

RESEARCH ARTICLE

Thermal physiology of the fingered limpet *Lottia digitalis* under emersion and immersion

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SUMMARY

Marine animals living high in the rocky intertidal zone experience long durations of aerial emersion, sometimes enduring rapid increases in temperature. To date, much of our understanding of the thermal physiology of intertidal organisms comes from studies in which organisms are exposed to increasing temperatures when immersed, with the added effect of aerial emersion rarely considered. In this study, we examined the physiological response of the finger limpet, *Lottia digitalis*, to increases in temperature under both immersed and emersed conditions. We investigated the thermal sensitivity and upper temperature tolerance of limpets through assessment of cardiac performance, metabolic rate, glycogen depletion and maintenance of protein integrity. Cardiac performance in response to ecologically relevant increases in temperature was similar in emersed and immersed limpets from 15 to 35°C and showed multiple break patterns in heart rate as temperature was increased. Overall, emersed limpets had a greater upper thermal limit on cardiac performance, with the ability to maintain heart rate at a temperature 3–5°C higher than that for immersed limpets. Metabolism in limpets also differed significantly between emersion and immersion, where a significant depression in aerobic metabolic rate was observed under immersion with increasing temperature. Greater levels of ubiquitin-conjugated proteins were found under emersed conditions compared with immersed limpets. Maintaining cardiac performance and aerobic metabolism to higher temperatures under emersed conditions is likely reflective of physiological adaptations to live in an aerially exposed environment. Measured field temperatures where fingered limpets were collected demonstrated that limpets have a narrow thermal safety margin for aerobic performance, and currently experience multiple days where summer temperatures might exceed their threshold limits.

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INTRODUCTION

Increases in mean global temperatures and the frequency of heat wave events are forecasted by the end of the century (Meehl et al., 2007). The ecological impacts of climate change have already been documented through changes in the distribution and abundance of species as they move poleward (Parmesan and Yohe, 2003; Root et al., 2003). It remains unclear what physiological mechanisms are underlying the sensitivity to increases in temperature that are ultimately leading to these changes in distribution and abundance (Hofmann and Todgham, 2010). It is predicted that rocky intertidal species will be particularly vulnerable to climate change because of the limited capacity of these organisms to increase their temperature tolerance (Stillman, 2003; Tomanek, 2010; Somero, 2010; Kelly et al., 2012). Inherent to the rise and fall of tides is daily immersion and aerial emersion, creating a highly variable habitat for intertidal organisms. During low tide emersion periods, these organisms potentially face large changes in abiotic factors such as rising temperatures, solar radiation and desiccation (Newell, 1979). Thermal stress is one of the dominant physical stressors in intertidal habitats (Helmuth and Hofmann, 2001) and is considered the primary limiting factor of latitudinal and vertical distribution patterns of organisms (Helmuth et al., 2002; Somero, 2002; Helmuth et al., 2005; Somero, 2005; Firth and Williams, 2009). Temperature stress also indirectly changes interspecific interactions within the

rocky intertidal zone as shown with *Pisaster ochraceus*, where physiological changes in response to warmer temperatures lead to different predator–prey relationships (Sanford, 1999; Sanford, 2002; Szathmary et al., 2009). It is thought that upper intertidal organisms may have already ‘stretched’ their physiology to the limits of acclimatization to live in such a variable environment and may possess a limited capacity to tolerate further increases in temperature (Somero, 2002; Stillman, 2003; Tomanek, 2008; Denny et al., 2009; Miller et al., 2009). For example, extreme heat waves in recent summers have caused mass mortality events in the rocky intertidal (Petes et al., 2007; Harley, 2008; Denny et al., 2009).

Until recently, much of the research into the thermal physiology of intertidal organisms and the mechanisms underlying temperature sensitivity has focused on temperature stress during immersion, even though intertidal organisms typically experience thermal stress when aerially exposed or emersed. Responses to increased temperature and upper temperature tolerance limits may differ in organisms under immersed and emersed conditions. When emersed, in addition to thermal stress, organisms face desiccation and changes in oxygen availability (Truchot, 1990; Verberk and Bilton, 2011). Intertidal organisms differ in their metabolic demands under emersed and immersed conditions in response to different temperature exposures (Sandison, 1967; Bannister, 1974; Stillman and Somero, 1996) and therefore may differ in the energy available to maintain the cellular

mechanisms underlying upper temperature tolerance (Pörtner, 2001). Previous studies that focused on assessing thermal tolerance under immersion may have overlooked important mechanisms underlying thermal tolerance in intertidal animals. Presently, there is little comparative information on the thermal sensitivity and physiological response of intertidal animals to increasing temperature under both emersed and immersed conditions.

Cardiac performance and oxygen consumption are commonly used as indices of whole-organism physiological performance in measuring a response to environmental stressors such as temperature (Stenseng et al., 2005; Dong and Williams, 2011), salinity (Marshall and McQuaid, 1993; Braby and Somero, 2006), oxygen limitations (Marshall and McQuaid, 1993; Frederich and Pörtner, 2000) and air exposure (Connor and Gracey, 2012; Marshall et al., 2011; Logan et al., 2012). Studies that have monitored heart rate under emersed and immersed conditions have shown significant effects of temperature on heart rate; however, the temperature exposures were acute exposures or changes in acclimation temperatures rather than simulating increases in temperature as would be experienced in nature with the onset of low tide (Trueman and Lowe, 1971; De Pirro et al., 1999). It is well established that rates of warming during a thermal performance trial can influence the upper temperature tolerance or critical thermal maximum (CT_{max}) of an organism (e.g. Peck et al., 2009), requiring experimental designs that closely mimic environmental conditions.

Intertidal organisms inhabiting the highest locations in the intertidal zone endure long exposures to warm aerial and substrata temperatures. The fingered limpet *Lottia digitalis* (Rathke 1833) inhabits the mid-to-upper intertidal zone and is one of only a few species within the Lottidae family found at the highest edge of the intertidal zone (Lindberg and Pearse, 1990). During low tides, fingered limpets can experience large fluctuations in environmental temperature over short periods of time, sometimes as much as a 24°C increase in temperature in 4 h during the summer (Fig. 1). Heat budget models for the owl limpet, *L. gigantea* demonstrate that limpet body temperature overlaps very closely with substrate temperature, suggesting the main driver for limpet body temperature is rock temperature (Denny and Harley, 2006), and during low tide, substrate temperatures can exceed aerial temperatures. *Lottia digitalis* provides an excellent opportunity to examine sensitivity to current environmental temperature increases under both emersed and immersed conditions and to assess whether this organism is currently living close to its thermal tolerance limits.

The main objective of this study was to investigate the thermal physiology of *L. digitalis* to natural rates of temperature increase under both immersion and emersion. Upper thermal limits of physiological performance were assessed by measuring cardiac and metabolic responses to increasing environmental temperatures in air and water. Additional investigations into metabolic fuel usage and cellular protein pool integrity were conducted in an effort to more broadly understand the differences in thermal physiology of *L. digitalis* under emersed and immersed conditions. By comparing physiological performance at different temperatures with current intertidal temperature profiles, this study provides insight into the thermal sensitivity of *L. digitalis* to increases in mean temperature and the increasing frequency of extreme heat waves projected to occur under future climate change scenarios.

MATERIALS AND METHODS

Limpet collection

Lottia digitalis were collected from the upper intertidal zone during low tide emersion periods at Fort Ross, CA, USA (38°30'45.79"N,

123°14'45.58"W). Limpets (length range 11.6–20.1 mm, mass range 271.5–1197.2 mg) were removed from rocks and immediately put in coolers to be transferred to the laboratory at the Romberg Tiburon Center for Environmental Studies, San Francisco State University, in Tiburon, CA, USA. Limpets were returned to the lab within 4 h of collection and put in temperature-controlled recirculating seawater tables. Limpets were acclimated for at least 14 days under stable conditions of 12.6±0.4°C, 34 p.s.u. salinity and a 14 h:10 h light:dark photoperiod, common summer coastal ambient ocean conditions in Northern California. Algae-covered rocks were also collected from Fort Ross, CA, USA, and placed in holding tanks to allow limpet grazing.

