiological performance can shift with temperature fluctuation.

Marine invertebrates, like bivalve molluscs, can respond to thermal stress by altering their physiology at the organismal and subcellular levels. Cardiac activity, or heart rate, is one organismal response indicative of respiratory function and physiological state (Bayne, 1971; Coleman, 1974; Depledge, 1985; Feder et al., 1987; Depledge and Andersen, 1990; Marshall and McQuaid, 1993). Heart rates of marine invertebrates can vary with multiple abiotic stressors, like combinations of thermal stress (Bayne et al., 1976a; De Pirro et al., 1999b; Pörtner et al., 2000; Stenseng et al., 2005; Dong and Williams, 2011), osmotic stress (Nicholson, 2002; Bakhmet et al., 2005), and desiccation stress (Bayne et al., 1976b; Marshall and McQuaid, 1993; De Pirro et al., 1999a; Connor and Gracey, 2012). Fluctuations in heart rate can reveal changes in metabolic rate (Marshall and McQuaid, 1992; Santini et al., 1999), as aerobic respiration and metabolic activity have a positive relationship (Dong and Williams, 2011; Bakhmet, 2017), and metabolic state, as heart rate tends to decrease for some organisms when they switch from aerobic to anaerobic metabolism (Connor and Gracey, 2012; Gracey and Connor, 2016). Respiration and oxygen availability directly impact metabolic activity, and changes at the level of the whole organism are often accompanied with subcellular changes such as increases in capacity to generate ATP via aerobic and anaerobic pathways (Hochachka and Somero, 2002). Citrate synthase (CS) catalyzes the reaction of acetyl CoA and oxaloacetate to produce citric acid in the TCA cycle, and it has been measured by others to investigate changes in aerobic capacity in mussels and other invertebrates (Yang and Somero, 1993; Lesser and Kruse, 2004; Doucet-Beaupre et al., 2010; Lockwood and Somero, 2011; Dowd et al., 2013; Drake et al., 2017). However, under anaerobic conditions, malate dehydrogenase (MDH), which is an indicator of potential anaerobic metabolic capacity (Dahlhoff et al., 2002; Ulrich and Marsh, 2006, Lockwood et al., 2012), plays an important role in the generation of energy using alternative metabolic pathways (e.g., glycolysis; Hochachka and Somero, 2002). MDH functions to re-oxidize cytoplasmic NADH and maintain redox balance in the cytosol by shuttling malate to the mitochondria (Livingstone, 1978; De Zwaan et al., 1982; Dahlhoff and Somero, 1991; Lockwood and Somero, 2011). Metabolic shifts between aerobic and anaerobic respiration are used as strategic responses by marine organisms to defend against environmental challenges that are complex, especially in response to thermal differences between submersion underwater and aerial exposure (Sandison, 1967; Stillman and Somero, 1996; Connor and Gracey, 2012; Gracey and Connor, 2016). Understanding organismal physiology with underlying metabolic mechanisms will help inform us how thermal stress affects important ecosystem engineer species and allow us to make predictions about energetic pathways that provide organisms with physiological strategies to withstand variable environments (Denny and Helmuth, 2009).

The mussel *Mytilus galloprovincialis* has invaded many shorelines around the world, from its native range in the Mediterranean Sea to Japan, South Africa, and the coast of California in North America (Seed, 1992). The propensity for *M. galloprovincialis* to invade new locations has made it a model organism for studying how organisms can respond and adapt to environmental challenges, like thermal stress (e.g., Braby and Somero, 2006). The mussel can survive in the subtidal and intertidal zones on rocky shores, so many investigations have assessed how thermal stress during periods of aerial exposure or submersion in water affect the mussel's survival and performance (e.g., Labarta et al., 1997; Schneider, 2008; Anestis et al., 2010; Tagliorolo et al., 2012). When thermally stressed in air, *M. galloprovincialis* can increase its rate of oxygen uptake with temperature (Tagliorolo et al., 2012), although its heart rate may not increase with temperature (Tagliorolo and McQuaid, 2015). Additionally, the mussel tends to open its valve during severe thermal stress in air, which is thought to help the mussel avoid additional physiological stress (e.g., hypoxia, reactive oxygen species) (Dowd and Somero, 2013). When thermally stressed in water, *M. galloprovincialis* can increase its rate of oxygen uptake and heart rate with temperature up to a point at which the mussel may close its valves, reduce its rate of oxygen uptake and heart rate, and switch from aerobic to anaerobic metabolism (Braby and Somero, 2006; Anesetis et al., 2007; Tagliorolo and McQuaid, 2015). Even without thermal stress, *M. galloprovincialis* tends to depress its metabolism when exposed to air (Olabarria et al., 2016), and in the long term this causes the mussel to grow more rapidly in subtidal conditions than in intertidal conditions (Schneider, 2008). Due to logistical constraints and location of pure populations, many studies on the thermal physiology of *M. galloprovincialis* have used experimental animals sourced from populations from one tidal zone (e.g., intertidal, subtidal) to assess the effects of thermal stress in contexts that do not match that population's history or ecology [e.g., exposing subtidal mussels to thermal stress in air, exposing intertidal mussels to thermal stress during constant immersion in water (Lockwood et al., 2010; Fields et al., 2012)]. While *M. galloprovincialis* can acclimate to new conditions over time (e.g., Anestis et al., 2007), the environmental conditions acting selectively on intertidal and subtidal populations in nature can be fundamentally different (Schneider and Helmuth, 2007; Astorga et al., 2018). Thus, the physiological responses – or the plasticity in physiological responses – of a mussel to thermal stress could be genetically-rooted and different between populations (Braby and Somero, 2006; Fields et al., 2006; Saarman et al., 2017; Vasquez et al., 2017).

