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RESEARCH ARTICLE

The role of stochastic thermal environments in modulating the thermal physiology of an intertidal limpet, *Lottia digitalis*

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ABSTRACT

Much of our understanding of the thermal physiology of intertidal organisms comes from experiments with animals acclimated under constant conditions and exposed to a single heat stress. In nature, however, the thermal environment is more complex. Aerial exposure and the unpredictable nature of thermal stress during low tides may be critical factors in defining the thermal physiology of intertidal organisms. In the fingered limpet, *Lottia digitalis*, we investigated whether upper temperature tolerance and thermal sensitivity were influenced by the pattern of fluctuation with which thermal stress was applied. Specifically, we examined whether there was a differential response (measured as cardiac performance) to repeated heat stress of a constant and predictable magnitude compared with heat stress applied in a stochastic and unpredictable nature. We also investigated differences in cellular metabolism and damage following immersion for insights into biochemical mechanisms of tolerance. Upper temperature tolerance increased with aerial exposure, but no significant differences were found between predictable treatments of varying magnitudes (13°C versus 24°C versus 32°C). Significant differences in thermal tolerance were found between unpredictable trials with different heating patterns. There were no significant differences among treatments in basal citrate synthase activity, glycogen content, oxidative stress or antioxidants. Our results suggest that aerial exposure and recent thermal history, paired with relief from high low-tide temperatures, are important factors modulating the capacity of limpets to deal with thermal stress.

KEY WORDS: Variability, Environmental predictability, Stress tolerance, Cardiac performance, Temperature

INTRODUCTION

Global climate change is projected to raise mean sea surface and air temperatures, as well as increase the frequency of extreme heat waves throughout the world (IPCC, 2013). With these changes occurring at unprecedented rates, it is important to understand whether contemporary animals have the capacity to cope with these changes, and to determine what physiological mechanisms define their ability to tolerate any further change in environmental temperatures (Helmuth et al., 2005; Pörtner and Farrell, 2008; Hofmann and Todgham, 2010; Somero, 2012). Essential to our capacity to predict vulnerability of species to future environmental change is a confidence that our estimates of current tolerance limits and response capacity are accurate. It is thought that more accurate predictions will be grounded in experiments that incorporate realistic variability and complexity in an animal's natural environment (Helmuth, 2002; Helmuth et al., 2014; Montalto et al., 2016). Additional empirical evidence is needed to understand to what extent incorporating realistic variability and complexity in an animal's natural environment into experimental design will improve our accuracy of predictions of thermal tolerance.

The rocky intertidal zone is a highly variable environment where organisms experience daily changes between marine and terrestrial conditions with the ebb and flow of tides. Several studies have demonstrated that intertidal organisms living high in the rocky intertidal are already experiencing temperatures close to their physiological tolerance limits (e.g. Somero, 2002; Stillman, 2003). In fact, in recent years, extreme heating events have caused mass mortalities of rocky intertidal organisms (Petes et al., 2007; Harley, 2008; Denny et al., 2009; Firth and Williams, 2009). Until recently, much of our understanding of the thermal physiology of intertidal organisms has come from experiments where animals were exposed to single, acute increases in temperature when submerged in water, an unrealistic environmental combination as intertidal organisms experience the greatest increases in temperature during low tide, when aerially emersed (Tomanek and Somero, 2000; Stenseng et al., 2005; Gardeström et al., 2007; Diederich and Pechenik, 2013; Madeira et al., 2014). To simplify experimental designs, many studies that have incorporated aerial emersion with heat stress have acclimated intertidal organisms under constant ambient ocean conditions rather than simulating tidal cycles (Helmuth et al., 2010; Logan et al., 2012; Dowd and Somero, 2013; Bjelde and Todgham, 2013; Zhang et al., 2014; Bjelde et al., 2015). Although there have been several experimental studies that have acclimated organisms to more realistic tidal cycles, our understanding of the role of repeated daily fluctuations in temperature, more indicative of natural conditions, is still deficient (McMahon et al., 1991; Marshall and McQuaid, 1992; Dong and Williams, 2011; Han et al., 2013; Dowd et al., 2015).

Currently, we have a limited understanding of what aspects of the environmental complexity of the thermal environment characterizing the rocky intertidal (e.g. repetitive nature of heat stress over low tide periods, unpredictable or stochastic magnitudes of temperature change) are important in structuring the thermal physiology of intertidal organisms (Denny et al., 2011; Montalto et al., 2016). Intertidal organisms can better tolerate heat stress during emersed conditions (low tide) compared with submersed conditions (high tide) (Wolcott, 1973; Jones et al., 2009; Bjelde and Todgham, 2013; Huang et al., 2015). These studies provide evidence that intertidal species may have the capacity to maintain or recruit physiological mechanisms when aerially emersed that better equip them to tolerate heat stress. Some intertidal organisms are also better able to tolerate heat stress if first exposed to a sublethal heat shock (Todgham et al., 2005; Dong et al., 2010;

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Giomi et al., 2016; Pasparakis et al., 2016). This phenomenon, known as heat hardening (Bowler, 2005), is a very important inducible stress tolerance mechanism in many organisms, both terrestrial and aquatic, inhabiting variable environments (Maness and Hutchinson, 1980; Rutledge et al., 1987; Middlebrook et al., 2008; Bilyk et al., 2012). Previous studies have shown fluctuating thermal environments increase thermal tolerance (Feldmeth et al., 1974; Otto, 1974; Threader and Houston, 1983; Woiwode and Adelman, 1992; Schaefer and Ryan, 2006; Oliver and Palumbi, 2011; Manenti et al., 2014; Kern et al., 2015), with intertidal species exposed to tidal cycle fluctuations being more stress-tolerant than those that are exposed to constant temperatures (Tomanek and Sanford, 2003; Podrabsky and Somero, 2004; Todgham et al., 2006; Giomi et al., 2016). Taken together, these studies suggest that the thermal physiology of intertidal organisms is likely modulated by the natural variability inherent with the ebb and flow of tides. The stochastic or unpredictable nature of temperature fluctuations over tidal cycles could also be playing a large but underappreciated role in the thermal tolerance of intertidal species (Denny et al., 2009; Denny and Dowd, 2012), as has been shown for model species including zebrafish (*Danio rerio*) and the fruit fly *Drosophila simulans* (Schaefer and Ryan, 2006; Manenti et al., 2014).

Although cellular stress response mechanisms to heat stress have been extensively studied in intertidal organisms (Hofmann and Somero, 1995; Roberts et al., 1997; Sokolova and Pörtner, 2001; Todgham et al., 2005; Gardeström et al., 2007; Dong et al., 2008a,b; Han et al., 2013; Zhang et al., 2014), only a few studies have considered the mechanistic strategies under unpredictable heating conditions associated with natural tidal cycles (Gracey et al., 2008; Connor and Gracey, 2011). Stress response mechanisms are energetically costly and require animals to increase metabolic rates (Sokolova et al., 2012). Under heat stress, the activity of metabolic enzymes, such as citrate synthase, can also increase, which allows for a higher aerobic capacity (Sokolova and Pörtner, 2001; Morley et al., 2009; Kern et al., 2015). Increased metabolic rates under stressful conditions can cause animals to deplete glycogen energy stores (Santini and Chelazzi, 1995; Lim et al., 1996; Leung and Furness, 2001; Palais et al., 2011; Bjelde and Todgham, 2013; Goh and Lai, 2014) and increase the production of reactive oxygen species (Abele et al., 2002; Kültz, 2005; Han et al., 2013; Zhang et al., 2014). Antioxidant defense mechanisms can be upregulated in order to deal with high levels of oxidative stress (Pannunzio and Storey, 1998; Abele et al., 2002; Malanga et al., 2004; Dong et al., 2008a; de Oliveira et al., 2015). Organisms have energetic and defensive cellular and physiological strategies to mitigate the impacts of stress; however, how animals prepare for the unpredictable nature of low tide periods in the intertidal zone is not well understood.