Temperature profile of the intertidal zone

Temperature data loggers (Maxim Integrated Products, Dallas, TX, USA) continuously recorded intertidal rock temperatures every 10 min at three different locations for the summer months of 2011 at Fort Ross, CA, USA (Fig. 1). Loggers were replaced on a monthly basis. Data loggers were secured in marine ZSPAR on rocks adjacent to where *L. digitalis* were collected. In addition to providing the thermal history of the collected limpets, these temperature data provided the natural rate of environmental temperature increase during a low tide period that was used for our ramping temperature profile.

Cardiac performance under ramping increases in temperature

To estimate the upper temperature tolerance limits of *L. digitalis*, heart rate was recorded as temperature was increased using a previously described methodology (Stillman, 2003) modified for limpets. Limpets were exposed to increases in temperature under both emersed and immersed treatment conditions (i.e. exposure treatments) in the laboratory using temperature ramping protocols designed to mimic environmental temperature increases during a low tide period from Fig. 1. The ramping protocol increased water or air (substrate) temperatures from 13°C (ambient summer ocean temperature) to 43°C (thermally stressed temperature), at a constant rate of 6°C h⁻¹. Heat budget models have demonstrated that limpet body temperature is primarily dependent on substratum temperature (Denny and Harley, 2006; Miller et al., 2009; Chapperton and Seuront, 2011). As a result, an aluminum, temperature-controlled heat block, in direct contact with the limpet's foot and covered with an overhead acrylic lid, was used for simulating emersion exposures. Preliminary trials confirmed that the recorded temperature of the limpet foot closely matched that of the heat block for the entire ramp (±1°C). Relative humidity in the aerial chamber was maintained at 50.7±0.11%, similar to humidity measurements recorded in the field next to *L. gigantea* at warmer temperatures (Miller et al., 2009). A circulating and aerated water bath was used to simulate immersion periods.

Twenty-four hours before a temperature ramp trial, two small holes were drilled through the limpet shell on either side of the heart in a horizontal plane close to the apex of the shell. Limpets were then returned to the sea tables overnight. Ceramic-coated 40 gauge copper wire was used to make impedance electrodes for monitoring cardiac signal (Belden, Richmond, IN, USA). The morning of the ramping experiment, electrodes were implanted through the predrilled holes and glued into the air cavity between the shell and the limpet, directly above the heart. Limpets were placed in the emersed ($N=20$) or immersed conditions ($N=29$) and allowed to recover from handling and placement of electrodes for 30 min at 13°C before temperature ramping began. For the duration of the experiment, each limpet's cardiac performance was recorded as

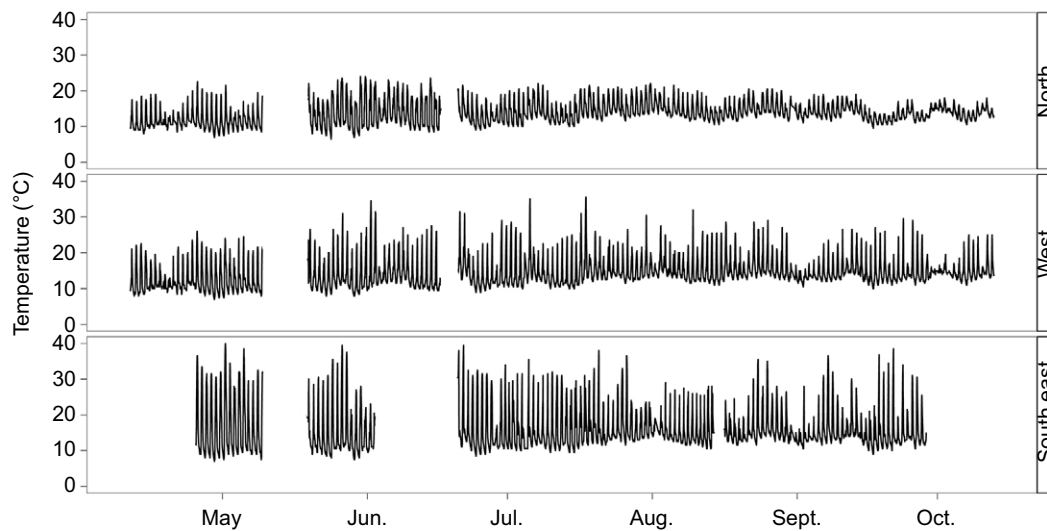


Fig. 1. Temperature profiles at Fort Ross, CA, USA, of upper intertidal rock temperatures recorded, from April to October 2011, every 10 min, at three different locations: site 1, north facing; site 2, west facing; and site 3, south-east facing. Limpets for this study were collected from all three sites.

impedance. Impedance converters were used to convert heart recordings to voltage signals using the PowerLab Chart 5 program (ADInstruments, Colorado Springs, CO, USA) and heart rate, in beats per minute, was calculated from the heart rate traces. Emersion and immersion temperature ramps were alternated daily.

Cardiac performance and tissue sampling under acute temperature exposure

In order to assess the cardiac response of limpets to an acute temperature increase, limpets ($N=15$) were exposed directly to one of six constant temperature treatments for 2.5 h under either emersed or immersed conditions. Exposure temperatures were 15, 20, 25, 30, 35 and 40°C. A 30 min period prior to each recording of limpet heart rate was provided to allow the limpets time to recover from handling and placement of electrodes before measurements began. Heart rate was then recorded for 2 h as described above. Immediately after each acute temperature exposure, limpet tissues were extracted, frozen in liquid nitrogen and stored at -80°C for later glycogen, total protein and ubiquitin (Ub)-conjugated protein analyses.

Cardiac performance analysis

The temperature sensitivity of heart function of limpets exposed to gradual increases in temperature was assessed by three measures of cardiac performance: (1) determining the highest temperature that caused rapid declines in heart rate (final break point temperature, final BPT), (2) calculating the slope of the decline in heart rate following the final break in heart rate and (3) characterizing multiple break patterns in heart rate as temperature was increased. Final BPT was calculated as described previously (Stenseng et al., 2005), by plotting heart rate (beats min^{-1}) against temperature and finding the inflection in the plot (sharp decrease in slope following a peak in heart rate). Two best-fit regression lines were drawn over the pre-BPT data (ascending) and the post-BPT data (descending). The intersection of the two lines was used to determine the final BPT. The rate of decrease (slope) in heart rate following the final BPT was calculated using the post-BPT linear regression described above. In addition to the final BPT and slope of decrease in heart function following the final break in heart rate, heart performance was analyzed for the total number of breaks experienced by limpets when they were ramped from 13°C to 43°C. A break in heart rate was defined as any inflection of the plot where heart rate steadily decreased following an increase in heart rate. The total number of

breaks in heart rate (1–3) and the temperature points at which these breaks occurred were calculated using the same regression line technique as for final BPT.

For cardiac performance under acute thermal exposure, heart rate was analyzed for each individual limpet ($N=8\text{--}13$ per temperature group) at 20 min intervals and averaged over the 2 h experiment under both emersed and immersed conditions (supplementary material Table S1). In order to compare cardiac performance of limpets under acute thermal exposures with that of limpets under ramping increases in temperature, performance curves of heart rate were generated for limpets exposed to ramping increases in temperature by capturing heart rate at 15, 20, 25, 30, 35 and 40°C during the ramping protocol. Thirty seconds of heart rate was captured at 30 s intervals for the duration of that temperature $\pm 0.5^{\circ}\text{C}$ (i.e. for 30°C, heart rate was taken from recordings at 29.5 to 30.5°C) and individual mean heart rate was then calculated from all the data points acquired for a single temperature. For limpets with irregular heart rates, only sections of the heart rate trace with detectable heart beats were used.

