

RESEARCH ARTICLE

Temperature and acidification variability reduce physiological performance in the intertidal zone porcelain crab *Petrolisthes cinctipes*

Adam W. Paganini¹, Nathan A. Miller¹ and Jonathon H. Stillman^{1,2,3,*}**ABSTRACT**

We show here that increased variability of temperature and pH synergistically negatively affects the energetics of intertidal zone crabs. Under future climate scenarios, coastal ecosystems are projected to have increased extremes of low tide-associated thermal stress and ocean acidification-associated low pH, the individual or interactive effects of which have yet to be determined. To characterize energetic consequences of exposure to increased variability of pH and temperature, we exposed porcelain crabs, *Petrolisthes cinctipes*, to conditions that simulated current and future intertidal zone thermal and pH environments. During the daily low tide, specimens were exposed to no, moderate or extreme heating, and during the daily high tide experienced no, moderate or extreme acidification. Respiration rate and cardiac thermal limits were assessed following 2.5 weeks of acclimation. Thermal variation had a larger overall effect than pH variation, though there was an interactive effect between the two environmental drivers. Under the most extreme temperature and pH combination, respiration rate decreased while heat tolerance increased, indicating a smaller overall aerobic energy budget (i.e. a reduced O₂ consumption rate) of which a larger portion is devoted to basal maintenance (i.e. greater thermal tolerance indicating induction of the cellular stress response). These results suggest the potential for negative long-term ecological consequences for intertidal ectotherms exposed to increased extremes in pH and temperature due to reduced energy for behavior and reproduction.

KEY WORDS: Ocean acidification, Global warming, Thermal tolerance, Comparative physiology, Metabolism, Marine

INTRODUCTION

Increased atmospheric CO₂ from anthropogenic activity is warming air and ocean temperatures (climate change, ocean warming), while also decreasing ocean pH and carbonate ion saturation (ocean acidification) (Solomon et al., 2007). Increased air temperatures from climate change are projected to include increased frequency, intensity and duration of heat waves (Solomon et al., 2007). Ocean warming will have additional global consequences including increased energy of oceanic storms, reduced ecosystem services from coastal marine habitats such as coral reefs, and shifts in species distributions and abundance (Solomon et al., 2007). Ocean acidification may also

reduce calcification in planktonic organisms (Bednaršek et al., 2014; Orr et al., 2005) though the greatest concern could be for coastal benthic calcifying organisms living in the intertidal zone (Feely et al., 2008; Kelly et al., 2013; Pespeni et al., 2013). Intertidal zone habitats already exhibit considerable variability in pH (Hofmann et al., 2011) and temperature (Stillman and Tagmount, 2009), but ocean acidification, ocean warming, climate change and hypoxia are likely to result in greater intertidal zone extremes in temperature and pH in the future (Doney et al., 2012; Melzner et al., 2013; Shaw et al., 2013; Solomon et al., 2007), which could pose a significant physiological stress on intertidal zone organisms (Waldbusser and Salisbury, 2014).

Physiological stress associated with increased environmental variability is likely to have repercussions for population distribution, growth rates, extinction risk and fitness (Boyce et al., 2006). There is evidence that pH and thermal variability can have significant effects on physiological and biochemical characteristics of marine invertebrates and fish (Alenius and Munguia, 2012; Feldmeth et al., 1974; Lowe and Heath, 1969; Otto, 1973; Podrabsky and Somero, 2004). Additionally, exposure to thermal variability can cause shifts in gene expression patterns that set limits for physiological function (Podrabsky and Somero, 2004), and exposure to increased pH variability can negatively affect performance (Alenius and Munguia, 2012). Because coastal environments are predicted to experience increases in the frequency and the magnitude of temperature and pH extremes as a result of increased atmospheric CO₂ (Doney et al., 2012; Shaw et al., 2013), there is a pressing need to determine how organisms within these ecosystems will respond to such variability.

Inferences regarding the impacts of ocean acidification and ocean warming on coastal marine organisms have chiefly been made from studies that expose animals to constant levels of temperature or pH (Daufresne et al., 2009; Whiteley, 2011) or the constant combination of the two environmental drivers (Arnberg et al., 2012; Matoo et al., 2013). However, during low tide, air temperatures in the intertidal zone fluctuate dramatically over short periods of time (e.g. 20°C in 6 h) (Stillman and Somero, 1996) and reach extremes when low tides coincide with hot days (Finke et al., 2007; Helmuth, 2002; Stillman and Somero, 2000). Marine intertidal organisms tolerate these natural temperature fluctuations, yet it is known that they are limited in their scope to tolerate future warming (Stillman, 2003). Additionally, pH routinely fluctuates by ≥0.5 pH units between day and night, and by ≥1 pH unit seasonally on temperate rocky shores (Wootton et al., 2008), pH on coral reefs varies by up to 0.5 pH units during day–night cycles (Birkeland et al., 2008), and pH changes rapidly by up to 0.5 pH units during upwelling of CO₂-rich water on the California coast (Feely et al., 2008; Hofmann et al., 2011). While many important inferences regarding organismal responses to ocean warming and ocean acidification have been made in studies that use constant pH and temperature, those inferences may not be ecologically relevant for intertidal zone organisms, for whom daily

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and tidal variability in temperature and pH are the norm (Waldbusser and Salisbury, 2014). Such studies must be refined to better reflect both the natural variation in these environments and the predicted changes in near-shore habitats.