The overall objective of this study was to investigate how the predictability of temperature change during daytime low tide periods modulated the upper thermal tolerance and constitutive cellular mechanisms of energy metabolism and antioxidant defense of the limpet *Lottia digitalis* (Rathke 1833). *Lottia digitalis*, the fingered limpet, is a rocky intertidal species that is found in the middle to upper intertidal zone and is routinely exposed to large fluctuations in environmental variables during low tide periods (Wolcott, 1973). Specifically, we examined how repeated heat stress of constant and predictable magnitude versus that which is stochastic or unpredictable in nature modulated the temperature sensitivity and upper temperature tolerance of limpets to an extreme heat wave. We measured cellular mechanisms underlying stress tolerance including glycogen levels and citrate synthase activity to better understand energy availability, and antioxidant and oxidative stress levels to give insight into cellular defense mechanisms available immediately prior to a midday low tide period. We hypothesized that a stochastic tidal regime would increase protective mechanisms that would provide limpets with reduced temperature sensitivity and higher upper temperature tolerance. We predicted that *L. digitalis* acclimated to stochastic tidal regimes would be less sensitive and have a higher tolerance to heat stress than limpets exposed to predictable temperatures. The variability in the magnitude of temperature increase during periods of emersion would prime the limpets to maintain protective mechanisms when exposed to the highest temperatures; however, emersion periods with low to moderate increases in temperature would provide limpets with reprieve from consistently high low-tide temperatures, which could minimize accumulated damage from heat stress and improve efficiency in the recruiting of protective mechanisms (Hofmann and Somero, 1996; Gracey et al., 2008; Denny et al., 2011; Zhang et al., 2014; Huang et al., 2015).

MATERIALS AND METHODS

Limpet collection

Lottia digitalis were collected during low tide from the mid-upper intertidal zone at Fort Ross, CA, USA (38°30′45.79″N, 123°14′45.58″W). Limpets (length range: 14.7–20.5 mm; mass range: 486.3–1771.6 mg) were removed from rocks gently, inspected for foot damage and undamaged limpets were put into a cooler to be transported back to San Francisco State University's Romberg Tiburon Center for Environmental Studies in Tiburon, CA, USA. Only limpets that could be removed on the first try, before they could secure themselves tightly to the rock surface, were collected. Collection and transport lasted no longer than 3 h. Limpets were held in 13°C recirculating rectangular tanks for at least 24 h before the start of the acclimation trials.

Acclimation to simulated tidal conditions

Tanks were built to simulate circa-tidal water levels and temperatures to mimic natural intertidal conditions during high and low tides. Tanks were designed to be flow through and continuously flushed with fresh seawater during high and low tides. Heat budget models for limpets have shown that substrate temperature is the primary driver of limpet body temperature (Bjelde and Todgham, 2013; Denny and Harley, 2006) and therefore substrate temperatures were manipulated to modulate limpet body temperature during low tide periods (Bjelde and Todgham, 2013; Bjelde et al., 2015; Pasparakis et al., 2016). Substrate temperature and water height were manipulated using Arduino microcontrollers (Arduino YUN, Adafruit, New York, NY, USA; Miller and Long, 2015). Limpets were confined to a 15×15 cm aluminum block heated by an internal silicon rubber heater sheet (180 W, McMaster-Carr, USA), and covered with 3 mol l^{-1} Safety Walk Tread Tape. The Arduino controller monitored temperature of the heat block surface through a temperature sensor encased in epoxy within a limpet shell, attached to the heat block surface (similar construction to 'Robolimpets', Lima and Wethey, 2009). The temperature of heat block surface (and limpet body temperature) was regulated and ramped at specified rates. Limpets were randomly divided between one of five different acclimation treatments and held under these conditions for 2 weeks (Figs 1 and 2): (1) unpredictable: variable (unpredictable and stochastic) tidal regime with ambient seawater conditions (~13°C) and varying aerial conditions within the range of 13 to 32°C (based on intertidal data loggers, see below); (2) predictable moderate: consistent/predictable tidal regime of

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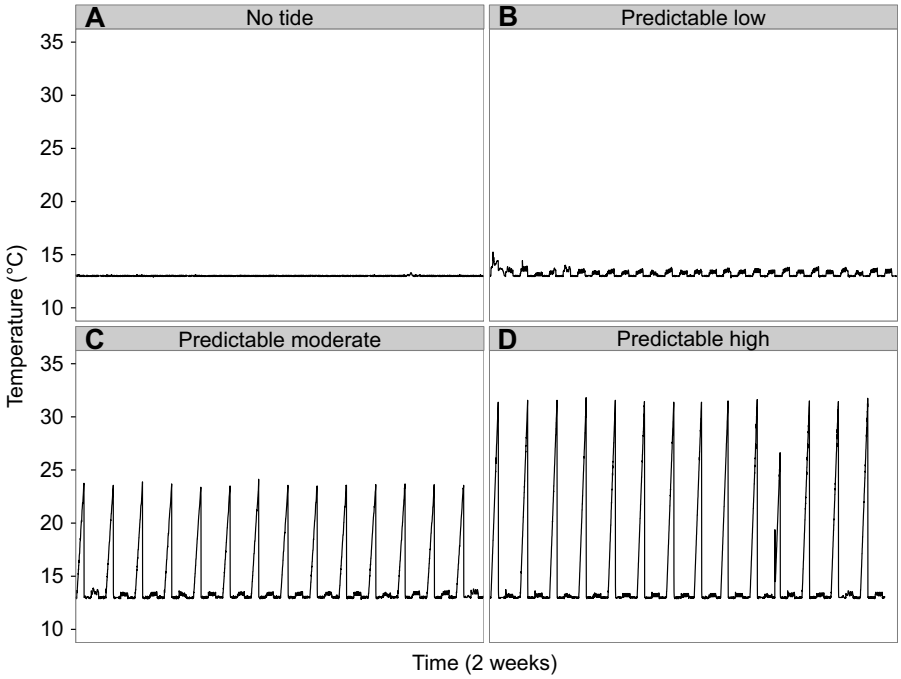

Fig. 1. Temperature profiles for predictable acclimation treatments over the 2-week acclimation period. Temperature measurements were taken every 5 min. Temperature data shown are from one trial, but the same acclimation treatments were repeated in all three trials. Each trial was a technical replicate. (A) Limpets in the no tide treatment were submersed in water during the entire acclimation period. (B) The predictable low treatment had low tide periods twice daily, but no heating occurred during low tide. (C) The predictable moderate treatment had low tide periods twice daily, with heating to 24°C during daytime low tide. (D) The predictable high treatment had low tide periods twice daily, with heating to 32°C during daytime low tide.

ambient seawater conditions (~13°C) and aerial conditions at a moderately elevated temperature (~24°C) every day equal to the total heating budget of the unpredictable tidal regime during the acclimation period; (3) predictable high: consistent/predictable tidal regime of ambient seawater conditions (~13°C) and aerial conditions at a highly elevated temperature equal to the highest temperature reached in the unpredictable tidal regime (~32°C) every day; (4) predictable low: consistent/predictable tidal regime of ambient seawater conditions (13°C) with ambient aerial exposure accompanied by no heating (13°C); and (5) no tide: no tidal pattern with ambient seawater conditions (~13°C) and no aerial exposure.