Final BPT in heart rate under emersion and immersion was analyzed by a one-way ANOVA. To determine the effect of the number of breaks in heart performance of a limpet on its final BPT under both emersed and immersed conditions, a two-way ANOVA was performed followed by a variance test to determine differences in the variability between final BPT and the number of breaks in heart performance, Bonferroni-corrected for multiple comparisons ($P<0.008$). To determine whether there was consistency in the temperatures at which breaks in heart function occurred for limpets with multiple breaks, temperatures where breaks occurred before the final BPT were compared by a two-way ANOVA followed by a Tukey HSD test. A Kruskal–Wallis rank test was used to analyze the slopes (rate of decrease) following final BPTs. Cardiac performance curve data from acute thermal exposures were analyzed separately by emersion and immersion treatment using a Kruskal–Wallis rank test followed by a Wilcoxon rank test between emersion and immersion heart rate at each temperature, Bonferroni-corrected for multiple comparisons ($P<0.008$). Performance curve data generated for estimating thermal sensitivity of limpets exposed to ramping increases in temperature were analyzed using a generalized linear model (nlme package) with limpet as a random factor accounting for repeated measures, followed by a Tukey HSD for *post hoc* comparisons. All statistical analyses were completed using R (R Development Core Team, 2008).

Metabolic rates under acute temperature exposure

Twenty-four hours prior to respirometry experiments, limpets were weighed (pre-mass, $M_1=599.29\pm 15.96$ mg), placed into the 30 ml respirometry vials and returned to the sea tables for overnight acclimation. Open vials were covered with a small-gridded rack to prevent escape but still allowed for adequate water flow and aeration overnight. Prior to experimentation, immersed experimental chambers containing limpets were filled with fresh, fully aerated seawater at one of six experimental temperature treatments (15, 20, 25, 30, 35 or 40°C). For emersed trials, water was removed from the respirometry vials containing limpets. Care was taken not to dislodge the limpets. Limpets were then allowed to recover with the caps removed from the vials for 30 min before trials were started in a water bath at the designated experimental temperature. This procedure assured that the temperature within the vial stabilized at the experimental temperature before metabolic trials. The vials were then capped and O_2 consumption was recorded using Presens Fibox optical sensor spots (accuracy of $\pm 0.4\%$ at 20.9% O_2) and an oxygen meter (Presens, Regensburg, Germany) that measured changes in percentage air saturation at 20 min intervals for 2 h. Chambers were mixed for 15 s before each measurement using a magnetic stirrer attached to the cap of the vial. In preliminary trials we confirmed that mixing for 15 s every 20 min over the 2 h period or having continuous mixing for 2 h resulted in no difference in oxygen consumption of the limpets. Following each respirometry trial, limpets were reweighed ($M_2=583.67\pm 15.88$ mg), extracted from their shells, dried at 60°C for 24 h and placed in a desiccator for an additional 24 h to attain dry mass (52.08 ± 1.37 mg). Shell mass (313.89 ± 8.60 mg) was also determined for each limpet. Mass-specific metabolic rate was then calculated in $\mu\text{mol } O_2 \text{ h}^{-1} \text{ g}^{-1}$ dry mass. Percentage body water was calculated to determine the amount of tissue water loss after all acute temperature exposures, where % body water = $\{1 - [\text{dry mass}/(M_2 - \text{shell mass})]\} \times 100$.

Oxygen consumption data, even when transformed, failed normality and homogeneity of variance tests. As a result, the data were separated into emersion and immersion trials and two Kruskal–Wallis tests were performed to test the effect of increasing acute temperature exposures on oxygen consumption in each trial independently. Wilcoxon rank tests were used to determine significant differences between metabolic rates at specific acute temperatures and emersion and immersion. Multiple comparisons were adjusted using the Bonferroni correction ($P < 0.008$). Percentage body water following temperature exposures was first analyzed by a two-way ANOVA with temperature and exposure treatment (i.e. emersion *versus* immersion) as factors, then separated by treatment and analyzed by a one-way ANOVA, followed by a Tukey HSD for the emersed treatment.

Glycogen content

Frozen foot samples from the cardiac acute thermal exposure experiments were ground into a fine powder under liquid nitrogen in an insulated mortar and pestle. Glycogen content was measured as described elsewhere (Fangue et al., 2008) with slight modifications. Glycogen was extracted by adding 1 ml of cold 8% HClO_4 to ~20 mg of powdered foot tissue, which was then homogenized on ice for ~20 s with a Pro200 Bio-Gen Series homogenizer (PROScientific, Oxford, CT, USA). A sample of the homogenate (200 μl) was put in a separate tube and frozen at -80°C for later glycogen quantification. The remaining homogenate was centrifuged at 10,000 g for 10 min at 4°C and the supernatant was extracted and neutralized with 3 mol l^{-1} K_2CO_3 . The neutralized solution was centrifuged at 10,000 g for 10 min at 4°C and frozen at -80°C for

later free glucose assays. Samples for glycogen determination were enzymatically digested following previous methods (Hassid and Abraham, 1957), and all samples were analyzed for glucose following a method (Bergmeyer, 1983) modified for spectrophotometry. To determine the effects of temperature and exposure treatment on foot glycogen, a two-way ANOVA was conducted, followed by a Tukey HSD multiple comparison test.

Sample preparation for total protein and dot blots

Frozen foot samples from the cardiac acute thermal exposure experiments were prepared for total protein and Ub-conjugated protein analyses as described previously (Todgham et al., 2007). Samples were homogenized on ice for ~20 s in homogenization buffer (50 mmol l^{-1} Tris base, 1 mol l^{-1} EDTA, 4% SDS w/v, pH 6.8) containing a cocktail of protease inhibitors including pepstatin A (0.7 $\mu\text{g ml}^{-1}$), leupeptin (0.5 $\mu\text{g ml}^{-1}$), aprotinin (1 $\mu\text{g ml}^{-1}$) and PMSF (20 $\mu\text{g ml}^{-1}$) (Sigma, St Louis, MO, USA) at a ratio of ~100 mg foot tissue to 500 μl of buffer. Homogenates were heated at 100°C for 5 min and centrifuged at 13,000 g for 10 min. The supernatant was transferred to a fresh microcentrifuge tube and total protein concentration of the tissue homogenates was determined using the bicinchoninic acid method (Smith et al., 1985) (Thermo Fisher Scientific, Rockford, IL, USA). The remaining homogenate was diluted to 50 μg total protein in 1 ml Tris-buffered saline (TBS: 20 mmol l^{-1} Tris-HCl, 140 mmol l^{-1} NaCl, pH 7.6) and stored at -20°C until dot blot analysis.

Dot blot analysis

Levels of Ub-conjugated proteins were measured in limpet foot tissue using immunochemical analysis following modified methods outlined elsewhere (Todgham et al., 2007). Nitrocellulose membranes (0.2 μm pore size) were pre-hydrated for 2 h in TBS on a gyrotory shaker. Equal amounts of total protein (10 μg) were blotted onto the wet nitrocellulose membrane by gravity filtration for 2 h using a BioDot dot blotter (BioRad, Hercules, CA, USA). Wells were washed twice with 200 μl TBS, and heat fixed for 20 min at 65°C. Following heat fixing, the nitrocellulose membrane was blocked in 5% non-fat milk powder in Tween-20 Tris-buffered saline (TTBS: 0.1% Tween-20 in TBS) for 1 h at room temperature. After blocking, the membranes were rinsed and washed 3 times for 5 min in TTBS. Membranes were then incubated in Ub-conjugated protein-specific primary antibody that detects poly-ubiquitinated proteins (1:5000 in 5% blocking solution, rabbit polyclonal antibody produced by Cocalico Biologicals, Reamstown, PA, USA) on the shaker for 1.5 h at room temperature. Following three 5 min washes in TTBS, membranes were incubated in horseradish peroxidase-conjugated protein A secondary antibody (1:5000 in 5% blocking solution, BioRad) for 1 h at room temperature. The nitrocellulose membrane was then washed 3 times in TTBS for 5 min and developed with chemiluminescent Supersignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) for 3–5 min. Chemiluminescence was detected and quantified using a Kodak Molecular Imager and included software (Kodak MI, Carestream Health, Woodbridge, CT, USA). Ubiquitin-conjugated protein values were standardized using dot intensity values from one standard reference sample that consisted of a mixture of five random samples of limpet foot tissue blotted twice in triplicate on every nitrocellulose membrane.