Along the California coast, *M. galloprovincialis* has displaced the native mussel *Mytilus trossulus* from the southern extent of its range (Baja Mexico) to northern California (Braby and Somero, 2006). Many studies have examined the thermal physiology of *M. galloprovincialis* along the California coast (Hofmann and Somero, 1996; Braby and Somero, 2006; Saarman et al., 2017; Dowd and Somero, 2013; Vasquez et al., 2017) but only examined individuals from a single population (i.e., either intertidal or subtidal). In the current study, we assess the physiological response to thermal stress of *M. galloprovincialis* from the California coast, using individuals from both subtidal and intertidal populations. The physiological processes we measure are (1) cardiac activity because it is an indicator of metabolism at the level of the organism, (2) citrate synthase activity because it indicates changes in aerobic capacity at a subcellular level, and (3) malate dehydrogenase activity because it indicates changes in anaerobic capacity at a subcellular level. The two populations that we examine originate from different geographic regions in California, so we do not statistically compare the populations but rather assess the qualitative differences in their responses to their respective thermal stress regimes. Our objective was to understand how tidal habitat affects mussel physiology by measuring within-population responses to thermal stress when exposed to air and submerged in water. While we cannot directly compare the responses of these two separate populations to each other, these data highlight whether or not populations from different geographic regions share similar responses to aerial exposure and submersion in water regardless of potential confounding effects such as genetic variants and local adaptions.

2. Materials & methods

2.1. Collection sites

Mytilus galloprovincialis was collected from two sites. Subtidal mussels (shell lengths from 50 to 70 mm, mean = 61 mm, n = 183) were taken from floating docks at Spud Point Marina in Bodega Bay, CA (38.33 °N, 123.06 °W) from May to August 2018, and intertidal mussels (shell lengths from 51 to 70 mm, mean = 60, n = 185) were collected from wharf pilings in the mid-intertidal zone in Santa Barbara, CA (34.41 °N, 119.69 °W) from July to August 2018. Separate sites were used for each population because to the best of our knowledge there was no single location where both subtidal and intertidal *M. galloprovincialis* cohabitated in enough abundance to provide mussels for the experiment. The collection sites are on either side of a major biogeographic barrier along the California coast (Point Conception, CA) and can therefore experience vastly different environmental conditions, including temperature (Fenberg et al., 2015). We controlled for the different thermal conditions at each site across this biogeographic barrier by using acclimation and experimental temperatures that were relevant to the respective sites (see below; Fig. 1). Immediately after collection, mussels were transported to Sonoma State University (Rohnert Park, CA) and held submerged in a recirculating holding tank with unfiltered seawater at either 12 ± 1 °C (mean ± sem; for mussels collected from Bodega, CA) or 16 ± 1 °C (mean ± sem; for mussels collected from Santa Barbara, CA). All mussels were tank-acclimated for 48 h prior to experimentation.

2.2. Mussel body temperature

Mussel body temperatures were recorded at each collection site in 30-min intervals using a clean, empty *M. galloprovincialis* shell (length = 50–70 mm) filled with silicone epoxy and an iButton (Maxim Integrated, DS1922L & DS1921G, San Jose, CA) with ±0.5 °C accuracy. These mussel-mimic loggers (robo-mussels) provide an accurate representation of 'real' mussel body temperature through time (Helmuth and Hofmann, 2001; Jost and Helmuth, 2007). We deployed loggers within mussel patches at each site for 6 months (March to August 2018).

2.3. Experimental design

Mussels from each population were subjected to either constant temperatures or increasing temperatures, while submerged in water or exposed to air. These four treatments are described in Table 1. From March to August 2018, the average mussel-mimic temperature at the subtidal site was 14.1 ± 1.5 °C (mean ± sd), while the average mussel-mimic temperature at the intertidal site was 17.5 ± 3.2 °C (mean ± sd). Thus, the maximum temperatures used in the heating experiments (Table 1) were approximately 10 °C warmer than the average temperatures experienced by the mussel populations in their respective habitats.

Table 1
Experimental treatments, temperatures, and sample sizes. Temperatures in heating treatments were increased at a rate of 4 °C h^{-1}, and temperatures in control treatments were held constant.

Subtidal mussels (acclimated at 12 °C)			Intertidal mussels (acclimated at 16 °C)		
Treatment	**Temperature range**	**n**	**Treatment**	**Temperature range**	**n**
Submerged, heated	8–24 °C	64	Submerged, heated	12–28 °C	50
Aerial, heated	8–24 °C	64	Aerial, heated	12–28 °C	75
Submerged, control	12 ± 1 °C	25	Submerged, control	16 ± 1 °C	30
Aerial, control	12 ± 1 °C	30	Aerial, control	16 ± 1 °C	30

At the start of each heating experiment, 30 mussels from each habitat were moved from the holding tank and placed into glass containers in an environmental chamber (KB034-ER-DM-DG, Darwin Chambers, St. Louis, MO). Containers were either filled with unfiltered seawater (submerged treatment) or not (aerial treatment). The temperature inside the chamber was increased so that the mussels' body temperatures increased at a rate of 4 °C h^{-1} over 5 h with enough free water in the chamber to maintain relative humidity near 100%. This heating rate is commonly experienced by intertidal mussels along the west coast of North America (Somero, 2002; Mislan et al., 2014). The 5-h heating period is within the range of aerial exposure time that the intertidal mussels at the collection site regularly experience in nature. To ensure that mussels reached the targeted body temperatures (Table 1), we monitored the body temperature of a live mussel in the chamber with an EasyLog type-K thermocouple temperature logger (DATAQ Instruments, Akron, OH) at 10-min intervals with ±0.5 °C accuracy. Temperatures in the environmental chamber were also measured with a robo-mussel at 10-min intervals (section 2.2). In each treatment, heart rates were continuously measured in 8 mussels per habitat – these continuous measurements, in combination with the records of the chamber's and experimental animals' temperatures, allowed us to isolate heart rate data from specific temperatures (section 2.4). Additionally, tissue