Temperature acclimation treatments were based on data from temperature loggers (Maxim Integrated Products, Dallas, TX, USA) embedded in the rock next to *L. digitalis* that continuously monitored temperature every 10 min from April 2011 to March 2013 at Fort Ross. The temperature data set provided two summers of temperature profiles indicative of the variable heating regimes experienced by limpets in nature at a upper mid-intertidal location (Bjelde and Todgham, 2013; Pasparakis et al., 2016). The average degree of heating during the low tide periods of the summer months of 2011 and 2012 for each 2-week time interval was calculated. Within the temperature logger data, a 2-week period of natural cycles in environmental temperature was found that that matched the average total degree heating for summer 2011 and 2012. This 2-week period of variable and unpredictable changes in daily temperature was used for our unpredictable treatment trial 1. To ensure testing of stochasticity, the order of peaks in daily temperatures of the unpredictable treatment was randomized

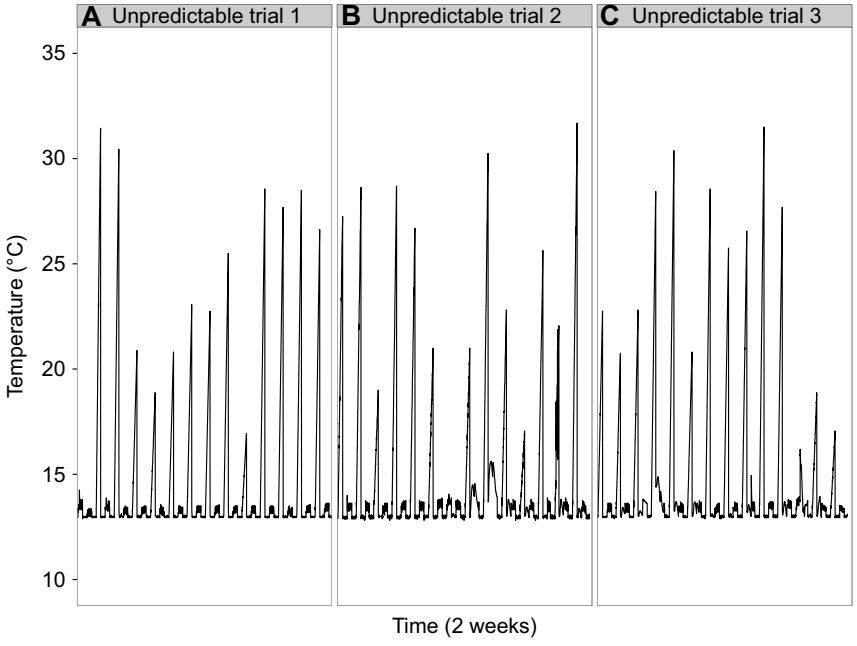

Fig. 2. Temperature profiles for unpredictable acclimation treatments over the 2-week acclimation period. All unpredictable acclimation treatments had two low tide periods daily, with heating only occurring during the daytime low tide. Temperature measurements were taken every 5 min. (A) Unpredictable trial 1 was taken directly from temperature data calculated as the average week at Fort Ross, CA, USA. (B,C) Unpredictable trials 2 and 3 included the same temperature peaks from unpredictable trial 1, but they were randomized in two different orders to test for the effects of stochasticity.

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(trials 2 and 3) so that for each trial the limpets experienced the same temperature peaks during low tide emersion, but in a different order. The predictable moderate treatment was designed to expose limpets to the same degree of heating throughout the 2 weeks as the unpredictable treatment (462°C h^{-1} over 14 days), but in a predictable manner (i.e. 24°C every daytime low tide period). The predictable high treatment was chosen by taking the hottest low tide period from the unpredictable treatment (i.e. 32°C) and providing that magnitude of temperature increase during every daytime low tide period for the 2-week period. In order to test the role of aerial exposure alone with no temperature increases, a predictable low treatment was designed. Lastly, as a control, limpets were held submersed under water for the entire 2-week period under constant temperature (i.e. no tide treatment). Each acclimation treatment, except the no tide treatment, also experienced a nighttime low tide; however, there was no heating associated with nighttime low tide periods in any of the acclimation treatments. Each low tide period occurred at the same time of day for the entire 2-week acclimation, lasted for 6 h, and the two low tides were separated with 6 h of high tide. Temperatures for all acclimation treatments throughout the 2-week acclimation periods were recorded on an SD card by the Arduino every 10 s, as well as uploaded onto a Google spreadsheet every 10 min.

All five acclimation treatments were conducted simultaneously and repeated three times in succession. Acclimation treatments were rotated so that treatments occurred in different tanks each trial. During acclimation trials, water quality parameters including temperature, salinity, dissolved oxygen, nitrate, nitrite and ammonia were checked twice a week to ensure acceptable conditions for the limpets. Temperature, salinity and dissolved oxygen were monitored using a YSI Model 85 m (YSI Incorporated, Yellow Springs, OH, USA) and nitrate, nitrite and ammonia were checked using an API saltwater test kit (API, Chalfont, PA, USA).

Limpet feeding

Limpets were fed by applying an agar/algae mixture to the tread tape on the surface of the aluminum heat block 1 day prior to limpets being placed in the tank, in order for limpet grazing to occur with a consistent amount of food across acclimation treatments (Hiratsuka and Uehara, 2007). *Ulva lactuca* (sea lettuce) was collected and 5 g (wet mass) of *U. lactuca* and 50 ml of seawater was ground in a blender until the algae was liquefied. The algae solution was then mixed well with heated agar solution (800 µl of algae solution and 120 mg agar in 4 ml of seawater) and the mixture was evenly spread across the surface. Preliminary trials were conducted to determine how much mixture to apply to the surface to allow for feeding over the entire 2-week experimental trial. The agar/algae mixture was only applied once prior to the start of the experiment. Throughout the 2-week acclimation periods, limpets in all acclimation treatments were observed to be grazing and at the end of each trial there was still a small amount of remaining algae.

Cardiac performance under ramping increases in temperature

Changes in upper temperature tolerance were evaluated by examining upper critical thermal limits of cardiac performance. Heart rates were monitored and analyzed following methods modified for limpets (Bjelde and Todgham, 2013). Fifteen limpets from each acclimation treatment were exposed to ramped increases in temperature until heart function drastically declined (defined as break point temperature, BPT) and then ceased (defined as flat line temperature, FLT). Exposure of limpets to the lethal heat ramp was timed so that it would occur at the start of the scheduled daytime low tide period during acclimation. Two small holes were drilled into the limpet shell the day before the cardiac performance trials, 1 h after the end of the daytime low tide in the acclimation treatments. The morning of the heat ramp, impedance electrodes were implanted into the air cavity between the shell and limpet, directly above the heart. Limpets were then placed on a temperature-controlled aluminum block in air. After a 30-min period at 13°C, temperature of the aluminum block was increased at a rate of 0.1°C min^{-1} (6°C h^{-1}), calculated from field data as the average heating rate in nature during summer daytime low tide periods. Cardiac performance was assessed by measuring beats per minute throughout the entire heat ramp using PowerLab and Chart 8 software (ADInstruments, Colorado Springs, CO, USA).

Cardiac performance analysis

Multiple measures of cardiac performance were used to determine upper critical thermal limits of cardiac performance and temperature sensitivity of heart rate. All analyses were performed using R (www.R-project.org). Final BPT was measured as the highest temperature at which heart rate drastically decreased and is considered to be the upper critical thermal limit of cardiac performance of intertidal organisms, including limpets (Stillman and Somero, 1996; Bjelde and Todgham, 2013). To calculate final BPT, individual limpet heart rates (beats min^{-1}) were plotted against temperature and best-fit regression lines were found for the ascending portion of heart rate and for the descending portion of the heart rate as described in Bjelde and Todgham (2013). The final BPT of each limpet was determined to be the intersection between the two best-fit regression lines (Fig. S1). FLT was measured as the highest temperature at the point where heart rate ceased, and determined by the temperature at which the last heartbeat was recorded. The difference (FLT–BPT) and ratio (FLT/BPT) of FLT and BPT were calculated to determine the temperature range of suboptimal performance past the final BPT. Maximum heart rate (V_{max}) for each individual limpet was measured as the highest heart rate recorded during the lethal heat ramp. Lastly, temperature sensitivity of heart rate was examined using thermal performance curves.