Levels of Ub-conjugated proteins were analyzed using a two-way ANOVA, with temperature and exposure treatments as main factors, followed by a Tukey HSD test to distinguish differences in Ub-conjugated protein levels within emersed and immersed

exposure treatments. All data were tested for normality (Shapiro–Wilks) and homogeneity of variance (Levene test). All data are reported as means \pm s.e.m. unless otherwise stated.

RESULTS

Cardiac performance during ramped increases in temperature

Upper thermal limits of cardiac performance, measured by exposing limpets to a 6°C h^{-1} thermal ramp, revealed that limpets exposed to increasing temperatures during emersed periods were more thermally tolerant with significantly higher final BPTs in heart rate than limpets exposed to increasing temperatures submerged in water (Fig. 2; one-way ANOVA, $P=0.001$). The mean final BPT of emersed limpets was $37.9\pm 0.8^{\circ}\text{C}$ compared with $34.8\pm 0.4^{\circ}\text{C}$ measured in immersed limpets. Limpets exposed to increasing thermal stress under both aerial emersed and immersed conditions exhibited inconsistent patterns in heart function reported as multiple breaks in heart rate, where heart rate increased and decreased periodically in response to increasing temperature until the final steep decline in heart function. These irregular breaks in heart rate ranged from a single break in heart rate as the limpet approached its upper thermal limit of cardiac performance to three breaks in heart function in response to increasing temperature (Fig. 3). Two-way ANOVA revealed that the number of breaks in heart performance did not ultimately affect final BPT ($P=0.125$); however, variance tests revealed that the variability in final BPT of limpets decreased from a single break to two breaks (Levene, $P<0.001$) in heart rate as well as from a single break to three breaks in heart rate (Levene, $P=0.001$) under emersion conditions (Fig. 4). In limpets with multiple breaks in heart rate, there were no distinct temperatures at which these breaks occurred. For example, limpets with two breaks in heart rate did not experience their first break at the same temperature that limpets with three breaks in heart rate experienced either their first or second break in heart rate (two-way ANOVA, supplementary material Table S2).

There was a significant difference in the rate of decline (slope) in heart rate following the final BPT under emersed and immersed conditions, with emersed limpets having a greater mean slope in declining heart rate of -53.4 ± 8.7 beats $\text{min}^{-1}\text{ }^{\circ}\text{C}$ beyond their final BPT compared with a more gradual decline of heart rate (mean slope $=-31.6\pm 5.3$ beats $\text{min}^{-1}\text{ }^{\circ}\text{C}$) in immersed limpets (Kruskal–Wallis, $\chi^2=6.2$, d.f.=1, $P=0.012$, data not shown). In emersed limpets, attenuation of heart function (i.e. when heart rate approached 0) occurred within $1\text{--}3^{\circ}\text{C}$ of their final BPT, whereas immersed limpets demonstrated a $5\text{--}7^{\circ}\text{C}$ difference between their final BPT and attenuation of heart function.

Cardiac performance during acute temperature exposures

Acute thermal exposures affected cardiac performance of limpets under both emersed and immersed conditions (Kruskal–Wallis; emersion: $\chi^2=30.2$, d.f.=5, $P=0.0001$; immersion: $\chi^2=19.6$, d.f.=5, $P=0.001$) (Fig. 5A). Under emersed conditions, the heart rate of limpets increased steadily from 15 to 30°C and then began to decline until 40°C . Limpets acutely exposed to 25 , 30 and 35°C had significantly higher heart rates than control limpets at 15°C . The heart rate of limpets exposed to 40°C did not differ from the heart rate of limpets at 15°C or from heart rates of limpets in the other four temperature groups. Wilcoxon rank tests revealed that immersed limpets maintained significantly higher heart rates than emersed limpets under both ambient exposures at 15°C ($Z=-3.82$, $P<0.001$) and extremely warm thermal exposures at 40°C ($Z=-2.66$, $P=0.008$). Under immersed conditions there was a significant increase in the heart rate of limpets at 30°C compared with that of limpets at 15°C , and heart rate declined at temperatures greater than 30°C such that

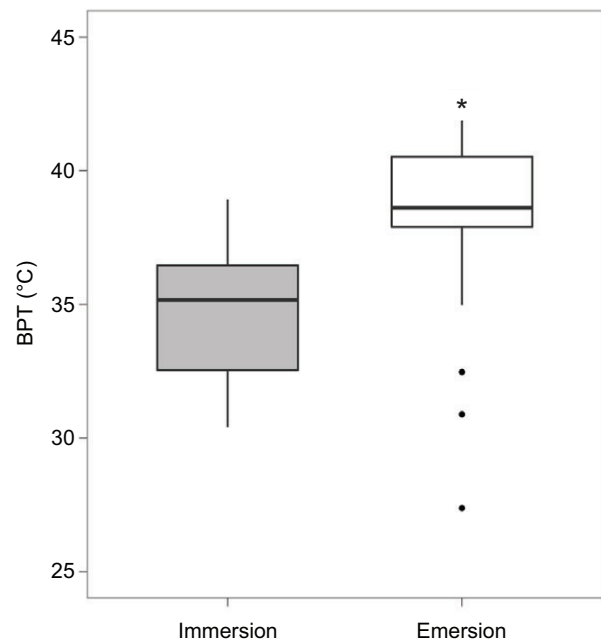


Fig. 2. Final break point temperature (BPT) in heart rate of immersed (gray, $N=29$) and emersed (white, $N=20$) limpets. The line on the boxplots represents the median, the box represents the inter-quartile range (IQR), the whiskers extend 1.5 times IQR. Points beyond the whiskers are outliers. Asterisks represent a significant difference between emersed and immersed conditions ($P<0.05$).

the heart rate of limpets at 40°C was back to levels measured for limpets exposed to 15°C for 2 h.

Cardiac performance curves of limpets exposed to acute increases in temperature (Fig. 5A) compared with cardiac performance curves generated from limpets exposed to a ramping temperature regime when heart rate was captured at 15 , 20 , 25 , 30 , 35 and 40°C (Fig. 5B) were similar with significant differences at 15 and 40°C . In both temperature exposure trials (i.e. ramped *versus* acute), the highest heart rate was recorded at 30°C under both emersed and immersed conditions, with heart rate decreasing as temperatures approached 15 and 40°C . Under immersed conditions in acute temperature exposure experiments, mean heart rate was 71.65 ± 3.74 beats min^{-1} at 30°C and under ramping temperature exposures mean heart rate was 72.17 ± 3.53 beats min^{-1} at 30°C . Under emersed conditions at 30°C , mean heart rate of limpets in acute temperature exposure trials was 69.92 ± 2.66 beats min^{-1} and heart rate under ramping temperature exposures was 63.07 ± 6.61 beats min^{-1} . There were notable differences in the heart rate of limpets at 15 and 40°C depending on whether limpets were acutely exposed to these temperatures or whether temperature was ramped from 15 to 40°C at a rate of 6°C h^{-1} . A generalized linear model, with limpet as a random factor accounting for repeated measures, followed by a Tukey HSD test showed that under ramping of temperatures, immersed and emersed limpets exhibited similar heart function at an ambient temperature of 15°C (Fig. 5B; $Z=-1.16$, $P=0.989$). However, a Wilcoxon rank test showed that when limpets were exposed to acute temperatures at 15°C , immersed limpets maintained significantly higher heart rates than emersed limpets (Fig. 5A; $Z=-3.82$, $P<0.001$). When temperatures were ramped to 43°C and heart rate was determined for limpets at 40°C , emersed limpets maintained higher heart rates compared with limpets immersed in water (Fig. 5B; $Z=4.55$,