Because temperature is arguably the most important factor influencing the physiology and distribution of ectothermic organisms (Angilletta, 2009; Cossins and Bowler, 1987) and as crustaceans are efficient acid–base regulators (Melzner et al., 2009), we hypothesized that exposure to daily variation in temperature would elicit a larger physiological response than exposure to daily variation in pH, but that there would be an interactive effect of the two environmental drivers because of the effect of temperature on acid–base regulation processes (e.g. ion pumping). Levels of experimental variability were determined by present-day (Hofmann et al., 2011; Stillman and Tagmount, 2009) and future temperature and pH scenarios for coastal regions following models of the Intergovernmental Panel on Climate Change (IPCC) and coastal oceanographic studies (Feely et al., 2008; Waldbusser and Salisbury, 2014).

The porcelain crab *Petrolisthes cinctipes* (Randall) was used as a model intertidal organism to identify the physiological consequences of increased environmental variability on an organism that already experiences considerable variability in nature. We used respiration rate as a proxy for the total energy available for biological processes, and changes in upper thermal tolerance to identify shifts in the cellular stress response (CSR), a critical component of basal maintenance (Sokolova, 2013). We observed a reduced metabolic rate accompanied by increased thermal tolerance under future temperature and pH variability, indicating a potential decline in ecological performance of these crabs due to energetic constraints.

RESULTS

Exposure to the combination of increasing extremes of pH and temperature reduced respiration rates by up to 25% (ANOVA, $F_{2,57}=3.17$, $P<0.05$, Fig. 1A). Exposure to increasing levels of pH variability in isolation did not affect respiration rates (ANOVA, $F_{2,55}=0.08$, $P=0.96$, Fig. 1A), while exposure to increasing temperature extremes in isolation reduced respiration rates by up to 17%, though that reduction was not statistically significant (ANOVA, $F_{2,58}=1.69$, $P=0.17$, Fig. 1A).

Exposure to the combination of the greatest extremes of pH and temperature variation had the strongest effect on thermal tolerance limits, increasing the critical thermal maximum (CT_{max}) by nearly 2°C above controls (ANOVA, $F_{2,56}=10.9$, $P<0.001$, Fig. 1B). Thermal variability in isolation increased CT_{max} (ANOVA, $F_{2,55}=3.16$, $P<0.05$, Fig. 1B) by up to ~1°C at 25°C and no further increases in CT_{max} were observed at 30°C exposure (Fig. 1B). Increasing pH variability in isolation also had a positive effect on CT_{max} (ANOVA, $F_{2,59}=3.15$, $P<0.05$, Fig. 1B) by up to 1°C between pH 8.1 and 7.15 (Fig. 1B).

The striking, interactive physiological effect of temperature and pH variability is apparent in the plots of respiration rate versus thermal tolerance (Fig. 2). Increased pH variability in isolation had a positive effect on the relationship between these two response variables, whereas increased temperature variability in isolation had the opposite effect (Fig. 2). In combination, these effects did not offset, but instead, the combined effect of pH and temperature variability was distinct and greater at both levels of variation (present day and projected future) (Fig. 2).

DISCUSSION

We observed a synergistic interaction of pH and temperature variability on decreasing energy production (respiration rate) and an