Limpet tissue sampling

Following the 2-week acclimation, foot tissue samples were dissected from five limpets from each acclimation treatment for each replicate trial. Foot tissue was dissected from limpets immediately prior to cardiac performance trials to assess the physiological condition, through biochemical assays, of the limpets in each treatment immediately prior to being assessed for upper temperature tolerance. Limpets sampled for biochemical analyses did not undergo the cardiac performance trials. Tissue samples were immediately frozen on dry ice and stored at −80°C until analyses were performed.

Glycogen content

Glycogen content was measured to quantify energy stores in limpet foot tissue as described by Bjelde and Todgham (2013). Briefly, foot tissue was frozen in liquid nitrogen, powdered, and then approximately 20 mg was homogenized in 1 ml of cold 8% $HClO_4$ on ice using a tissue homogenizer (PROScientific, Oxford, CT, USA). The homogenate was split into two tubes – 200 µl was used to measure glycogen, and free glucose was measured in the remaining homogenate. The glycogen homogenate was broken down enzymatically using methods from Hassid and Abraham

(1957). Once the glycogen samples were broken down, glucose was measured in all samples using methods from Bergmeyer (1983), modified for a microplate spectrophotometer (Synergy HT, Biotek, Winooski, VT, USA).

Citrate synthase activity

Enzymatic activity of citrate synthase was quantified in limpet foot tissue to assess cellular aerobic capacity (Morley et al., 2009). Limpet foot tissue frozen in liquid nitrogen, powdered, and then approximately 15 mg was homogenized by hand in 200 µl of ice-cold 50 mmol l^{-1} potassium phosphate buffer (pH 6.8). Samples were then centrifuged at 1000 ***g*** for 10 min at 4°C and the supernatant was transferred to a new microcentrifuge tube. Samples were diluted fivefold in order to best capture citrate synthase activity. On a clear, polystyrene 96-well plate, 10 µl of each sample was added in triplicate and 200 µl of citrate synthase buffer (50 mmol l^{-1} imidazole pH 8.2, 1.5 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} Elman's reagent and 0.12 mmol l^{-1} acetyl CoA) containing 0.5 mmol l^{-1} oxalacetic acid was quickly added. A second set of triplicates was used as a blank and 200 µl of citrate synthase buffer without the substrate was added to measure background activity. Enzymatic activity was measured using a microplate spectrophotometer (Biotek Synergy HT) set to read at 412 nm at 25°C for 2 h using a kinetic sweep, and the maximum slope of change in absorbance was calculated (Biotek Gen5 software). Citrate synthase activity was calculated by subtracting the mean background rate from the mean enzyme rate for each sample. Total protein concentration of foot tissue samples was measured using the bicinchoninic acid assay (Smith et al., 1985) with bovine serum albumin as a protein standard (Thermo Fisher Scientific, Rockford, IL, USA). Citrate synthase specific activity (in micromoles of oxaloacetate oxidized per minute) is expressed as international units (U) per gram of wet mass.

Superoxide dismutase activity

Superoxide dismutase (SOD) enzymatic activity was quantified in limpet foot tissue following the manufacturer's instructions (19160-1KT-F, Sigma-Aldrich, St Louis, MO, USA). Approximately 25 mg of foot tissue was weighed and homogenized in 250 µl of 100 mmol l^{-1} phosphate buffer (pH 7.8) using a tissue homogenizer (PROScientific, Oxford, CT, USA). Samples were centrifuged at 1500 ***g***, at 4°C, for 10 min and the supernatant was transferred to a new microcentrifuge tube. SOD activity was determined by measuring the absorbance change of the conversion of Dojindoo's water-soluble tetrazolium salt {WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]} to a water-soluble formazan dye during reduction with a superoxide anion in a microplate spectrophotometer (Biotek Synergy HT). One unit of SOD was assessed as the amount of enzyme necessary for 50% inhibition in activity of the formazan dye. SOD activity of the samples was normalized per milligram of wet mass (U mg^{-1} wet mass).

Carbonylated proteins

Carbonylated proteins were quantified as a measure of accumulated cellular oxidative damage in limpet foot tissue (Han et al., 2013) and were measured using discontinuous sodium docecyl sulfate (SDS) polyacrylamide gel electrophoresis followed by chemiluminescence staining of the secondary antibody according to the methods of Castegna et al. (2003). Approximately 25 mg of foot tissue was homogenized in 200 µl of a 50 mmol l^{-1} Tris lysis buffer, pH 7.5, using a tissue homogenizer (PROScientific, Oxford, CT, USA). The samples were then centrifuged at 14,000 ***g*** for 10 min at 4°C and the supernatant was transferred to new tubes. Protein concentrations of the samples were determined using a bicinchoninic acid assay, as above. Samples were diluted to ensure that detection fell within the linear range of detection (2–6 µg $µl^{-1}$) for the carbonylated protein assay. Each sample had a positive and negative aliquot with equal amounts of protein (4 µg). Before gel electrophoresis, 3 µl of 12% SDS was added to each sample, and then 6 µl of 10 mmol l^{-1} 2-4 DNPH solution (Sigma-Aldrich; D199303, 0.198 g DNPH in 100 ml 2 mol l^{-1} HCl) was added to each positive aliquot and 6 µl of 2 mol l^{-1} HCl was added to each negative aliquot. The samples were incubated at room temperature for 15 min and then 4.5 µl of neutralization solution (20 mmol l^{-1} Tris-HCl, 36.7 mmol l^{-1} glycerol) was added to each sample. Samples were loaded and electrophoresed on a 7.5% polyacrylamide gel. Proteins were then transferred onto a nitrocellulose membrane using the semi-dry transfer method. Membranes were blocked with 2% non-fat milk in Tris-buffered saline with Tween-20 (TTBS; 20 mmol l^{-1} Tris-HCl, 140 mmol l^{-1} NaCl, 0.1% Tween-20, pH 7.6) and then incubated in a rabbit anti-DNP primary antibody for 1 h (1:5000 in 2% blocking solution, Sigma-Aldrich D9656) followed by three washes in TTBS. Membranes were then incubated in a goat anti-rabbit HRP secondary antibody for 1 h (1:10,000 in 2% blocking solution, ADI-SAB-300). After washing in TTBS twice and once in TBS, the western blot was developed using Supersignal (Life Technologies, 34080) and then exposed and quantified using a Bio-Rad Imager (Gel Doc XR+ and ChemiDoc XRS+ Systems with Image Lab Software, Bio-Rad Laboratories, Hercules, CA, USA). Carbonylated proteins were calculated by subtracting the intensity of the negative aliquot from the intensity of the positive aliquot. To standardize samples between different gels, a sample of heat-shocked limpet foot tissue was run on every gel to quantify levels of carbonylated protein relative to the same internal standard sample.

Statistical analysis

Statistical analyses were done using R. Technical replicates (i.e. different repeated trials) were first assessed for normality and equal variance and then a one-way ANOVA was performed to ensure there were no differences between trials. Replicates from the unpredictable acclimation treatment were kept separate for all analyses because of statistical differences between the three replicate trials in our chosen measures of performance. All combined data were visually assessed for normality and equal variance (residual and q–q plots) then analyzed using a one-way ANOVA with acclimation treatment as the main effect, unpredictable treatments separated by trial nested within the model, and trial as a blocking effect. A Tukey's HSD test was run to distinguish differences between acclimation treatments.