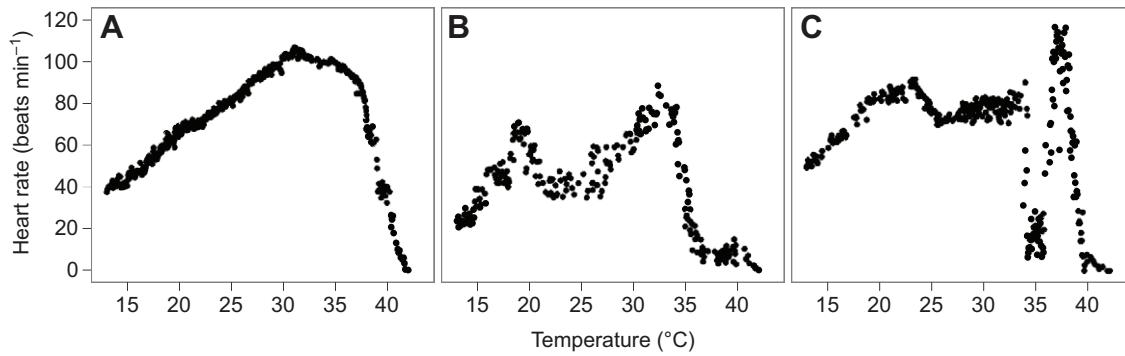


Fig. 3. Examples of limpet heart rate patterns observed in response to increasing temperature. Each image represents one of the patterns seen in cardiac performance experiments: (A) single break, (B) two breaks and (C) three breaks in heart rate. Limpets from both emersed and immersed conditions demonstrated these patterns in heart function.

$P < 0.001$). Conversely, in response acute thermal exposures to 40°C for 2 h, the reverse was seen; limpets under conditions of immersion maintained higher heart function than limpets that were emersed (Fig. 5A; $Z = -2.66$, $P = 0.008$).

Metabolic rate

Mass-specific metabolic rate (M_{O_2}) measured by oxygen consumption under acute temperature exposures demonstrated that limpets under emersed and immersed conditions have different oxygen consumption rates in response to increasing temperature exposures (Fig. 6). Increasing acute temperature exposures affected limpet oxygen consumption under both emersed and immersed conditions (emersion: $\chi^2 = 31.5$, d.f. = 5, $P < 0.001$; immersion: $\chi^2 = 47.4$, d.f. = 5, $P < 0.001$). Under immersed conditions, limpets at both 15 and 20°C steadily consumed oxygen at a rate of $\sim 20 \mu\text{mol O}_2 \text{h}^{-1} \text{g}^{-1}$ dry mass. Above 20°C, increasing temperature exposure resulted in a decrease in oxygen consumption, whereby at 30, 35 and 40°C, oxygen consumption was less than or equal to $10 \mu\text{mol O}_2 \text{h}^{-1} \text{g}^{-1}$ dry mass, significantly different from that of limpets at 15 and 20°C. In contrast, there was no significant difference in oxygen consumption of limpets under emersed conditions exposed to increasing temperatures from 15 to 35°C. In fact, there was a trend of increasing oxygen consumption with increasing temperature over this range. Oxygen consumption of aerial emersed limpets at 40°C was less than $10 \mu\text{mol O}_2 \text{h}^{-1} \text{g}^{-1}$ dry mass, significantly different from limpet oxygen consumption at all other acute temperature exposures except 30°C. Wilcoxon rank tests were run to detect any differences between emersed and immersed oxygen consumption at given temperature exposures. Oxygen consumption of limpets under emersed conditions was higher than that of limpets under immersed conditions at 25°C ($Z = 3.11$, $P = 0.002$) and 35°C ($Z = 3.58$, $P < 0.001$).

Air to water oxygen consumption ratios were calculated by comparing the mean oxygen consumption values of limpets under emersed and immersed conditions at each acute temperature exposure. Air to water oxygen consumption ratios increased with increasing temperature, with oxygen consumption of emersed limpets at 25°C roughly 2 times higher than that of immersed limpets. At 30 and 35°C, oxygen consumption of emersed limpets was roughly 3–4 times higher than that of water-immersed limpets (Fig. 6, inset).

Percentage body water was calculated to test the effect of increasing temperature exposures under both emersed and immersed conditions (Fig. 7) on tissue water loss (i.e. a significant decrease in wet mass:dry mass ratio). A two-way ANOVA showed a

significant effect of temperature on percentage body water ($P < 0.001$), no effect of exposure conditions (i.e. emersion *versus* immersion, $P = 0.061$), and no interaction between temperature and exposure conditions ($P = 0.088$). Data were split into emersed and immersed exposure conditions and two one-way ANOVA were run to test for the effect of temperature on each trial. Limpets under emersion conditions showed a significant water loss with increasing temperature ($P < 0.001$). A Tukey HSD test showed that there was no significant decrease in percentage body water from 15 to 35°C; however, there was a significantly lower percentage body water at 40°C ($P < 0.001$) in comparison to all other temperature exposure groups, suggesting tissue water loss at 40°C. As expected, limpets exposed to a temperature increase under conditions of immersion showed no change in percentage body water.

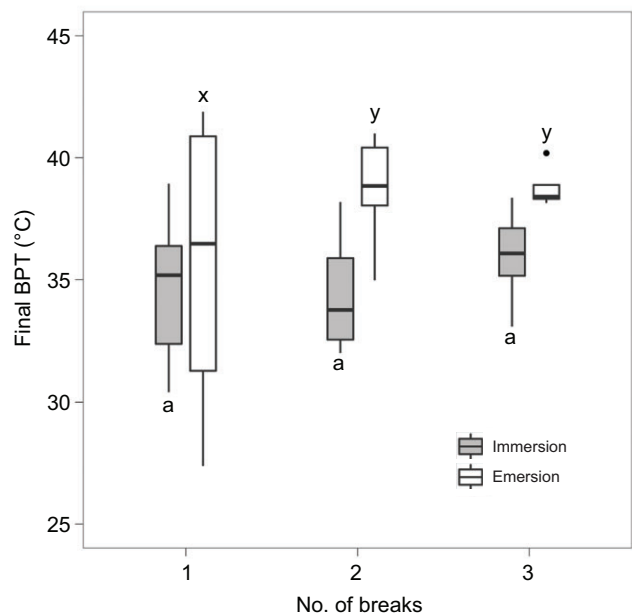


Fig. 4. Final BPTs in heart rate separated by the total number of breaks in heart rate that limpets exhibited throughout the complete temperature ramp. Boxplots as in Fig. 2. Within immersion and emersion groups, different letters indicate statistical differences ($P < 0.008$) in variability (Levene test) between the number of breaks in heart rate and the final BPTs.

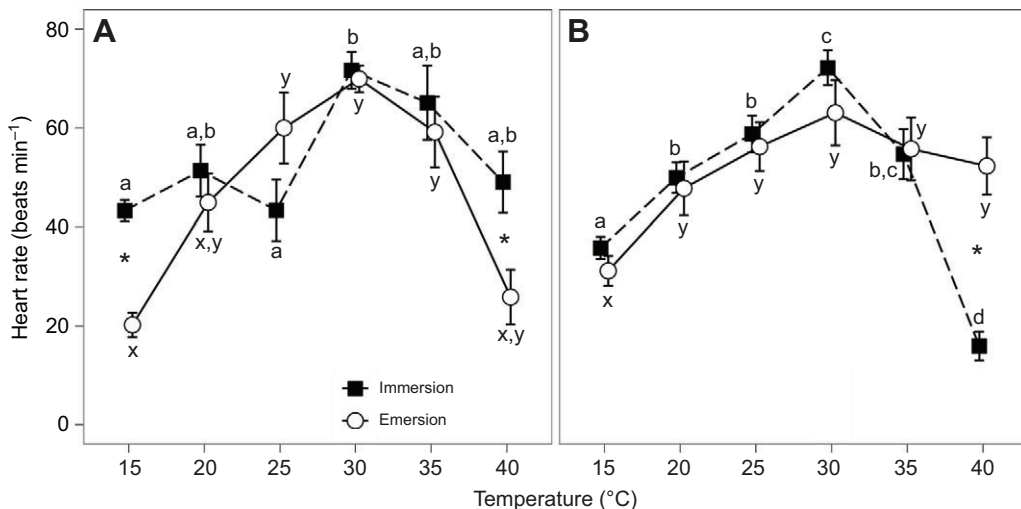


Fig. 5. Cardiac performance curves of immersed and emersed limpets in response to increasing temperatures under acute (A) and ramped (B) increases in temperature. (A) Heart rate of limpets in response to an acute temperature exposure of 15, 20, 25, 30, 35 or 40°C for 2 h. Data are presented as means \pm s.e.m. for $N=11-13$ limpets for each data point. (B) Heart rate of limpets at 15, 20, 25, 30, 35 and 40°C when exposed to increasing temperature at a rate of 6°C h⁻¹ from 13 to 40°C. Data are presented as means \pm s.e.m. for $N=31$ (emersion) and $N=20$ (immersion) limpets. Different letters indicate statistical differences ($P < 0.05$) between temperatures within emersed and immersed conditions. Asterisks represent a significant difference between emersion and immersion at a given temperature (A: $P < 0.008$, B: $P < 0.05$).