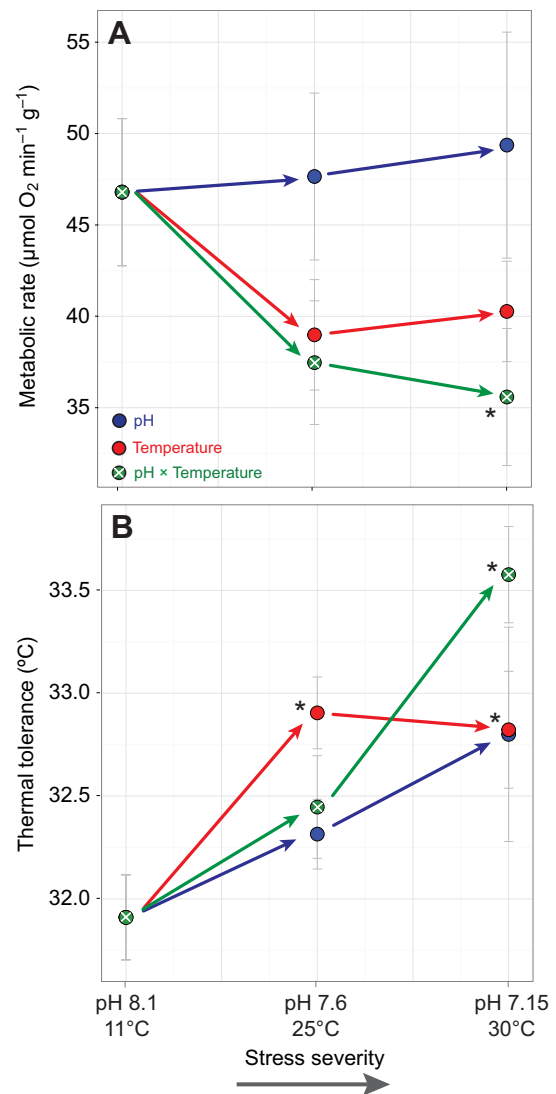


Fig. 1. The effect of increasing pH and temperature stress severity on *Petrolisthes cinctipes*. (A) Metabolic rates; (B) thermal tolerance limits. Increasing pH variability is shown in blue, increasing temperature variability is shown in red, and increasing variability of both is shown in green. Each point is the mean \pm 1 s.e. of $N=17$ – 20 crabs. Asterisks indicate a significant difference from controls (pH 8.1, 11°C), where $\alpha=0.05$.

additive relationship between the two on increasing basal maintenance (i.e. survival, via the CSR). Reduced constant pH alone has been shown to reduce the metabolism of embryonic and larval porcelain crabs (Carter et al., 2013; Ceballos-Osuna et al., 2013) and larval urchins (Dorey et al., 2012), resulting in reduced scope for growth. Yet there are complexities in metabolic responses whereby not all individuals reduce metabolism to the same degree, if at all (Carter et al., 2013; Ceballos-Osuna et al., 2013), suggesting the potential for adaptive responses to ocean acidification exists. Meta-analyses across many taxa indicate reduced calcification and survival in response to ocean acidification (Kroeker et al., 2013). In combination with increased constant temperature, reduced pH has been shown to increase the metabolism of coastal marine organisms such as oysters (Matoo et al., 2013), clams (Matoo et al., 2013), shrimp (Arnberg et al., 2012) and coral (Cumbo et al., 2013). Our results indicate that the addition of environmentally relevant variability of pH and temperature could alter the trajectory of metabolic responses from elevation to

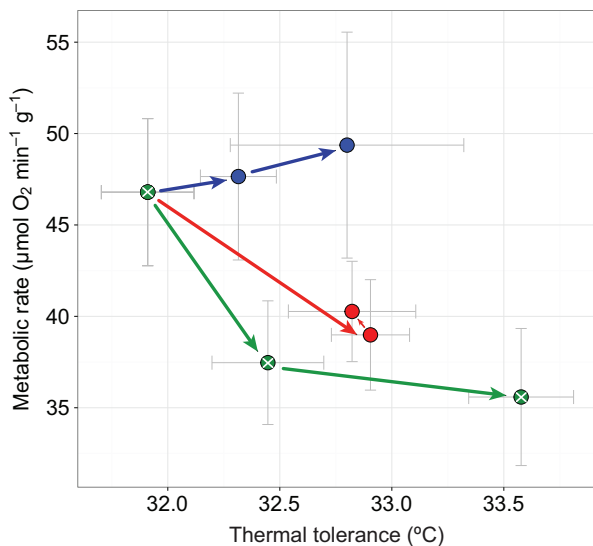


Fig. 2. Relationship between thermal tolerance and metabolic rate.

Points are means \pm 1 s.e. of $N=17-20$ crabs. Blue arrows indicate the effect of variable pH in isolation and extend from pH 8.1 to pH 7.6 to pH 7.15. Red arrows indicate the effect of variable temperature in isolation and extend from daily aerial temperatures of 11°C to 25°C to 30°C. Green arrows indicate the combined effects of variability in pH and temperature and extend from the combination of pH/temperature of pH 8.1/11°C to pH 7.6/25°C to pH 7.15/30°C.

metabolic depression. Metabolic depression may be an energy-saving mechanism for these crabs (Sokolova, 2013) or a method to reduce oxidative damage (Teranishi and Stillman, 2007) during times of extreme environmental variability. Reduced aerobic metabolism can also be an indicator of reduced ecological performance (Sokolova et al., 2012).