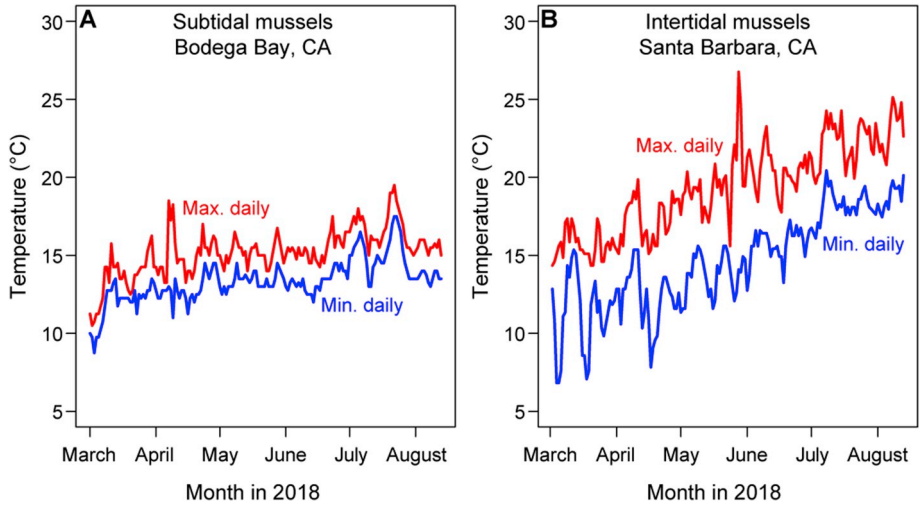

Fig. 1. Mussel body temperature at each site. Daily minimum and maximum temperatures recorded by sensors mimicking the thermal properties of mussels in the A) subtidal population and B) intertidal population in the months prior to collection. Values are the mean of two sensors.

samples were collected from 5 to 8 mussels at specific temperatures for genotyping and biochemical analyses (sections 2.5 to 2.7).

To control for time spent in the environmental chamber, mussels from subtidal and intertidal habitats were held either submerged in water or exposed to air at a constant temperature that was representative of their habitat (Table 1) for 5 h (i.e., the amount of time used in the heated treatments). Five mussels per hour were sampled from each treatment for genotyping and biochemical analysis.

2.4. Heart rate

To measure heart rate (HR) of mussels, we attached one non-invasive infrared HR sensor (NewShift, Portugal; Burnett et al., 2013) using Fun Tak mounting putty (Loctite, USA) and cyanoacrylate (Loctite, USA) to the outer shell near the dorsal edge of each mussel (n = 8) in each treatment, and allowed those mussels to recover for 12–18 h in their holding tanks before any experiments. Heart rates were monitored continuously during the experiment using the HR sensors and oscilloscope with a sampling rate of 750 Hz (Picoscope 2240 A, Pico Technology, St. Neots, UK). We used the psd package (Barbour and Parker, 2014) in R Statistical Software (https://www.r-project.org/) to calculate the mean HR (beats min^{-1}) in 10-min intervals at each treatment. The short duration of the measurement interval insured that the HR data were collected during a specific temperature (i.e., at a heating rate of 4 °C h^{-1}, the body temperatures of the mussels would change < 1 °C during the 10-min HR recording). All mussels outfitted with HR sensors were sacrificed at the end of the entire experiment, and tissues (gill and adductor) were immediately frozen in liquid nitrogen and stored for genotyping and biochemical analysis (sections 2.5 to 2.7).

2.5. Genotyping and biochemical analysis

In each treatment, 5 to 8 mussels from each habitat were sacrificed at 8 (subtidal only), 12, 16, 20, 24, and 28 °C (intertidal only) (Table 1), and the gill and adductor tissues were excised, immediately frozen in liquid nitrogen, and stored for further analysis.

2.6. Citrate synthase (CS) activity

Total citrate synthase (CS) activity, an enzyme commonly used to measure aerobic capacity in mussels and other invertebrates (Yang and Somero, 1993; Morley et al., 2009), was measured following published methods (Dahlhoff et al., 2002; Dalziel et al., 2012; Drake et al., 2017). Adductor tissues (mass = 0.07–0.1 g $mussel^{-1}$) from each combination of temperature (8, 12, 16, 20, 24, 28 °C), treatment (aerial or submerged) and population (subtidal and intertidal) was homogenized in a diluted 1:10 ratio of ice-cold 50 mM potassium phosphate buffer (pH 7.5) – first, by hand for 15–20 s with a pestle and then with an electric homogenizer (Bio-Gen Pro200, ProScientific, Oxford, CT) for 10 passes (~5 s total). Protein homogenates were centrifuged at 2000g at 4 °C for 10 min and supernatant was transferred to a new microcentrifuge tube. Samples (10 μl) were added in triplicate to a clear polystyrene 96-well plate and 200 μl of citrate synthase buffer (50 mmol l^{-1} Imidazole pH 8.2, 0.2 mmol l^{-1} Ellman's reagent and 0.3 mmol l^{-1} acetyl CoA) containing 1.0 mmol l^{-1} oxaloacetate was quickly added. A second set of triplicates was run as a blank and 200 μl of citrate synthase buffer without substrate was added to measure background activity. Enzymatic activity was measured with a pathlength of 1 cm in a microplate spectrophotometer (BioTek Synergy HT, Winookski, VT) set to read 412 nm at 15 °C (a temperature ecologically relevant and experienced by these mussels, see Fig. 1) for 20 min using a kinetic sweep to detect the production rate of 5-thio-2-nitrobenzoic acid (TNB), which has an extinction coefficient of 13.6 mM^{-1} cm^{-1}. The read started immediately after the oxaloacetate was added and shaken (5 s). Raw data files show the initial slope was steeper than the averaged slope and were linear. Temperature of the assays were maintained within ±0.3 °C by running them in a temperature-controlled room. The maximum slope of change in absorbance was calculated using BioTek Gen5 software and CS activity was calculated by subtracting the mean background rate from the mean enzymatic rate for each sample. CS specific activity (in micromoles of oxaloacetate oxidized per minute) is expressed as international units per gram of wet mass (U g^{-1} WM). To standardize samples between different plates, a sample of mussel adductor tissue was run on each plate (in triplicate) to quantify levels of CS activity relative to experimental samples.