Glycogen content

A two-way ANOVA revealed a significant effect of temperature on glycogen content in response to increasing temperature ($P < 0.001$); however, there was no effect on glycogen content of emersed and immersed conditions ($P = 0.445$), nor an interaction between

temperature and emersed/immersed ($P = 0.187$) conditions (Fig. 8). A Tukey HSD test revealed that limpet glycogen content under emersed conditions was not affected by increasing acute temperature exposures; however, foot glycogen levels under immersed conditions at 40°C were significantly lower in comparison to acute temperature exposures at 15°C ($P = 0.007$) and 25°C ($P = 0.016$).

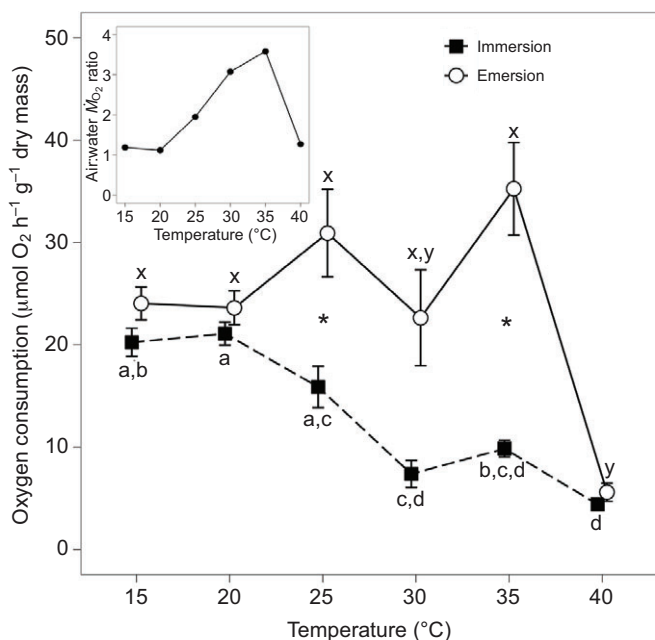


Fig. 6. Oxygen consumption of immersed and emersed limpets subjected to an acute temperature exposure of 15, 20, 25, 30, 35 or 40°C for 2 h. Data are presented as means \pm s.e.m. for $N=11-12$ limpets per data point. Different letters indicate statistical differences ($P < 0.05$) between temperatures within emersed and immersed conditions. Asterisks indicate significant differences ($P < 0.008$) in oxygen consumption between emersed and immersed conditions at a specific temperature. The inset depicts the ratio of oxygen consumption by limpets in air relative to water at each temperature.

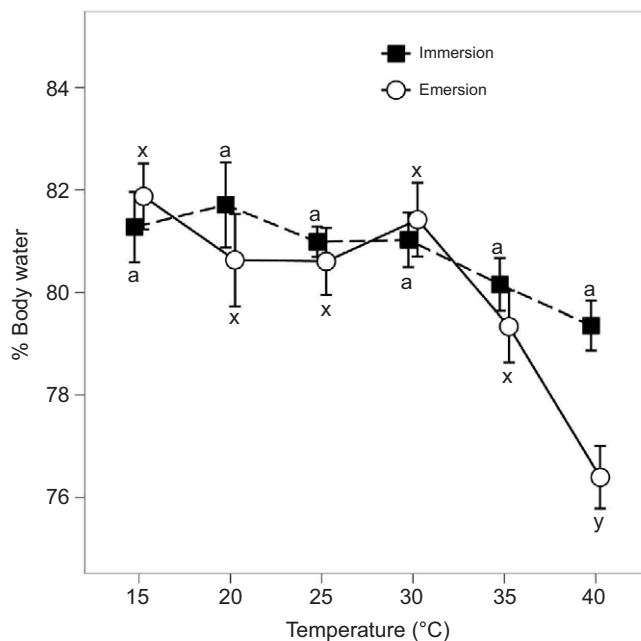


Fig. 7. Percentage body water of limpets following an acute temperature exposure of 15, 20, 25, 30, 35 and 40°C for 2 h under both emersed and immersed conditions. Percentage body water is expressed as $\{1 - [\text{dry mass}/(M_2 - \text{shell mass})]\} \times 100$. Data are presented as means \pm s.e.m. for $N=11-12$ limpets per data point. Different letters indicate statistical differences ($P < 0.05$) between temperatures within emersed and immersed conditions.

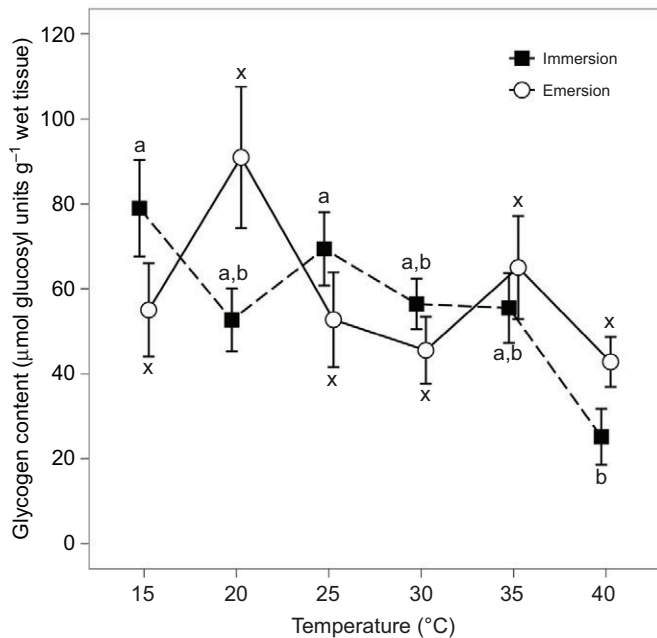


Fig. 8. Glycogen content of limpet foot tissue following an acute temperature exposure of 15, 20, 25, 30, 35 and 40°C for 2 h under both emersed and immersed conditions. Data are presented as means \pm s.e.m. for $N=12-15$ limpets per data point. Different letters indicate statistical differences ($P<0.05$) between temperatures within emersed and immersed conditions.

Ub-conjugated protein

Ub-conjugated proteins were present in all limpets for all acute temperature exposures under both emersed and immersed conditions (Fig. 9). A two-way ANOVA showed a significant effect of temperature on levels of Ub-conjugated proteins ($P<0.001$) in response to increasing acute thermal exposures. A significant effect of emersed and immersed conditions ($P=0.014$) as well as an interaction ($P=0.012$) between temperature exposures and treatment conditions on levels of Ub-conjugated protein were detected in response to increasing acute thermal exposures. Under immersed conditions, Ub-conjugated protein levels were not significantly affected by increasing acute thermal exposures. Ub-conjugated protein levels of emersed limpets were significantly affected by increasing temperature exposures ($P<0.001$); there were greater levels of Ub-conjugated proteins at 25°C ($P=0.019$) and 35°C ($P=0.001$) in comparison to those at ambient conditions of 15°C. Levels were also greater at 25°C ($P<0.001$) and 35°C ($P<0.001$) in comparison to those in limpets exposed to the most extreme temperature (40°C). Ub-conjugate protein levels were significantly higher in emersed limpets than in immersed limpets in response to acute temperature exposures at 25°C ($P=0.023$, Fig. 9).