Decapod crustaceans possess a well-developed ability to actively reduce hemolymph pH during acidification stress (Melzner et al., 2009). In addition to active acid–base regulation, some species of porcelain crabs in the genus *Petrolisthes* are capable of passively reducing hemolymph pH during times of aerial stress by means of leg membranes that aid in passive respiration (Stillman and Somero, 1996; Stillman and Somero, 2000) and acid–base regulation (Vargas et al., 2010). pH extremes in isolation did not alter metabolic rates of adult *P. cinctipes* in the present study, while early life history stages of *P. cinctipes* (lacking the leg membrane structures) showed negative effects on energetics in response to lowered pH (Carter et al., 2013; Ceballos-Osuna et al., 2013), indicating that leg membrane structures may represent an exaptation for withstanding pH stress in the intertidal zone, providing an energetically inexpensive mechanism for acid–base regulation.

Multi-stressor studies of pH and temperature have illustrated the complex interactions between these variables, which may depend on species, life history stage and geographic range studied (Harvey et al., 2013). In corals, the combined effects of decreased pH and increased warming (increased overall stress) increased rates of microbioerosion of skeletal structures (Reyes-Nivia et al., 2013). Intertidal zone organisms may be able to withstand increased warming and acidification, but with performance effects that could lead to reduced ecological resilience. The exposure to the combination of constant low pH and constant elevated temperature has been shown to reduce growth in intertidal snails (Melatunan et al., 2013) and activity in shrimp (Dissanayake and Ishimatsu, 2011). These kinds of organismal tradeoffs for stress tolerance would be expected to occur for *P.*

cinctipes if exposure to increased stress reduced the energy available for key processes such as growth or activity.

Mechanisms that underlie metabolic suppression during exposure to increased environmental variability are unknown, but may involve adjustments in cellular machinery that change mitochondrial abundance or capacity, enzyme activity, ion and gas transport, and membrane composition, as these are central to generalized responses to altered environments (Hochachka and Somero, 2002; Sokolova, 2013). The CSR is a generalized response to cellular perturbation, whereby separate stressors induce a common stress response pathway involving protein stabilization, DNA repair and free radical scavenging (Kültz, 2005). *Petrolisthes cinctipes* is known to respond to heat stress by upregulation of molecular chaperone genes (i.e. CSR) and downregulation of mitochondrial respiration genes (Teranishi and Stillman, 2007), and that response is known to shift with habitat temperature variability (Stillman and Tagmount, 2009). Following the initial stressor (e.g. thermal stress), the products of stress response pathways may remain, protecting against a subsequent, yet different, stressor (e.g. pH stress), resulting in cross-tolerance (Kültz, 2005). Acidification has been shown to induce CSR heat shock proteins *hsp70* and *hsp90* in gill tissue of zebrafish, demonstrating that cellular responses to acidification share signaling pathways and cross-talk with cellular responses to thermal stress (Sinclair et al., 2013; Tiedke et al., 2013). Even without clear mechanisms, the observed decreases in metabolism combined with increased upper temperature tolerance (i.e. basal maintenance/survival) suggest that limited energy may remain for discretionary metabolic demands [growth, reproduction and/or behavior (e.g. Sokolova, 2013)] and may result in negative ecological consequences due to increased variability in temperature and pH.

Our current findings suggest that acclimatization capacity may be beneficial in the short term (increased tolerance for extreme temperatures), yet detrimental in the long term (reduced overall energy). The potential for evolutionary adaptive responses to interacting stressors is unknown, but will depend on evolutionary capacity of the pathways and tolerance mechanisms involved (Sunday et al., 2014). Assessing the standing genetic diversity of genes involved in those pathways across populations from distinct thermal habitats [e.g. hotspots (Helmuth et al., 2011)] or pH gradients [e.g. upwelling centers (Feely et al., 2008)] could reveal the evolutionary potential that these crabs have to adapt to future change. The role of standing genetic diversity and local adaptation in responding to a single stressor, ocean acidification, has been demonstrated in the purple sea urchin, *Strongylocentrotus purpuratus* (Pespeni et al., 2013), red abalone, *Haliotis rufescens* (De Wit and Palumbi, 2013), and the intertidal zone carnivorous snail *Concholepas concholepas* (Lardies et al., 2014). Here, we highlight the need to understand the potential to adapt to multiple interacting stressors.

In conclusion, by highlighting the importance of environmentally realistic experiments to characterize organismal responses to ocean acidification and ocean warming, we have shown that the coastal impacts of ocean acidification and ocean warming will likely result in a physiological response that will reduce overall energy budgets and have ecological consequences as a result of reductions in energy available for behavior, growth and reproduction.