We used generalized additive mixed modeling (GAMM) to test for differences in thermal sensitivity of heart rates between acclimation treatments following Zuur et al. (2009) and Angilletta et al. (2013). To account for repeated measures, the identity of each limpet was included as a random factor. Analyses were performed with the mgcv (Wood, 2004) and nlme (Pinheiro et al., 2013) libraries in R.

RESULTS

Cardiac performance under ramping increases in temperature

Acclimation had a significant effect on limpets' final BPT, an estimate of the upper critical limit of cardiac performance (one-way ANOVA, $F_{6,119}$= 9.6423, P<0.0001; Fig. 3). Limpets in the no tide

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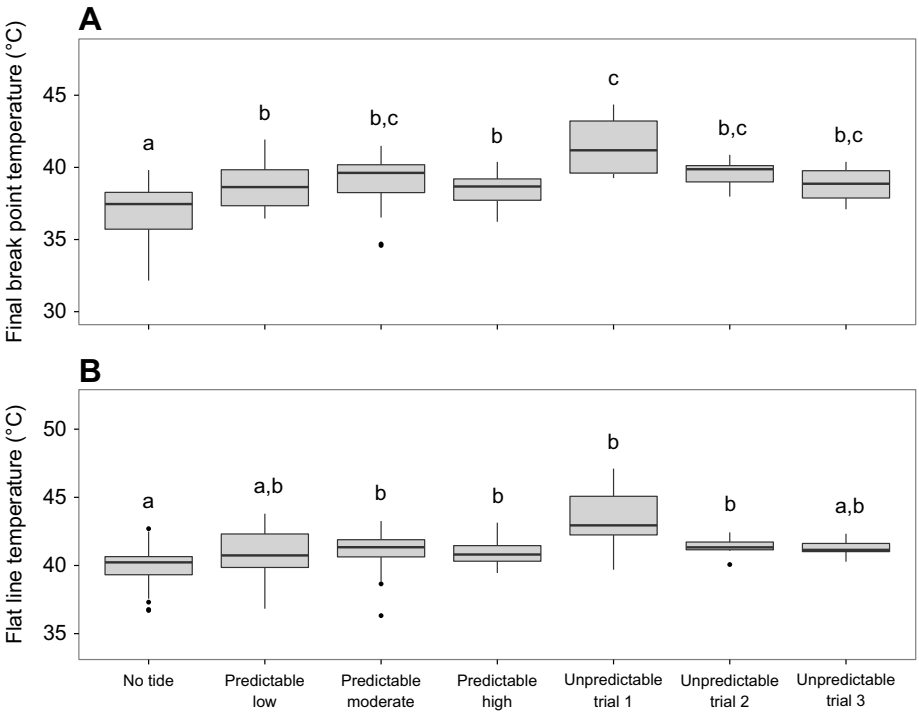

Fig. 3. Final break point temperature in heart rate for limpets and flat line temperatures from the no tide (*n*=29), predictable low (*n*=22), predictable moderate (*n*=25), predictable high (*n*=26) and unpredictable (trial 1: *n*=8; trial 2: *n*=10; trial 3: *n*=9) acclimation treatments. (A) Final break point temperature; (B) flat line temperature. For all predictable acclimation treatments, technical and biological replicates are grouped together. Because of statistical differences between replicate trial, each trial of the unpredictable treatment is presented separately and only biological replicates are grouped together. The line on the boxplots represents the median, the box represents the inter-quartile range (IQR), the whiskers extend 1.5 times IQR. Points beyond the whiskers are outliers. Differences in letters represent significant differences between acclimation temperatures (one-way ANOVA; Tukey's HSD, $P<0.05$).

treatment had the lowest final BPT of 36.96±0.34°C (mean±s.e.m.), statistically lower than all acclimation treatments. The highest final BPTs were observed in limpets from the unpredictable treatment trial 1 (41.49±0.75°C). Limpet final BPTs from the three different trials of the unpredictable treatment or from the predictable moderate treatment were not significantly different. There were no differences in final BPTs observed between predictable low, predictable moderate or predictable high treatments.

Acclimation treatment also had a significant effect on FLT, a proxy for upper temperature tolerance (one-way ANOVA, $F_{6,119}=6.0565$, $P<0.0001$; Fig. 4). Similar to final BPTs, limpets in the no tide treatment had the lowest FLT at 39.86±0.29°C (mean±s.e.m.), which was significantly lower than all acclimation treatments except predictable low and unpredictable treatment trial 3. Limpets from the unpredictable treatment trial 1 had the highest FLT (43.43±0.83°C), but this was only significantly higher than the no tide treatment.

Limpets from all acclimation treatments exhibited similar patterns in the temperature range between final BPT and FLT (FLT–BPT), an indication of how acutely cardiac function collapses following the initial decline in performance (one-way ANOVA, $F_{6,119}=1.4614$, $P=0.197$; Table 1). There was also no significant difference found among acclimation treatments if the temperature difference was reflected as FLT/BPT (one-way ANOVA, $F_{6,119}=1.9182$, $P=0.083$; Table 1). Lastly, there were no differences among acclimation treatments in the V_{max} the limpets were able to reach during the ramping protocol (one-way ANOVA, $F_{6,119}=1.938$, $P=0.080$; Table 1).

Performance curves of limpet heart rates from all acclimation treatments exhibited the expected non-linear response to warming, with an initial increase in heart rate with increasing temperature until an eventual plateau and ultimate decline (Fig. 4). GAMM analysis of the thermal performance curves showed that acclimation treatment had a significant effect on temperature sensitivity of limpet heart rates when compared with the no tide treatment (Table 2). Limpets acclimated to the unpredictable treatment trial 3 had performance curves the most similar to those of limpets from the no tide treatment, with limpets in both treatments experiencing increases in heart rate at the fastest rate, with heart rates peaking and

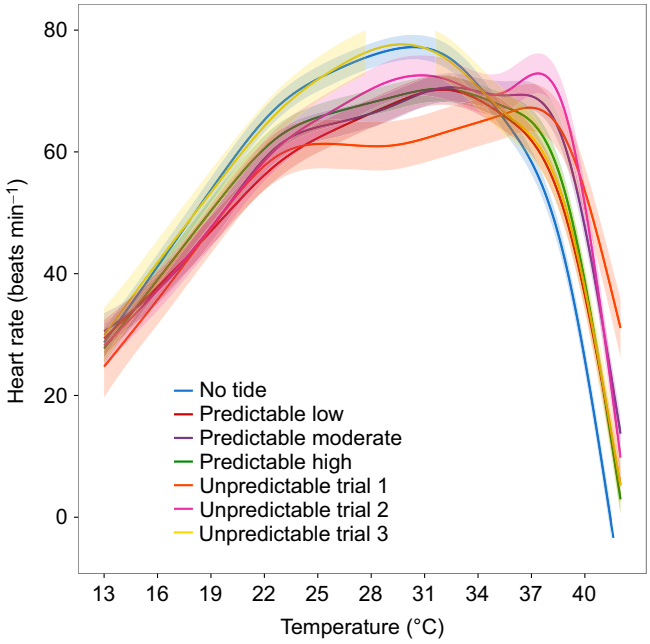

Fig. 4. Generalized additive mixed modeling (GAMM) for heart rates throughout the heat ramp for limpets from the no tide (*n*=29), predictable low (*n*=21), predictable moderate (*n*=25), predictable high (*n*=26) and unpredictable (trial 1: *n*=8; trial 2: *n*=10; trial 3: *n*=9) acclimation treatments. For all predictable acclimation treatments, technical and biological replicates are grouped together. Because of statistical differences between replicate trials, each trial of the unpredictable treatment is presented separately and only biological replicates are grouped together. Statistical differences are reported in Table 2.