DISCUSSION

Cardiac performance

Temperature sensitivity and upper thermal limits of cardiac performance of *L. digitalis* are significantly different under emersion and immersion. Limpets exposed to increasing temperature under emersed conditions exhibited a higher final cardiac BPT and were able to maintain heart function at higher temperatures compared with limpets exposed to thermal stress under immersed conditions. These differences in thermal tolerance suggest that the combination of aerial emersion and increasing temperature may confer an

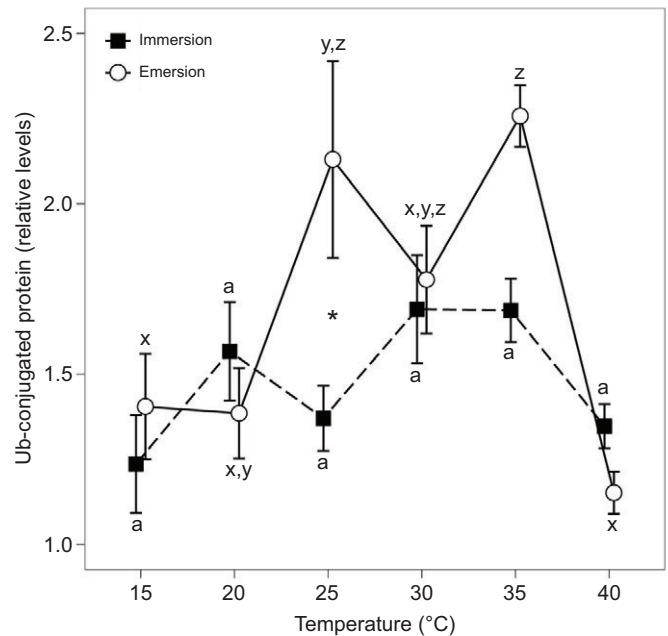


Fig. 9. Ubiquitin (Ub)-conjugated protein levels of limpet foot tissue following an acute temperature exposure of 15, 20, 25, 30, 35 and 40°C for 2 h under both emersed and immersed conditions. Ub-conjugated protein levels are expressed as relative values compared with the intensity of an internal reference sample. Data are presented as means \pm s.e.m. for $N=11-15$ limpets per data point. Different letters indicate statistical differences ($P<0.05$) between temperatures within emersed and immersed conditions. Asterisks represent a significant difference between emersion and immersion at a given temperature ($P<0.05$).

enhanced stress tolerance on limpets. It has previously been shown that animals living higher in the intertidal zone and those living at lower latitudes have a higher thermal tolerance and breadth of performance over a wider temperature range than congeners living lower in the intertidal zone or at higher latitudes (Stillman and Somero, 1996; Chelazzi et al., 2001; Stillman, 2003; Stenseng et al., 2005; Braby and Somero, 2006; Dong and Williams, 2011; Logan et al., 2012). These findings provide support that an animal's performance and tolerance limits typically reflect its natural habitat temperatures. It is perhaps not surprising that intertidal animals that are located high on the rocky shore and experience daily exposure to elevated temperatures during emersion have biochemical and physiological mechanisms in place that provide enhanced temperature tolerance under emersion compared with immersion. To date, there are limited studies that have investigated the thermal sensitivity of intertidal animals under conditions of emersion, the condition when they naturally experience the largest fluctuations in environmental temperature (but see Williams et al., 2005; Miller et al., 2009; Dong and Williams, 2011; Logan et al., 2012). In one study, the marbled rock crab, *Pachygrapsus marmoratus*, showed no difference in heart rate between emersion and immersion in response to increasing acute temperature exposures from 10 to 32.5°C (De Pirro et al., 1999). Unlike limpets that are sessile during a low tide period, crabs are mobile and can move to cooler, more shaded environments if environmental temperatures increase. Therefore, the need to have additional stress tolerance mechanisms during emersion may depend not only on location within the intertidal zone but also on the capacity to behaviorally thermoregulate.

Though differences in upper thermal limits of cardiac performance exist among limpets under emersed and immersed conditions, there were no significant differences in the heart rate of limpets under emersed and immersed conditions for temperatures from 15 to 35°C (Fig. 5). These results suggest that limpet heart rate has similar temperature sensitivity under the two conditions between 15 and 35°C and that differences in cardiac performance only emerged at temperatures greater than 35°C. It is noteworthy that the manner in which limpets were exposed to a 40°C heat shock (i.e. ramped *versus* acute change) significantly affected the cardiac performance of immersed and emersed limpets and their comparative sensitivities. Emersed limpets exposed to gradual increases in temperature maintained a significantly higher heart rate than did immersed limpets at 40°C; however, under acute exposures to elevated temperatures, immersed limpets had significantly higher heart rates than emersed limpets at 40°C. In nature, only emersed limpets would experience temperatures greater than 35°C; therefore, aerial exposure during gradual increases in temperature may recruit cellular defense mechanisms that are then in place to enhance tolerance to temperature extremes. Under immersion, there may not be the same stimulus or signal to upregulate similar cellular defenses. The nature and kinetics of these potential anticipatory mechanisms require further investigation in limpets.

Some limpets under both emersed and immersed conditions experienced multiple breaks in heart rate with increasing temperature, during which heart rate would drastically decrease but recover shortly afterward and continue increasing with increasing temperature. Breaks in heart function, other than final break points at their upper critical temperature threshold, have not previously been reported. Closer examination of data from previous studies suggests that other intertidal animals have demonstrated periodic heart rate suppression as well (Helm and Trueman, 1967; Trueman and Lowe, 1971; Bayne et al., 1976; Braby and Somero, 2006; Marshall et al., 2011). The tropical littoral-fringe snail *Echinolittorina malaccana* experienced decreased heart rate in response to increases in temperature (Marshall et al., 2011). Braby and Somero have also shown depressed heart rate in the blue mussel *Mytilus trossulus* in response to increasing temperature, followed by a return to normal heart rate when temperature was decreased (Braby and Somero, 2006). Multiple breaks in heart rate could represent active depression of heart rate in an effort to conserve energy in response to increasing thermal exposures, whereby heart rate can fluctuate between zones of temperature sensitivity and insensitivity. Because limpets under both emersed and immersed conditions experienced multiple breaks, the breaks were not a consequence of aerial exposure (Connor and Gracey, 2012) or immersion, but a response to the increase in temperature. Grigg and Seebacher have shown that lizard hearts act as thermoregulators in response to temperature changes (Grigg and Seebacher, 1999). Thermonegative metabolism has been observed in the intertidal snail *E. malaccana* where snails actively depress metabolism in response to warming between 35 and 46°C, suggesting efforts to conserve energy at higher temperatures to offset limitations on energy gain from living in a variable thermal environment (Marshall et al., 2011). It is noteworthy that the number of breaks in heart rate during increases in environmental temperature had no effect on the final break point temperature in heart function in either group of limpets. Therefore, it does not appear to be a physiological mechanism for extending upper temperature tolerance.

While limpets under emersed conditions were able to maintain heart function to higher temperatures than limpets that were immersed during heat stress, emersed limpets exhibited a faster rate

of decrease (slope) in heart rate following final BPT. This rapid decrease in heart rate was interpreted as a 'collapse' in heart function, demonstrating that emersed limpets were unable to control cardiac activity following their final BPT. In contrast, immersed limpets experienced a decline in heart rate 3–5°C earlier; however, overall heart function was maintained at a decreased level as temperatures continued to increase. Although we did not record exactly when heart function ceased in all limpets (referred to as flat line temperature) (Braby and Somero, 2006), based on our calculations of slope, it appears that flat line temperature did not differ between emersed and immersed limpets. Therefore, while the emersed limpets are able to maintain elevated heart function to a higher temperature than immersed limpets, there did not appear to be differences in the temperature resulting in cardiac arrest.