MATERIALS AND METHODS

Porcelain crab collection and maintenance

Male, adult *P. cinctipes* were collected from Fort Ross, CA, USA (38.5143°N, 123.2438°W) during low tides from September 2012 to January 2013 for a total of five collection dates. Specimens were immediately

transported to the Romberg Tiburon Center for Environmental Studies (Tiburon, CA, USA; transport time ~90 min), where they were placed in recirculating, 20 μm filtered, UV-sterilized seawater ($12\pm 0.5^\circ\text{C}$, salinity 33.3 ± 0.2 , ambient pH 8.09 ± 0.08) for 24 h. Animals were not fed during this 24 h period. On the second day, specimens were acclimated in one of nine combinations (see below) of present day and future variability in pH (CO_2 acidified water) and low-tide air temperature.

Seawater was obtained during high tide from a 15 m deep pipe in San Francisco Bay, settled in a stagnant tank for ≥ 1 week to remove sediment, filtered to 20 μm , treated with activated carbon, UV sterilized, and foam fractionated with a protein skimmer before being added to the recirculating system. In the recirculating system, water flowed continuously past a UV sterilizer before being delivered to any tank, and outflow from any tank was pumped past 100 μm and 20 μm mechanical filters, activated carbon, and a UV sterilizer before being returned to the reservoir. The total volume of the recirculating system was ~10,000 l and ~4000 l of new seawater was exchanged before each acclimation experiment.

Experimental aquaria

Acclimation experiments were performed in acrylic cylinders (one crab per cylinder, 7×15 cm diameter \times height) that had 5.3 cm of reticulated foam filter (Aquatic Ecosystems, PF7) as a base to prevent upwelling of water into the cylinder (Fig. 3). Cylinders were placed in insulated and lidded acrylic tanks (Aqua Logic, USA), where they were arranged in a 5×8 cylinder grid. Each tank had a standpipe to set an upper water height that submerged the crabs by 5 cm, as well as a lower standpipe to set a lower water level that exposed each cylinder to air, but left enough water to regulate the temperature of the air space in the tanks (see below). Each tank received a continuous flow of recirculated seawater. While crabs were immersed, the water in each tank was maintained at $11\pm 0.5^\circ\text{C}$. All animals were fed every other day with equal amounts of Shellfish Diet[®] (Reed Mariculture Inc., www.reedmariculture.com) containing a mixture of 30% *Isochrysis* sp., 20% *Pavlov* sp., 20% *Thalassiosira weissflogii* and 30% *Tetraselmis* sp.

Daytime emersion and temperature ramping

Petrolisthes cinctipes of Northern California (Fort Ross) usually experience a diurnal tidal cycle with low tides coinciding with dawn and dusk, while *P. cinctipes* that are ~450+ miles north (Cape Arago, OR, USA) usually experience low tides during peak sunlight hours (see Stillman and Tagmount, 2009). Crabs perch in moist areas under rocks during low tides, usually gregariously. We chose to expose the specimens in our study to air during a 'low tide' at 12:00 h each day (Fig. 3). Solenoid valves (Hayward

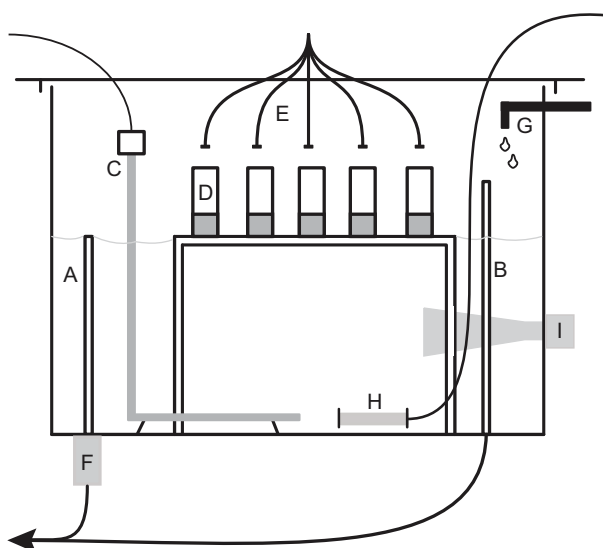


Fig. 3. Depiction of the experimental aquaria that were used in the 18 day acclimation period. (A) Lower standpipe, (B) upper standpipe, (C) titanium heater, (D) crab cylinders, (E) pH drippers, (F) solenoid valve, (G) seawater inflow, (H) air bubbler and (I) circulating pump.

PVC/CPVC) controlled by a timer opened to enable tanks to drain over 25 min, so that by 12:30 h each day the crabs were exposed to air. During low tide, air temperatures were regulated within each sealed tank by vigorously bubbling the water below the cylinders to thermally equilibrate the air and water (Fig. 3).