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Table 1. Differences between flat line temperature (FLT) and break point temperature (BPT), the ratio of FLT/BPT, and the maximum heart rate (V_{max}) of limpets from all acclimation treatments

Acclimation treatment	FLT–BPT	FLT/BPT	V_{max}	*N*
No tide	2.90±0.27	7.26±0.66	87.45±2.38	29
Predictable low	2.30±0.19	5.57±0.46	81.30±2.75	22
Predictable moderate	2.12±0.18	5.16±0.45	86.42±2.82	25
Predictable high	2.46±0.25	5.95±0.59	83.71±2.59	26
Unpredictable trial 1	1.94±0.30	4.43±0.69	99.31±5.42	8
Unpredictable trial 2	1.80±0.19	4.36±0.46	86.38±4.03	10
Unpredictable trial 3	2.54±0.42	6.13±1.01	86.89±2.03	8

Data are means±s.e.m.

Table 2. Comparisons of generalized additive mixed models of heart rate as a function of temperature, $f(T)$, referenced to the curve of no tide treatment

Acclimation treatment	e.d.f.	*F*-value	*P*-value
$f(T)$ for no tide	8.740	280.64	<0.0001
Deviation from $f(T)$ for predictable low	5.256	20.64	<0.0001
Deviation from $f(T)$ for predictable moderate	6.697	35.90	<0.0001
Deviation from $f(T)$ for predictable high	6.060	21.64	<0.0001
Deviation from $f(T)$ for unpredictable trial 1	6.078	38.81	<0.0001
Deviation from $f(T)$ for unpredictable trial 2	8.374	32.30	<0.0001
Deviation from $f(T)$ for unpredictable trial 3	4.179	17.05	<0.0001

e.d.f., effective degrees of freedom.

declining at lower temperatures than limpets in other acclimation treatments.

Glycogen content and citrate synthase activity

There was no significant effect of acclimation treatment on glycogen content, a measure of carbohydrate energy storage (one-way ANOVA, $F_{7,78}$ =1.3086, P=0.2575; Fig. 5A). The mean (±s.e.m.) glycogen content of all acclimation treatments was 34.18±3.03 µmol glucosyl units g^{-1} wet mass, with a range of 0.28–117.86 µmol glucosyl units g^{-1} wet mass. There were also no significant differences found in citrate synthase activity, a measure of cellular aerobic capacity (one-way ANOVA, $F_{7,80}$=0.5598, P=0.7862; Fig. 5B). The mean citrate synthase activity of limpets from all acclimation treatments combined was 0.86±0.04 U g^{-1} wet mass, with a range of 0.32–2.13 U g^{-1} wet mass.

Superoxide dismutase activity and carbonylated proteins

SOD activity, a measure of cellular antioxidant defense mechanisms, was similar in all acclimation treatments (one-way ANOVA, $F_{7,77}$=0.3638, P=0.9205; Fig. 6A) with a mean (±s.e.m.) of 0.65±0.02 U mg^{-1} wet mass and a range of 0.30–1.07 U mg^{-1} wet mass. There were also no differences found in relative levels of carbonylated proteins, a measure for accumulated oxidative stress (one-way ANOVA, $F_{7,80}$=0.2304, P=0.9768; Fig. 6B). The average relative intensity was 1.33±0.06, with a range of relative intensities of 0.74–3.59.

DISCUSSION

Climate change models suggest that intertidal organisms will experience increases in mean temperature as well as more extreme temperatures in the future (Harley et al., 2006; IPCC, 2013). To better understand how predicted future thermal environments will impact intertidal organisms, it is critical to capture present day thermal physiological plasticity under ecologically relevant conditions of repeated and stochastic fluctuations in environmental temperature during low tide periods (Helmuth, 2002; Helmuth et al., 2014; Montalto et al., 2016). We predicted that intertidal limpets would tailor their physiology to an unpredictable thermal cue and, consequently, by maintaining high levels of energy reserves and high antioxidant levels, be better prepared to cope with a large temperature increase. We also predicted that limpets acclimated to a predictable environment

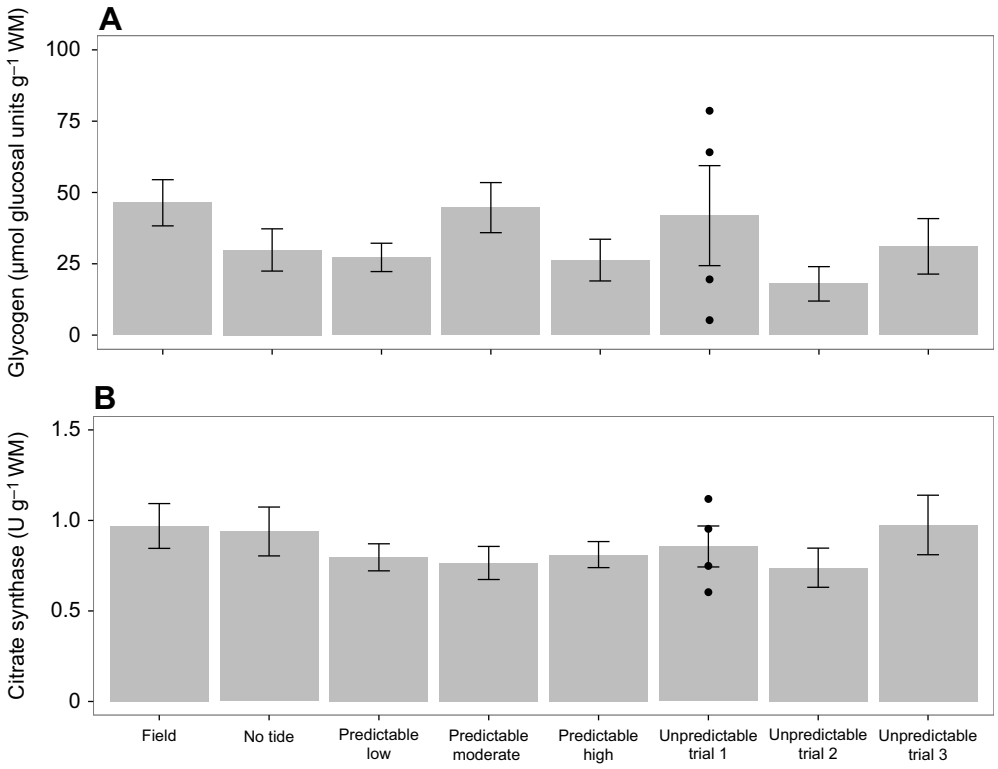

Fig. 5. Glycogen content and citrate synthase activity in limpet foot tissue from the field (*n*=15) and the no tide (*n*=15), predictable low (*n*=15), predictable moderate (*n*=15), predictable high (*n*=15) and unpredictable (trial 1: *n*=4; trial 2: *n*=5; trial 3: *n*=5) acclimation treatments. (A) Glycogen content; (B) citrate synthase activity. Data are presented as means±s.e.m. For all predictable acclimation treatments, technical and biological replicates are grouped together. Because of statistical differences between replicate trials, each trial of the unpredictable treatment is presented separately and only biological replicates are grouped together. No statistical differences were found (one-way ANOVA). WM, wet mass