2.7. Total malate dehydrogenase (MDH) activity

There are two isozymes of malate dehydrogenase: a mitochondrial form (mMDH) involved in the aerobic TCA cycle, and a cytosolic form (cMDH) that maintains redox balance and is a measure of anaerobic capacity. Cytosolic MDH is more abundant than mMDH in most invertebrate muscles (Lazou et al., 1987) and accounts for nearly 90% of all MDH found in the adductor tissue of *M. galloprovincialis* (Dahlhoff and Somero, 1991). To assess anaerobic capacity, we measured total MDH activity levels following published methods (Dahlhoff and Somero, 1991; Dahlhoff et al. 1991, 2002). The same protein homogenates and volumes were used as above (section 2.6) with the procedure being similar, except the MDH assay buffer differed in its composition (200 mmol l^{-1} imidazole pH 7.15, 0.2 mmol l^{-1} NADH) and 0.2 mmol l^{-1} oxaloacetate was quickly added to the 96-well plate. Samples were diluted sixteen-fold to improve the capture of MDH activity. Background and enzymatic activity were measured in the same way as above, except at a wavelength of 340 nm using a kinetic sweep to measure the rate of NADH oxidation, which has an extinction coefficient of 6.22 mM^{-1} cm^{-1}. The change in absorbance and specific activity (U g^{-1} WM) calculations for MDH were conducted similarly to those for citrate synthase (section 2.6).

2.8. Genotyping

All mussels were genotyped after experimentation to verify their taxonomy. Following manufacturer's instructions, DNA was extracted from mussel gill tissue using the Dneasy Blood and Tissue Kit (Qiagen, Hildon, Germany) and used for polymerase chain reaction (PCR) with a section of non-repetitive region of the byssal thread adhesive protein Me15: CCAGTA TAC AAA CCT GTG AAG A and Me16: TGT TGT CTT AAT AGG TTT GTA AGA as the oligonucleotide primers for determining species; product length *M. galloprovincialis* (126 base pairs), *M. trossulus* (168 base pairs), or a hybrid showing both bands (Inuoe et al., 1995; Dutton and Hofmann, 2008). PCR protocol denatured for 4 min at 94 °C, then amplification for 40 cycles at 94 °C (30 s), 60 °C (30 s), and 72 °C (30 s), followed by a final hold at 72 °C for 4 min. PCR products were visualized on a 2% agarose gel run at 90V in 1X TAE buffer (40 mmol l^{-1} Tris Base, 20 mmol l^{-1} Acetic acid and 1 mmol l^{-1} EDTA sodium salt dihydrate, pH 8.0) for 1 h. If any hybrids (*M. galloprovincialis*/*M. trossulus*) or *M. trossulus* were found, they were eliminated from the dataset and analysis.

2.9. Statistical analysis

To compare HR between treatments, we first separated control data from heating data (Table 1). Heart rates of *control* mussels were analyzed using a first-order autoregressive linear mixed model (AR(1)) with time, treatment, and time by treatment as fixed effects. This model was selected to detect if there was a significant effect of time spent in the chamber on HR, and because the data were heteroscedastic. Heart rates of the *heated* mussels were not heteroscedastic and were therefore analyzed using a linear mixed model (LMM) with treatment, temperature, and treatment by temperature as fixed factors. For control and heated mussels, we monitored the same individuals over time, so we included the mussels' identities as a random factor in the respective

models.

Enzyme activities for all mussels were not heteroscedastic. Therefore, we analyzed data for *control* mussels using a general linear model (GLM) with time, treatment, and time by treatment as fixed effects, and we analyzed data for *heated* mussels using a GLM with temperature, treatment, and temperature by treatment as fixed effects. We did not account for individual identities because each mussel was measured only once.

Independence, normality, and heteroskedasticity assumptions were met prior to all statistical analysis. Outliers (n = 2 to 4 for each treatment-temperature combination) were detected with Cook's D analysis and removed. The excluded HR data were physiologically unrealistic and were likely caused by technical problems with the HR sensors. Since this was not a common garden experiment, there were several confounding factors (site location, acclimation temperature, and range of temperatures) that could limit our ability to infer the mechanisms driving trends in the data. Therefore, mussel populations (subtidal *vs.* intertidal) were analyzed separately. The *control* mussels were analyzed independent of the *heated* mussels and split by population to control for the effect of time. *Post hoc* analyses (Student's t-test, $p < 0.05$ for significance) were run when appropriate. All means reported are mean ± sem and statistical analyses were performed with JMP Pro 13 (SAS Institute Inc., UK).

3. Results

3.1. Heart rate

Heart rates (HR) of subtidal mussels were affected by temperature ($F_{1,147.4} = 10.03$, $p < 0.0001$; Fig. 2A) but not by exposure to air ($F_{1,38.41} = 2.62$, $p = 0.1135$). Subtidal mussels in both treatments (submerged and aerial) had higher HR at 8 °C than between 16 and 24 °C. The mean HR of submerged subtidal mussels decreased by 29% from 8 to 24 °C, while the mean HR of air-exposed subtidal mussels decreased by 50% over the same temperature range (Fig. 2A). There was, however, a temperature*treatment interaction in the aerial exposed mussels at 12 and 20 °C ($F_{4,147.4} = 6.20$, $p = 0.0001$, Fig. 2A).

Temperature ($F_{4,69.98} = 0.85$, $p = 0.4967$), treatment (submersion or aerial; $F_{1,18.43} = 0.04$, $p = 0.8459$), and the temperature*treatment interaction ($F_{4,69.98} = 1.04$, $p = 0.3907$; Fig. 2B) did not affect the HR of intertidal mussels. In fact, between 12 and 28 °C, the mean HR was almost identical between aerial (43.0 ± 2.0 beats min^{-1}) and submerged intertidal mussels (43.1 ± 2.0 beats min^{-1}; Fig. 2B).