To increase air temperature during low tide, immersion heaters in the water, regulated by temperature controllers whose temperature sensors were in the air, heated the air at 2.6 or 3.7°C h^{-1} for 5 h (maximum air temperature of 25 and 30°C , respectively, Fig. 4B). Water was chilled back to $11\pm 0.5^\circ\text{C}$ before the crabs were resubmerged. At 18:30 h, the solenoid valves closed to fill the tanks, submerging the crabs and ending the low tide period (Fig. 4B). Crabs in the tank that had no heating experienced $11.0\pm 0.5^\circ\text{C}$ air temperatures during aerial exposure (Fig. 4B).

Night-time pH variability

To alter the pH each crab experienced, water was pumped from reservoirs containing ambient or CO_2 -acidified water. Water was delivered through vinyl tubing with 0.94 l h^{-1} irrigation drippers (Netafim 71) that were threaded through the lids of the tanks and suspended above each cylinder (Fig. 4A). The irrigation drippers ensured equal flow rates to each cylinder. Pumps delivered water for 7 h (03:00 h to 10:00 h) from the ambient pH water tank ($\sim\text{pH } 8.09\pm 0.08$), or for 5 h (04:00 h to 09:00 h) from the low pH water tank ($\text{pH } 7.59\pm 0.08$ or 7.14 ± 0.07 , depending on experimental treatment, Fig. 4A). Water in all cylinders was automatically re-equilibrated to ambient conditions through air exposure and through vigorous aeration of the circulating water by air pumps and protein skimmers by 10:30 h; thus, all crabs experienced the same pH and temperature conditions prior to the

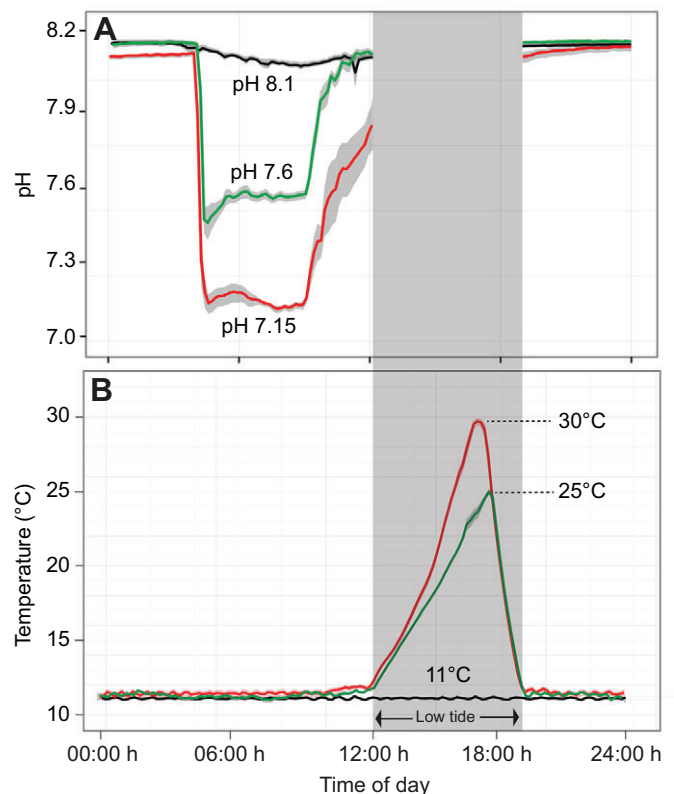


Fig. 4. Conditions of pH and temperature variability during the 18 day acclimation period. (A) pH variability occurred from 04:00 h to 09:00 h with pH values of 8.1, 7.6 and 7.15. Lines represent mean pH during the acclimation period (across all days) and shaded areas represent 95% confidence intervals. (B) Temperature variability occurred from 12:00 h to 19:00 h with temperatures of 11, 25 and 30°C . Lines represent mean temperature during the acclimation period (across all days) and shaded areas represent 95% confidence intervals. Crabs experienced aerial exposure 'low tide' between 12:00 h and 19:00 h, indicated by the shaded box.

period of emersion (Fig. 4). pH was monitored in randomly chosen cylinders at 1 min intervals throughout each experiment using an Omega pH electrode attached to an Omega pH/H-SD1 pH data logger. The electrode was moved to a different cylinder each day. The probe was calibrated every 5 days with NBS buffers 4, 7 and 10 (Fisher, SB101, SB110, SB115). In addition to logging pH with the probe, pH was also determined spectrophotometrically biweekly during the experiment (see below).