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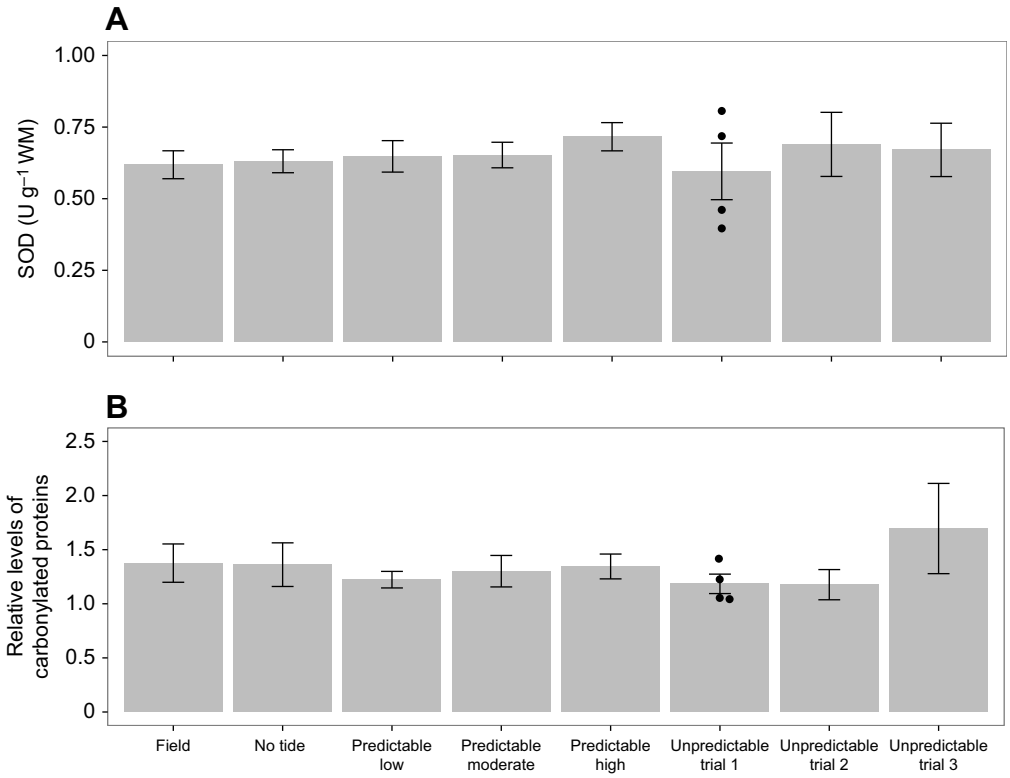

Fig. 6. Superoxide dismutase (SOD) activity and relative amounts of carbonylated proteins in limpet foot tissue from the field (*n*=15) and the no tide (*n*=15), predictable low (*n*=15), predictable moderate (*n*=15), predictable high (*n*=12 for A, *n*=15 for B) and unpredictable (trial 1: *n*=4; trial 2: *n*=5; trial 3: *n*=5) acclimation treatments. (A) SOD activity; (B) carbonylated proteins. Data are presented as means±s.e.m. For all predictable acclimation treatments, technical and biological replicates are grouped together. Because of statistical differences between replicate trials, each trial of the unpredictable treatment is presented separately and only biological replicates are grouped together. No statistical differences were found (one-way ANOVA).

would tailor their physiology to the consistent heating regime experienced, be less able to respond to an unpredicted high temperature exposure, and therefore have a lower upper temperature tolerance. Our results suggest that repeated aerial exposure alone (the most predictable aspect of the tidal cycle), regardless of the magnitude of temperature increase, had the largest effect on maintaining a high upper temperature tolerance in limpets. No significant differences in thermal tolerance were found between predictable treatments with considerable different daily maxima (13, 24 and 32°C), providing evidence that recovery from low tide periods can be sufficient to maintain thermal tolerance even when continually exposed to very high low-tide temperatures. Lastly, there were subtle but significant (in some comparisons) increases in thermal tolerance in one of the unpredictable treatment trials, suggesting that recent thermal history and perhaps unpredictability can modulate small adjustments in upper temperature tolerance.

Daily low tide aerial exposure alone acts as an important modulator of upper temperature tolerance in limpets. Previous studies have shown that experiencing thermal stress while emersed increased the thermal tolerance of limpets (Bjelde and Todgham, 2013; Huang et al., 2015). In the present study, upper thermal tolerance of all limpets was assessed during emersion and, therefore, our results expand our understanding of the role of repeated aerial emersion in priming the limpet to better tolerate a severe heat stress, compared with limpets that have been acclimated to completely submersed conditions (e.g. no tide). Considering that limpets in nature predictably experience aerial exposure with low tide and typically experience thermal stress coupled with aerial exposure, it is perhaps not surprising that acclimation to repeated aerial exposure confers an increased resistance to elevated temperatures. The link between aerial exposure and increased temperature tolerance could be due to differences in metabolic demands in air versus water. Limpets living higher in the intertidal zone can have higher metabolic rates in air than when submerged in water (Bannister, 1974; Branch and Newell, 1978; McMahon, 1988; Marshall and McQuid, 1992; Bjelde and Todgham, 2013). This may be an important adaptive strategy to increase energy available during stressful heating periods when emersed, and repeated emersion may reinforce metabolic activity during low tide periods. The results from the present study suggest that aerial exposure is likely the strongest cue to prime limpets for warm low tide temperatures, and that the heating during low tide is less important.

Peak acclimation temperature did not extend the upper temperature tolerance of limpets acclimated to a predictable environment within our range of temperatures (i.e. 13°C versus 24°C versus 32°C). Limpets that were exposed to repeated and predictable low, moderate or high heating patterns had similar upper critical thermal limits in cardiac performance (Fig. 3) as well as thermal cardiac performance curves through GAMM analyses (Fig. 4). Our results are at odds with previous studies that have found higher thermal tolerance of organisms when exposed to greater magnitudes of fluctuating temperatures (Schaefer and Ryan, 2006; Oliver and Palumbi, 2011; Kern et al., 2015; Giomi et al., 2016). Temperature tolerance was only increased with acclimation to unpredictable heating regimes in one trial of the unpredictable treatments, but only in comparison to the predictable low and predictable high treatments. Notably, there were no significant differences found in unpredictable treatments in comparison to the predictable moderate treatment, which experienced the same degree of heating throughout the acclimation period. Patterns of intertidal thermal stress characterized along a latitudinal gradient from California to Washington State demonstrated that average daily maximum temperature at many of the sites (six of eight) was between ca. 24°C and 31°C (see fig. 1 in Helmuth et al., 2002). The similarity in the average patterns of thermal stress calculated by Helmuth and colleagues (2002) from field measurements and our experimental heating regimes suggests that the results from our study could likely be extended to limpet populations along the west

coast of the United States, although this would have to be investigated. Other studies have demonstrated that unpredictable versus predictable environments modulate developmental differences, including variables such as body size, developmental time and egg to adult viability in zebrafish (*Danio rerio*) and a fruit fly (*Drosophila simulans*) (Schaefer and Ryan, 2006; Manenti et al., 2014). These studies and ours provide evidence that the element of environmental unpredictability likely plays a role in modulating the mechanisms of stress resistance in ectotherms; however, it is complicated because an unpredictable environment is a series of specific heating patterns that change, and animals might be responding to a particular pattern and not just the unpredictable nature of the pattern.