3.1.1. Control heart rate

Time did not affect the HR of subtidal ($F_{1,126} = 0.10$, $p = 0.7481$) or intertidal mussels ($F_{1,116} = 0.59$, $p = 0.4424$; data not shown), while exposure to air increased HR for all mussels (subtidal: $F_{1,126} = 34.24$, $p < 0.0001$, submersion mean HR = 43.2 ± 2.9 beats min^{-1}, aerial mean HR = 72.8 ± 3.5 beats min^{-1}; intertidal: $F_{1,116} = 19.02$, $p < 0.0001$, submersion mean HR = 42.8 ± 2.5 beats min^{-1}, aerial mean HR = 60.3 ± 3.4 beats min^{-1}). There were no treatment*time interactions (subtidal: $F_{1,126} = 0.002$, $p = 0.9583$; intertidal: $F_{1,116} = 0.09$, $p = 0.7677$).

3.2. Citrate synthase activity

In each population, the average of the total citrate synthase (CS) activity was higher in water than in air (Fig. 3A). However, there were no statistical effects of temperature, treatment, or interaction between temperature and treatment on total CS activity in subtidal mussels ($F_{1,117} < 1.85$, $p > 0.18$; Fig. 3A) or in intertidal mussels ($F_{1,116} < 1.78$, $p > 0.10$) (Fig. 3A).

3.2.1. Control citrate synthase activity

Time did not affect total CS activity for subtidal ($F_{1,51} = 1.52$, $p = 0.2235$; data not shown) or intertidal mussels ($F_{1,56} = 0.08$, $p = 0.7811$; data not shown). Exposure to air significantly affected total CS activity in subtidal mussels ($F_{1,51} = 4.7539$, $p = 0.0339$; submerged mean = 7.3 ± 0.5 U g^{-1} WM, aerial mean = 6.0 ± 0.3 U g^{-1} WM; data not shown) but not in intertidal mussels ($F_{1,56} = 0.1000$, $p = 0.7530$; submerged mean = 4.7 ± 0.2 U g^{-1} WM, aerial mean = 4.6 ± 0.3 U g^{-1} WM; data not shown). The time*treatment interaction did not affect total CS activity in either population (subtidal $F_{1,51} = 0.8849$, $p = 0.3513$; intertidal $F_{1,56} = 0.5717$, $p = 0.4528$; data not shown).

3.3. Total malate dehydrogenase activity

In each population, the average of the total malate dehydrogenase (MDH) activity was higher in air than in water (Fig. 3B). There was no statistical effect of temperature, treatment, and their interaction on total MDH activity in subtidal mussels ($F_{1,117} < 0.81$, $p > 0.37$; Fig. 3B) and no effect of temperature and the temperature*treatment interaction on total MDH activity in intertidal mussels ($F_{1,115} < 1.11$, $p > 0.2952$; Fig. 3B). However, aerial exposure of intertidal mussels significantly increased their total MDH activity by 15% relative to the submerged treatment ($F_{1,115} = 9.36$, $p = 0.0028$; Fig. 3B).

3.3.1. Control total malate dehydrogenase activity

Time did not affect total MDH activity in subtidal ($F_{1,51} = 1.08$, $p = 0.3043$; data not shown) or intertidal mussels ($F_{1,56} = 0.8574$, $p = 0.3584$; data not shown). Exposure to air affected total MDH activity for subtidal mussels ($F_{1,51} = 7.4418$, $p = 0.0087$; submerged mean ± SEM = 1.8 ± 0.1 U g^{-1} WM, aerial = 2.2 ± 0.1 U g^{-1} WM; data not shown), but not intertidal mussels ($F_{1,56} = 0.3874$, $p = 0.5362$, submerged = 1.9 ± 0.1 U g^{-1} WM, aerial = 2.0 ± 0.1 U g^{-1} WM; data not shown). Neither group of mussels was affected by time*treatment interactions (subtidal: $F_{1,51} = 0.4477$, $p = 0.5065$; intertidal: $F_{1,56} = 0.1144$, $p = 0.7364$; data not shown).

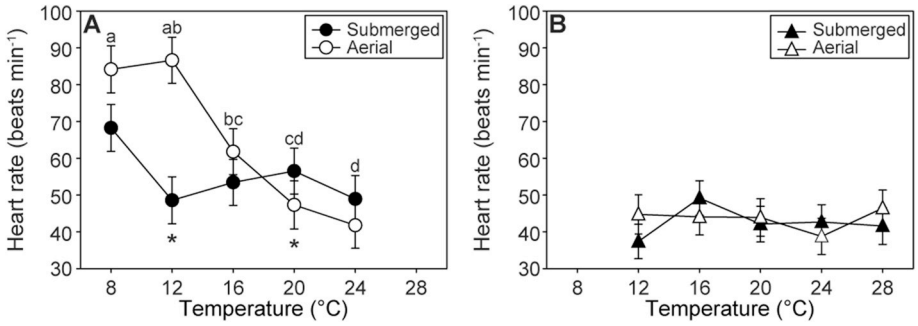

Fig. 2. Tidal habitat impacts mussel heart rate. Mussel heart rate from A) the subtidal population (n = 15 to 17) and B) the intertidal population (n = 7 to 8). In (A) the sample size of submerged mussels ranged from 16 to 17 at each temperature and the sample size of aerial mussels ranged from 15 to 17 at each temperature; in (B) the sample size of submerged mussels ranged from 7 to 8 at each temperature and the sample size of aerial mussels ranged from 7 to 8 at each temperature. The closed symbols represent submerged treated mussels and open symbols represent aerial treated mussels. Values are least square means ± sem. Letters represent significant differences between temperatures for the aerial treatment only. Asterisks represent significant differences between treatments at a given temperature.