Water was acidified to pH 7.6 and 7.15 using compressed CO₂ gas (Praxair). A pH electrode (Fisher Scientific, Accumet pH Submersible Combination Electrode no. 13-620-Ap56) and pH controller (Professional, pH-ORP 2002C controller) activated a solenoid valve on the CO₂ tank regulator (Widdicombe and Needham, 2007). The pH electrode was placed in an insulated and covered ~600 l CO₂ mixing tank containing water held at 12±1°C. When the pH was above the setpoint of the pH controller, CO₂ gas was introduced into the mixing tanks using a wooden air diffuser. To prevent background respiration from altering the P_{CO₂}, the water was continuously circulated through a 0.2 µm filter. Electrodes were calibrated once per week during experiments using a two-point calibration with NBS buffers 7 and 10 (Fisher, SB110, SB115). The CO₂ mixing tank was additionally bubbled with ambient air for oxygenation. Each day, the mixing tank was filled with recirculated seawater between 10:00 h and 12:00 h, CO₂ acidified, and pumped into the cylinders 16–18 h later (see above). Salinity did not change during the short period that the water was being acidified in the tank.

Water chemistry

Seawater samples to determine pH were collected bi-weekly from random cylinders between 08:45 h and 09:00 h when pumps were delivering acidified or ambient water to the cylinders. Seawater pH was monitored daily with electrodes and measured spectrophotometrically using *m*-Cresol Purple sodium salt dye (PharmaSpec UV-1700, Shimadzu, Columbia, MD, USA; Sigma-Aldrich no. 211761, St Louis, MO, USA) following the modified protocol of DOE (DOE, 1994). Seawater samples for alkalinity were collected at the same times as pH. Total alkalinity by mass (µmol kg⁻¹ seawater) was calculated through a linear Gran procedure using a Metrohm dosimat 765 pH-Meter (Metrohm Herisau Switzerland) (Gran, 1952). Nitrogen species (nitrate, nitrite and ammonium) were analyzed by colorimetric assay using test kits (Salifert, American Pharmaceuticals), and were consistently undetectable above 0 mg l⁻¹.

Additional parameters of the seawater carbonate chemistry including [HCO₃⁻], [CO₃²⁻], P_{CO₂}, Ω_{calcite} and Ω_{aragonite} (where Ω is the saturation state) were calculated using SeaCarb 2.4.8 (Lavigne et al., 2013) in the statistical computing program R v 3.0.1 (R Development Core Team, 2013). Seawater salinity, temperature, pH and alkalinity were used as input parameters (flag 8) using the total pH scale (pH_T). Total phosphate and total silicate were assumed to be 0 mol kg⁻¹, though measurements were not taken. Total alkalinity was high relative to open ocean water for our study in all pH treatments (3133±133 µequiv kg⁻¹ at pH 8.1, 2702±360 µequiv kg⁻¹ at pH 7.6 and 2873±235 µequiv kg⁻¹ at pH 7.15, Table 1), reflecting the influence of terrigenous input in our coastal/estuarine water source (Duarte

et al., 2013). Calcite and aragonite saturation states did not fall below 1 at pH 8.1 and 7.6, though aragonite did fall to 0.6 at pH 7.15 (Table 1).

Oxygen consumption rate measurements

Oxygen consumption rate measurements were conducted after 17 days of exposure to the experimental treatments, using closed respirometry vials (*N*=17–20 crabs per treatment). Metabolic rates were measured at 18°C, the maximal average body temperature of *P. cinctipes* in nature (Stillman and Tagmount, 2009). Crabs were collected from the acclimation tanks at 10:00 h, following the low pH exposure, and were immediately placed in 70 ml glass vials (one crab per vial, Fisher Scientific) containing 18°C, 0.2 µm filtered seawater at ambient P_{CO₂} (salinity 33, pH 8.05) and a 4×4 cm piece of mesh to stabilize the crabs and minimize disturbance during measurement. Vials were sealed with a plastic screw lid containing a gas-tight rubber gasket. A magnetic impeller built into the vial lid help to ensure the water in the vial was well mixed and prevented the formation of oxygen concentration gradients. Planar optode spots (diameter 0.5 cm; PreSens Precision Sensing GmbH) were glued to the inside of each vial and calibrated using O₂-saturated and O₂-free sterile seawater prior to each sampling experiment. A Fibox T3 oxygen meter (PreSens Precision Sensing GmbH) was used to measure the oxygen saturation in each vial using PST6v541 software (PreSens Precision Sensing GmbH). Once sealed, the vials were placed in a dark, recirculating water bath at 18°C for a 10 min recovery period. Oxygen was measured every 10 min for 40 min in a custom-milled aluminium block that maintained temperature and aligned the O₂ probe and sensor spot. Oxygen saturation of the water never went below 75–100% O₂ saturation. A ‘blank’ vial containing no crab was run alongside each set of animals. After respirometry, each crab was towel dried and fresh mass was measured to the nearest 0.1 g. Slopes were calculated by the decrease of oxygen in the vial per hour and normalized to fresh mass to get respiration rates in units of µmol O₂ min⁻¹ g⁻¹.