Specific heating patterns during consecutive low tide periods likely play a role in fine-tuning the upper temperature tolerance of limpets. In our study, each unpredictable trial had the same set of daily maximum temperatures, but their pattern was manipulated in order to investigate the role of stochasticity. Despite experiencing the same degree of heating over the 2-week period, the upper temperature tolerances of limpets from different unpredictable trials were different and appear to reflect the heating patterns during the last few emersion periods prior to experiencing a lethal heat stress. It is of note that cardiac performance curves were also very distinct between unpredictable treatments, with unpredictable trial 2 having a similar curve to the no tide treatment and the other two unpredictable trials being distinct from all other treatments. Although thermal tolerance did not differ significantly between limpets in the different unpredictable treatments, unpredictable trial 1 had the highest thermal tolerance of all the treatments and it also represented the unpredictable regime that ended with a series of warming days. Similarly, limpets from unpredictable trial 3 had the lowest thermal tolerance of the unpredictable treatments and the low tide temperatures immediately prior to assessing thermal tolerance were cooler. Limpets from unpredictable trial 2 ended with single warm day of 32°C, but exhibited intermediate thermal tolerance in comparison to limpets from unpredictable trial 1. Although speculative, the response to elevated temperatures may integrate over recent thermal history, with 'recent' likely representing a period shorter than our entire 2-week experiment but greater than simply the last midday low tide temperature exposure. It has been shown in the low intertidal snail *Tegula brunnea* that when the range of temperatures experienced in the field increased, there was a 4-day lag of an increase in heat shock proteins (Tomanek, 2002).

The natural pattern of increases and decreases of temperature during the monthly progression of low tides may be a factor in understanding the thermal tolerance differences documented between unpredictable treatment trials. Unpredictable trial 1 represented a heating pattern drawn directly from field data. Although unpredictable trials 2 and 3 had the same temperature peaks, the sequence of peaks, and therefore the pattern of temperature fluctuations, was artificial. There may be more predictability in the heating patterns during low tide than is apparent from a cursory examination of the temperature data, and this may be important in preconditioning a limpet for upcoming heat stress. In future studies, it will be important to understand the degree of environmental predictability when designing unpredictable treatments (Burgess and Marshall, 2014). Species living in the intertidal zone may be tuned to the temporal progression of tidal cycles, and this represents an important area of additional research.

Counter to our predictions, no constitutive differences were found in citrate synthase activity, glycogen content, oxidative stress or antioxidants in limpets acclimated to different heating regimes. All tissues used for the biochemical assays were taken from limpets immediately prior to the daytime low tide period. Therefore, our biochemical assays only take into account differences in constitutive mechanisms between acclimation treatments in how prepared limpets were for the next heating event. Previous studies have found that animals have the potential to 'frontload' cellular stress response mechanisms in order to quickly handle stress (e.g. Barshis et al., 2012). Basal citrate synthase activity and glycogen content were measured to determine whether acclimation to unpredictable heating regimes increased the metabolic capacity of limpets or whether acclimation to different heating regimes resulted in depleted energy stores over the 2-week period. Responding to elevated temperatures can be energetically costly for ectotherms (Sokolova et al., 2012). Constitutive levels of metabolic enzymes are indicative of temperature stress experienced in the environment by the marine snails *Littorina saxatilis* and *L. obtusata*, such that higher temperatures increased enzyme activity until heat denaturation occurs (Sokolova and Pörtner, 2001). No acclimation treatments in the present study altered glycogen stores or changed the specific activity of citrate synthase; therefore, this suggests that limpets in all acclimation treatments were equally able to mobilize energy at the start of the low tide period. We also measured SOD and carbonlyated proteins to determine whether any acclimation treatment had enhanced antioxidant defense or accumulated damage from oxidative stress. Previous work has shown that SOD increases with heat stress in other species of limpets (Pöhlmann et al., 2011), and it has been shown that fluctuating temperatures increased SOD levels in sea cucumbers (Dong et al., 2008a). In contrast, in the present study, there were no differences between acclimation treatments with higher magnitudes of heat stress or between fluctuating and non-fluctuating environments in foot SOD levels. There was also no evidence of greater oxidative damage of proteins, in contrast to previous work that has shown that elevated temperatures increased levels of carbonlyated proteins in limpets (Han et al., 2013; Zhang et al., 2014). Intertidal animals tailor their cellular stress response mechanisms to be quickly initiated following the onset of high tide such that recovery is complete before the start of the next low tide period (Hofmann and Somero, 1996; Tomanek and Somero, 2000; Schill et al., 2002; Clark et al., 2008; Zhang et al., 2014). Transcriptomics of *Mytilus californianus* have also shown upregulated gene expression of recovery mechanisms, including antioxidants, to return to cellular homeostasis during the high tide period before the next low tide period (Gracey et al., 2008; Place et al., 2012). It may have been that the periods of immersion during high tide were sufficient to mitigate any acquired stress during the daytime low tide periods of the acclimation treatments by available antioxidants. We targeted four commonly examined biochemical indices of energy metabolism and oxidative stress; however, there are numerous other aspects of the cellular stress response to be explored (e.g. heat shock proteins) that may be primed to be constitutively expressed in response to acclimation to different heating patterns (Tomanek and Somero, 2002; Dong et al., 2008a,b). Furthermore, differences found in upper temperature tolerance may be due to differences in the capacity to induce cellular defense or protective mechanisms upon heating during low tide. Several studies have shown that intertidal organisms start producing cellular chaperones once heating begins (e.g. Tomanek and Somero, 2002; Huang et al., 2015). It has been previously shown that different limpet species all in the genus *Lottia* have unique strategies involving heat shock proteins (Dong et al., 2008b). For example, *L. scabra* maintains high levels of constitutive heat shock proteins whereas *L. austrodigitalis*, *L. scutum* and

L. pelta rely more on inducing heat shock proteins when exposed to elevated temperatures (Dong et al., 2008b). It could be that *L. digitalis* relies more on inducible stress response mechanisms in order to increase thermal tolerance during a specific low tide period as temperatures approach their thresholds of sensitivity. Future studies looking to understand the mechanisms underlying increases in thermal tolerance of *L. digitalis* should consider measuring inducible stress response mechanisms and how recruitment of these mechanisms might vary between limpets acclimated to predictable compared with unpredictable heating patterns.

Environmental complexity and, specifically, the repeated nature of aerial exposure and unpredictable magnitude of temperature increase are important aspects of the thermal physiology of *L. digitalis* and modulate upper temperature tolerance. Studies that have not incorporated these aspects of the intertidal environment may not be accurately capturing the current sensitivity of intertidal organisms to projected changes in temperature under different climate change scenarios. Our study highlights the critical importance of repeated aerial exposure for increasing the upper temperature tolerance of *L. digitalis*. If aerial exposure is the predominant factor driving thermal tolerance of intertidal limpets, and perhaps intertidal organisms more broadly, it suggests that organisms inhabiting the low intertidal are likely sensitive to warming not only as a result of a thermal history of lower temperatures but also as a result of not predictably being exposed to air during low tide periods. We also provide evidence that thermal history from a few days prior may be important in fine-tuning upper temperature tolerance in unpredictable fluctuating environments. Timing of low tides (i.e. during midday versus early morning in summer months) has been predicted to be an important factor defining risk of thermal stress from extreme events in intertidal organisms (Helmuth et al., 2002). Therefore, opportunities exist to better understand the link between aerial exposure and heating in defining the thermal physiology of organisms by examining populations of limpets along a latitudinal gradient, such as the west coast of the United States. Additional studies are needed to determine the cellular mechanisms conferring higher thermal tolerance from heating regimes that are unpredictable in nature. Moving forward, physiological studies should incorporate environmental unpredictability rather than just focusing on the magnitude of heat stress to fully understand the thermal physiology of animals living in variable environments. More studies are needed to identify which aspects of environmental complexity are the key drivers of organisms' physiological responses. Identifying these key components of thermal variability will lead to more informed experimental designs that will improve our predictions regarding how intertidal organisms will respond to climate change (Helmuth et al., 2014).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.A.M., A.E.T.; Methodology: M.J.D., N.A.M., A.E.T.; Software: N.A.M.; Formal analysis: M.J.D.; Investigation: M.J.D., N.A.M.; Resources: A.E.T.; Writing - original draft: M.J.D. Writing - review & editing: N.A.M., A.E.T.; Supervision: A.E.T.; Project administration: A.E.T.; Funding acquisition: A.E.T.

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