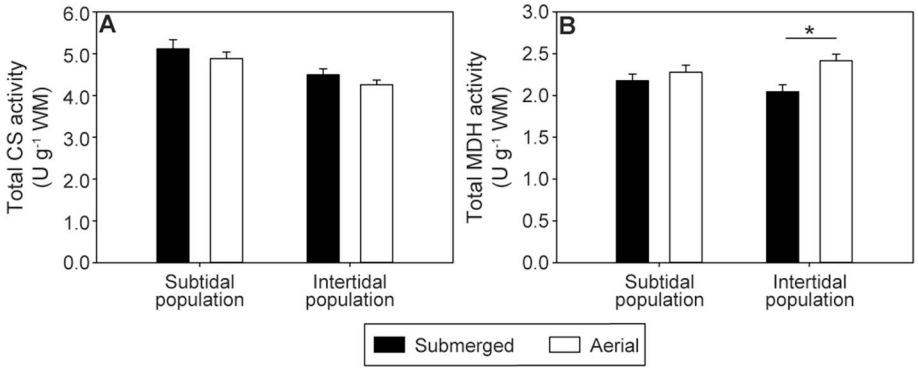

Fig. 3. Effects of aerial and submerged treatments on enzymatic activities of subtidal and intertidal mussels. (A) Total citrate synthase activity of *M. galloprovincialis* from the subtidal population with aerial (n = 61) and submerged (n = 60) treatments, and intertidal population with aerial (n = 72) and submerged (n = 48) treatments; B) total malate dehydrogenase activity from *M. galloprovincialis* subtidal mussels with aerial (n = 61) and submerged (n = 60) treatments, and intertidal mussels with aerial (n = 72) and submerged (n = 47) treatments. Values are means ± sem. Asterisk notes significant differences between treatments.

4. Discussion

Our study demonstrates that there are multiple factors driving aerobic and anaerobic capacity of *Mytilus galloprovincialis*. Intertidal mussels can experience rapid changes in body temperature during a normal tidal cycle, and mussels are known to respond to these temperature fluctuations by adjusting their heart rate (HR) independent of their subcellular changes (Coleman and Trueman, 1971; Bayne et al., 1976b; Moyen et al., 2019). In contrast to previous studies on the aerobic metabolism of *M. galloprovincialis* (Braby and Somero, 2006; Tagliorolo et al., 2012), temperature had no effect on the HR of intertidal mussels. The HR of intertidal mussels ranged from 37 to 48 beats min^{-1} across temperatures from 12 to 28 °C, which is slightly faster than the resting HR of the subtidal *M. galloprovincialis* (26 beats min^{-1} in 21 °C seawater; Braby and Somero, 2006). In total, our results show that *M. galloprovincialis* did not exhibit a physiological response to the elevated temperatures used in this study which aligns with its reputation as one of the most physiologically plastic *Mytilus* species (Braby and Somero, 2006). For instance, *M. galloprovincialis* displays a higher induction temperature necessary to trigger the synthesis of heat shock proteins compared to their native congener, *M. trossulus* (Hofmann and Somero, 1996). Thus, the lack of sensitivity to temperature displayed by the intertidal population of *M. galloprovincialis* compared to previous studies on *Mytilus* congeners may be further indicative of their adaptation to warmer environments. While *M. galloprovincialis* appears relatively insensitive to temperature, we identified an increase in total malate dehydrogenase (MDH) activity during aerial exposure that might be driven by oxygen limitation, however we cannot say for certain because our current experiment did not test this parameter. Furthermore, our study also highlighted the potential role that tidal habitat plays in setting the sensitivity of *M. galloprovincialis* to temperature and exposure to air.

Although the HR of intertidal mussels were not affected by temperature, the HR of subtidal mussels decreased with increasing temperatures, and they were also greatly reduced when subjected to warm temperatures (>12 °C) in air (Fig. 2A). This pattern is similar to other marine mussels that reduce HR and oxygen consumption when exposed to air but then increase their HR when re-submerged in water (Coleman and Trueman, 1971, Bayne et al., 1976b; Widdows and Shick, 1985). On the other hand, the unheated, control mussels from subtidal and intertidal populations showed elevated HR during exposure to air, which indicates that the HR of *M. galloprovincialis* is sensitive to aerial exposure (i.e., regardless of the mussel's tidal zonation), but that the mussel's thermal stress response can dominate over the response to aerial exposure (i.e., as seen in the heated mussel's responses). Although we did not assess the response to re-immersion in water, the intertidal *M. galloprovincialis* that were exposed to air showed little variation in HR over the 5 h experiment, further suggesting unique strategies of each population for coping with temperature and aerial exposure. These differences may be based on the mussels' acclimatization to diurnal tidal cycles, which expose intertidal mussels to rapid and extreme environmental changes and may diminish the mussels' sensitivity to aerial exposure and thermal stress (Fraenkel, 1968; Huppert and Laudien, 1980; Zippay and Helmuth, 2012; Sokolova, 2013). The mussels' physiological response to warming could also have been accompanied by valve closure or gaping (e.g., Dowd and Somero, 2013). For instance, in warming water *M. galloprovincialis* can spend a large portion of time with its valves closed (Anestis et al., 2010) and this is correlated with a drop in the metabolic rate – potentially to limit the mussel's depletion of oxygen in the mantle tissue – and a switch from aerobic to anaerobic metabolism (Widdows and Shick, 1985). This pattern could explain why the intertidal mussels in our study increased MDH with exposure to warming in air (Fig. 3B). The mussel may also open its valves, such as after extreme warming (Dowd and Somero, 2013), and this is thought to help the mussel avoid hypoxic conditions and cellular damage within its tissues by permitting aerial respiration (Widdows and Shick, 1985; Tagliorolo et al., 2012). However, our study did not use the experimental temperatures that have previously been reported to induce prominent valve gaping in air (33 °C; Dowd and Somero, 2013). We did not monitor gaping behavior in the current study, but it could have explained the unexpected patterns in HR and enzyme activity. Finally, the sizes of the mussels used in this experiment could have affected our results. Previous studies on *M. galloprovincialis* that reported thermal sensitivity of aerial respiration, HR, and growth rates all used mussels that were smaller (i.e., minimum sizes in each study ranged from 20 to 40 mm) than the mussels used here (Braby and Somero, 2006; Schneider, 2008; Anestis et al., 2010; Schneider et al., 2010; Tagliorolo et al., 2012; Tagliorolo and McQuaid, 2015), with some exceptions (Anestis et al., 2007; Dowd and Somero, 2013). The size of a mussel can affect its physiological response to temperature (Tagliorolo et al., 2012), suggesting that our results may have been different (i.e. shown larger effects of immersion, aerial exposure, and temperature) if we used a smaller size range of mussels. Regardless of valve closure and mussel size, the thermal insensitivity of the mussels' metabolic rates at warm temperatures suggests that *M. galloprovincialis* – between 50 and 70 mm long – may be capable of surviving thermal stress by reducing their metabolic capacity and "waiting out" the unfavorable temperatures (Widdows and Shick, 1985), and this strongly points toward the existence of an energetic constraint on physiological responses (Fitzgerald-Dehoog et al., 2012).