Upper thermal tolerance limits of cardiac performance (CT_{max})

After 18 days, thermal tolerance limits were indexed by cardiac CT_{max} (Stillman, 2003). Specimens were collected from the acclimation tanks at 10:00 h immediately following low pH exposure. A small piece of cork (1.5×1.9 cm) was glued to the carapace of each crab using cyanoacrylate glue (superglue) as a handle for immobilizing each crab. Pinholes through the carapace were made laterally on either side of the heart and 25 µm diameter impedance electrodes (ceramic coated copper wire) were inserted into each hole and glued into place with cyanoacrylate glue. Specimens were immersed in an aerated, temperature-controlled seawater bath at 12°C. Impedance electrodes were connected to a PowerLab 16sp data acquisition (ADInstruments) unit and crab heart rates were recorded continuously using the Chart v. 5 recording software (ADInstruments). Surgery never resulted in mortality and specimens were given a 30 min recovery period at a constant temperature of 12°C in which heart rates decreased to a baseline rate (see supplementary material Fig. S1) before ramping began. Temperature was increased at 0.1°C min⁻¹ for 5 h, a rate determined to be environmentally realistic for a thermally extreme day (Stillman and

Table 1. Parameters of the carbonate system measured or calculated throughout the duration of the experiments

Parameter	Ambient	Moderate	Extreme	<i>F</i> _{d.f.}	<i>P</i>
Salinity	33.6±0.2	33.2±0.4	33.8±0.6		
Temperature (°C) ¹	21.3±0.9	21.3±0.6	22.0±0.01		
pH _T ²	8.12±0.02	7.60±0.04	7.15±0.10	128 _{2,10}	<0.001
TA (µequiv kg ⁻¹) ²	3133±133	2702±360	2873±235	1.1 _{2,10}	0.38
DIC ^{2,3}	2752±125	2616±329	2967±202	0.5 _{2,10}	0.63
P _{CO₂} (µatm) ^{2,3}	461±50	1476±15	4801±668	116 _{2,10}	<0.001
[HCO ₃ ⁻] (µmol kg ⁻¹) ^{2,3}	2443±116	2475±304	2781±212	0.7 _{2,10}	0.54
[CO ₃ ²⁻] (µmol kg ⁻¹) ^{2,3}	294±15	95±25	38±10	51.8 _{2,10}	<0.001
Ω _{calcite} ^{2,3}	7.1±0.4	2.3±0.6	0.9±0.3	50.9 _{2,10}	<0.001
Ω _{aragonite} ^{2,3}	4.6±0.2	1.5±0.4	0.6±0.2	48.9 _{2,10}	<0.001

ANOVA were used to assess statistical differences among treatments. Values represented are means ± 1 s.e.

pH_T, total pH scale; TA, total alkalinity; DIC, dissolved inorganic carbon; Ω, saturation state.

¹Average temperature across the entire acclimation period. ²pH and other measurements of the carbonate system reflect water conditions at the peak of the pH variability (Fig. 4). ³Parameter calculated in SeaCarb package for R statistical computational program.

Tagmount, 2009). Heart rate was monitored for a total of 5 h and was expressed as beats min^{-1} or transformed to the natural logarithm of beats min^{-1} for Arrhenius plots. An Arrhenius break temperature (ABT) represents the temperature during a thermal ramp at which heart rate dramatically changes (typically from a steady increase with temperature to an abrupt decrease). ABTs were determined in Microsoft Excel by fitting linear regressions to ascending and decreasing heart rate data (pre- and post-ABT). The temperature at which these regressions intersected was considered the ABT (Stillman, 2003). ABTs were cross-checked using the *segmented* package in the statistical computing program R (v 3.0.1), which generated ABTs identical to those generated in Microsoft Excel. Data shown were calculated from Microsoft Excel.

Statistical analyses

All statistical analyses were carried out using the R statistical program (v 3.0.1). Metabolic rate and CT_{max} data were analyzed by two-way ANOVA (with nightly pH spike and daily temperature spike as independent or combined fixed factors depending on best-fit modeling). Planned comparisons for specific pairwise analysis were done with one-way ANOVA. All residual errors were tested for normality using QQ normality plots, and data were log transformed to meet assumptions of normality or non-parametric Kruskal–Wallace analysis was applied. Tukey HSD *post hoc* comparisons were made to identify significant differences between treatments. Values reported in the text are means \pm 1 s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.H.S. and A.W.P. conceived the study. A.W.P. designed and constructed the experimental apparatus and collected the data. A.W.P., N.A.M. and J.H.S. analysed and interpreted the data and wrote the manuscript.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.109801/-DC1>

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