Subtidal mussels experience less temperature variation over time than do intertidal mussels (e.g., Helmuth and Hofmann, 2001), which may explain the different physiological responses to temperature between the two populations. The HR of subtidal mussels, regardless of treatment, were highest in the cool temperatures of the experiment (8 and 12 °C), and this pattern may be explained by the relatively stable water temperature of the subtidal mussels' habitat (Fig. 1A). Overall, *M. galloprovincialis* are a warm-adapted bivalve and may be cold-limited in the northern part of their range (Braby and Somero, 2006). As such, the reduced HR of subtidal mussels in warm temperatures (Fig. 2A)

could be because the warm temperatures in the experiment represented a temperature range that was more optimal than the cool temperatures. However, this would be surprising given that increases in temperature are generally associated with increases in metabolic rate until further increases in temperature become physiologically suboptimal (Hochachka and Somero, 2002). The decreased HR at warmer temperatures in the heating rate experiment (16–24 °C) reaches just above the resting HR observed for this genus (26 beats min^{-1} in 21 °C seawater; Braby and Somero, 2006), suggesting these mussels are tolerant of warm water temperatures, as previously documented (Seed, 1992; Hofmann and Somero, 1995; Lockwood and Somero, 2011). These results suggest that the subtidal population of *M. galloprovincialis* may have reduced physiological plasticity for cold.

Many marine ectotherms increase aerobic and anaerobic enzyme activities with elevated temperatures (Crockett and Sidell, 1990; Sokolova and Pörtner, 2003; Lesser and Kruse, 2004), but the *M. galloprovincialis* populations used in the present study did not show any effect of temperature on enzyme activities. However, we did find habitat-specific physiological responses to increased temperatures that were similar to patterns found for other marine mussels (Doucet-Beaupre et al., 2010). While previous studies have found that subtidal *M. galloprovincialis* exhibit a strong subcellular response to acute thermal stress in the 24 h following the stress (Tomanek and Zuzow, 2010), our data suggest that subtidal *M. galloprovincialis* do not mount a large and *instantaneous* subcellular response to thermal stress. Instead, they may be reducing energy expenditure and oxygen consumption, as well as gaping (see discussion above) to minimize the harmful consequences of thermal stress as it occurs (Bayne, 1971; Widdows and Shick, 1985; Nicholson, 2002).

Aerobic metabolism requires the presence of oxygen and can therefore be very susceptible to changes in oxygen availability, especially in marine ecosystems (Sokolova and Pörtner, 2003). Citrate synthase (CS) activity, a common indicator of aerobic capacity in invertebrates, increases with temperature in many organisms (Lesser and Kruse, 2004; Morley et al., 2009, Doucet-Beaupre et al., 2010), but several species of marine molluscs do not show this pattern (Bayne et al., 1976a,b, Bjelde and Todgham, 2013). Thus, the patterns of CS activity in *M. galloprovincialis* that are reported here (i.e., unaffected by temperature) are not unusual. Although aerobic metabolism can show a strong dependence on temperature, *M. galloprovincialis* tends to have low metabolic rates – lower than a congener on the west coast of North America, *M. trossulus* (Braby and Somero, 2006), and a mussel from the same taxonomic family (Mytilidae) in the Mediterranean Sea, *Modiolus barbatus* (Anestis et al., 2008). The low enzyme activities of *M. galloprovincialis* that are reported in the literature and in the present study (Fig. 3) may have masked any effect of warming temperatures by lowering the overall maintenance energy costs and ATP-generation (Lockwood and Somero, 2011). Aerial exposure also had no significant effects on aerobic activity in either mussel population. This could, again, be a mechanism to conserve energy by reserving energy stores in anticipation of aerial exposure during low tides (Connor and Gracey, 2012). Shick et al. (1986) found that intertidal mussels continue to use aerobic pathways while aerially exposed to meet energy demands that are not met by anaerobic pathways in certain tissues (e.g., digestive gland). We only measured enzyme activities in adductor tissue, the main muscular tissue that functions in valve closure, similar to other studies (Livingstone, 1978; Churchill and Livingstone, 1989; Dahlhoff and Somero, 1991; Brown et al., 2004). However, our results for CS activity in air-exposed mussels may have been different if we analyzed other tissues from the mussels.

Although temperature did not affect anaerobic capacity in either mussel population, aerial exposure increased total MDH activity for the intertidal mussels (Fig. 3B). For *M. edulis*, cMDH activity was unaffected by temperature alone but was affected by a combination of thermal stress and hypoxia (Livingstone, 1978), thus our results align with previous work. Additionally, many other intertidal animals show increases in anaerobic activity with exposure to air, and this trait allows them to be prepared to switch metabolic pathways when re-submerged in water (Anestis et al., 2008; Connor and Gracey, 2012; Bjelde and Todgham, 2013). The total MDH activity of subtidal mussels was not affected by exposure to air, and this may be due to metabolic depression caused by hypoxia in the air-exposed mussels. Hypoxic depression is a response to minimize dependence on anaerobic metabolism (Guppy and Withers, 1999), which is energetically costly for such a low ATP turnout and can be a common response for molluscs with substantial environmental changes (Pörtner, 2010). Due to several confounding factors in this study (collection location, acclimation temperature, and thermal history), we cannot determine the sole driver of changes in anaerobic metabolism. Additionally, tidal cycle and circadian rhythm has been shown to influence activity levels of certain enzymes and mRNA expression in *Mytilus* (Simpfendörfer et al., 1997; Gracey et al., 2008; Connor and Gracey, 2011). However, when Dowd and Somero (2013) examined the periodicity of MDH and CS enzyme activities from field collected *M. californianus*, they found little evidence for periodic variation in activity of these two proteins. By having a short acclimation period of 48 h while continuously submerged, it is unlikely this short window of time could impact or disrupt the enzyme activity, however we cannot say for certain since this was not tested. Other population factors at the genetic, geographic, and environmental levels, although beyond the scope of our study, could have influenced our results.

Genetic differences, including epigenetic and phenotypic plasticity, could be contributing to changes in HR and metabolic enzymatic responses in each population (Hedgecock, 1986; Helmuth et al., 2006; Hofmann and Todgham, 2010; Logan et al., 2012). Due to the large distance between these populations (~625 km), the populations could be genetically distinct (Geller et al., 1994). As of 1999, there has been little evidence of gene flow between hybrids (*M. galloprovincialis*/*M. trossulus*) in the California hybrid zone (Monterey Bay to Bodega Bay; McDonald and Koehn, 1988; Lockwood and Somero, 2011) and bordering parental populations (Rawson and Hilbish, 1999). Considering the subtidal site (Spud Point Marina in Bodega Bay, CA) is the northern-most point of the hybrid zone and the southern intertidal Santa Barbara site is outside of the hybrid zone, there is likely limited gene flow across the hybrid zone. However, it has been shown that epigenetic variation can compensate for founder effects and lower genetic variation, especially in invasive populations (Ardura et al., 2017). All the mussels for the controlled and heating experiments were genotyped to eliminate hybrids and *M. trossulus* from our study. In the subtidal population (n = 183) only 5 mussels were hybrids and the remainder were *M. galloprovincialis*. In the intertidal population (n = 185), all mussels were *M. galloprovincialis*. It is also possible that these sites could be experiencing local genetic adaptation by changing subcellular phenotypes since Dutton and Hofmann (2009) found heat shock response to be linked to latitudinal acclimatization for *M. galloprovincialis*.

Acclimation temperature can play an important role in shaping an organism's physiological response to thermal stress (e.g., survival – Oliver and Palumbi, 2011, Giomi et al., 2016; critical thermal maximum – Kern et al., 2015; heart rate – Braby and Somero, 2006). One study found that both resting HR and critical temperature (the temperature where HR decreases dramatically) increased with higher acclimation temperature in *M. galloprovincialis,* where resting HR and critical temperature differed in *Mytilus* species reflective of the temperatures they were adapted to evolutionarily (Braby and Somero, 2006). Our results were likely affected by acclimation temperature, as reported in many other studies (Davis and Parker, 1990; Stillman and Somero, 2000; Stillman, 2002, 2003), although at least one study has found that acclimation temperature does not affect thermal physiology of marine molluscs at the organismal level (i.e., the intertidal limpet *Lottia digitalis*; Drake et al., 2017). However, we used acclimation temperatures that were representative of the temperatures experienced by the mussels in their respective habitats rather than combining all mussels under a

common, unrepresentative acclimation temperature. As a result, our experiments captured the mussels' physiological responses to thermal stresses that were ecologically relevant for each population.

5. Conclusions

Our data show that the northern mussel population (i.e., subtidal) has a physiological response (i.e., elevated heart rate) to cold temperatures (Fig. 2) and experience a narrower range of temperatures in their habitat as compared to the southern (i.e., intertidal) mussel population (Fig. 1). These data suggest that variation in a mussel's thermal history plays an important role in their response to thermal stress. Therefore, periods of cold temperatures may have a greater effect on the northern population, however, it is unclear if this is a result of genetic differences between these two populations or related to the habitat of the respective populations are acclimatized to (i.e., subtidal vs. intertidal populations). To understand organismal and subcellular responses of animals living in a variable environment, future comparative studies should investigate physiological performance with common garden designs that incorporate ecologically relevant field conditions as treatments. Habitat temperature, in particular, can vary geographically in ways that are unexpected (Helmuth et al., 2002) and marine organisms can acclimatize to their habitats with several physiological mechanisms (Harley et al., 2006). Therefore, comparing responses of different populations to warming temperatures should account for the normal magnitude of and variation in temperature in each population's habitat. Identifying the metabolic pathways and strategies that different populations use in response to thermal stress can provide valuable habitat-specific information about how climate change will affect the metabolism and physiological performance of marine organisms (Somero, 2010). However, our study emphasizes the point that generalizing physiological responses across populations that vary both in geographic and habitat distribution will require simultaneous consideration of differences in both genetic and acclimatization history of the organisms.

Authors contribution statement

CL Collins-conceptualization, methodology, validation, formal analysis, investigation, writing-original draft, visualization, funding acquisition.

NP Burnett-software, formal analysis, writing-review and editing, visualization.

MJ Ramsey-investigation.

K Wagner-investigation.

ML Zippay-conceptualization, methodology, validation, formal analysis, resources, writing-review and editing, visualization, supervision, project administration, funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2019.104849.

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