Metabolism

Metabolic rate (\dot{M}_{O_2}) of limpets under emersion and immersion showed greater differences in temperature sensitivity than did cardiac performance. When exposed to elevated temperatures between 25 and 35°C, \dot{M}_{O_2} was higher in emersed limpets compared with those under immersion. Emersed limpet \dot{M}_{O_2} was variable with slight but non-significant fluctuations in O_2 consumption in response to increasing temperature from 15 to 35°C, with a drop in \dot{M}_{O_2} at 40°C. Immersed limpet \dot{M}_{O_2} steadily decreased with increasing temperature exposures from 20 to 40°C. These results are similar to what has been seen in other studies that compared oxygen consumption of intertidal animals under conditions of emersion and immersion, although there is substantial variability in the literature. Specifically, air to water \dot{M}_{O_2} ratios between 1 and 5 have been recorded for other gastropods and intertidal organisms (Wolcott, 1973; Branch and Newell, 1978; McMahan, 1988; Truchot, 1990) and in the present study air to water \dot{M}_{O_2} ratios varied between 1 and 4 depending on the temperature, with the greatest difference at seen 35°C (see Fig. 6, inset). It is noteworthy that although there existed large differences in oxygen consumption in emersed and immersed limpets at 35°C, cardiac performance of the two groups was similar. Within the literature, studies document a variable effect of emersion on the oxygen consumption of intertidal organisms, suggesting that elevated aerobic metabolism in air when exposed to increases in temperature is not a unified stress tolerance or response strategy in all intertidal organisms. In two closely related limpets, *Patella caerulea* and *P. lusitanica* from the Mediterranean sea, *P. caerulea* had higher O_2 consumption when submerged in water than in air in response to acute changes in temperature, whereas *P. lusitanica* consumed a greater amount of O_2 in air than in water (Bannister, 1974). In the shore crab, *Carcinus maenas*, no difference in metabolic rate was observed between emersed and immersed conditions (Taylor and Butler, 1978), whereas in adult porcelain crabs, *P. cintipes* and *P. eriomerus*, metabolic rate was higher under immersed conditions than emersed conditions in response to increased temperature exposure (Stillman and Somero, 1996). Vertical location within the intertidal zone may be an important determinant of whether an organism relies on increased aerobic metabolism during emersion, such that this phenomenon is more dominant in organisms in the higher regions of the intertidal zone that spend long periods out of water exposed to high temperature (Branch, 1979). Higher O_2 consumption in emersed *L. digitalis* could reflect additional metabolic demands associated with increasing temperature during aerial exposure that may not be present under immersion, such as having to lift the edge of the shell to expose the mantle and gills to the air for gas exchange (Wolcott, 1973; McMahan, 1988). Though desiccation is often attributed as

an additional factor under emersion, in the current experiment, percentage body water was measured in limpets sealed in a closed respirometer and therefore exposed to 100% relative humidity, preventing us from testing the effect of desiccation. It is interesting to note that even though emersed limpets were exposed to 100% relative humidity at 40°C, a significant decrease in percentage body water was measured. The mechanisms underlying this water loss are unclear but may be associated with changes in membrane lipid composition that have been shown to occur over a tidal cycle in response to elevated temperature in intertidal mussels (Williams and Somero, 1996). A second factor to consider is differences in oxygen availability in terrestrial and aquatic environments. Limpets exposed to temperature increases while aerially emersed have ~30 times greater oxygen availability than limpets that are immersed (Truchot, 1990; Verberk and Bilton, 2011) and gas exchange therefore may be more efficient in air than in water, and this is represented by a higher \dot{M}_{O_2} .

At temperatures greater than 20°C, immersed limpets appear to be suppressing their metabolic rate as temperature is increased. This suggests immersed limpets may not be able to maintain the oxygen supply necessary to meet the metabolic demand of increasing temperature (Pörtner, 2001) and may be relying increasingly on anaerobic metabolism. Glycogen levels measured in the present study in foot tissue, the primary site of glycogen storage (Santini and Chelazzi, 1995), did not change significantly in response to increasing temperature under immersion until 40°C, where they were significantly lower than levels at 15 and 25°C. Therefore, it does not appear that immersed limpets are increasing anaerobic pathways and depleting glycogen at temperatures where they have reduced \dot{M}_{O_2} . Our results are consistent with a previous study in the intertidal snail *E. malaccana* in response to increasing temperature stress under emersion (Marshall et al., 2011). *Echinolittorina malaccana* depressed aerobic metabolism in response to prolonged temperature exposures to conserve energy; however, they avoided undergoing anaerobic metabolism (Marshall et al., 2011). Though patterns are variable, some animals have been shown to partition energy demands between aerobic and anaerobic metabolism using anaerobic pathways that lead to end products of succinate, alanine and propionate (Truchot, 1990; Santini et al., 2001). The ribbed mussel *Mytilus californianus* has been shown to oscillate between aerobic and anaerobic metabolites in relation to the tidal cycle, where anaerobic metabolites and catabolism of fatty acids increase and aerobic metabolites decrease during low tide emersion (Connor and Gracey, 2012).

Emersed limpets demonstrated relatively wide temperature-insensitive regions in \dot{M}_{O_2} for temperatures that they would see regularly during summer midday low tide periods (Fig. 1). Many other intertidal animals such as the eastern oyster, *Crassostrea virginica* (Davies and Tribe, 1969), limpet *Patella granularis* (Marshall and McQuaid, 1991), marine pulmonate snail *Siphonaria oculus* (Marshall and McQuaid, 1991), intertidal snail *E. malaccana* (Marshall et al., 2011) and brackish and coastal killifish *Fundulus heteroclitus* (Fangue et al., 2008) exhibit temperature-insensitive physiological performance in response to increases in environmental temperature. Marshall and McQuaid suggest that metabolic rate depression and temperature-independent metabolism are energy-conserving strategies (Marshall and McQuaid, 2011). Typically, the thermally insensitive regions of physiological performance are closer to an animal's upper critical body temperature (Davies and Tribe, 1969; Marshall et al., 2011). Metabolic stability of intertidal animals has been suggested to be adaptive and important for terrestrial survival of marine species (Marshall and McQuaid, 1991). Presently, it is unclear whether factors in addition to temperature mediate

temperature insensitivity of metabolic rate. Results from the current study suggest that emersion and the associated changes in other abiotic factors, such as oxygen and water availability, are likely important considerations.

Cellular protein integrity

To better understand the temperature sensitivity of emersed and immersed limpets at the cellular level, Ub-conjugated proteins were quantified as a measure of the magnitude of protein misfolding and damage that may occur in response to increasing temperature. Overall levels of Ub-conjugated proteins in limpet foot tissue were significantly higher in emersed limpets than in immersed limpets; however, they were only significantly different at 25°C. It is possible that emersed limpets are experiencing a higher level of protein denaturation in response to elevated temperature than immersed limpets and, as a result, have more proteins tagged for degradation by the Ub-proteasome proteolytic pathway (Glickman and Ciechanover, 2002). The higher temperature sensitivity at the cellular level in emersed limpets is in contrast to the cardiac performance data, which demonstrate that emersed limpets have a higher upper temperature tolerance. A possible alternative explanation for the elevated levels of Ub-conjugated protein is that emersed limpets have a greater capacity to upregulate cellular defense mechanisms to deal with the accumulation of denatured proteins, such as the Ub-proteasome pathway, and the elevated levels of Ub-conjugated proteins is an indication of a more effective cellular stress response. If this is the case, then immersed limpets could have as many denatured proteins but have a lower capacity to tag and shuttle these for breakdown, and risk the cytotoxic aggregation of misfolded proteins (Hightower, 1991). As Ub conjugates were measured immediately following a 2h heat shock of varying degrees and not during recovery, when compensatory mechanisms might be upregulated (Kültz, 2005), it is not possible to determine whether differences exist in the kinetics of the Ub-proteasome response in emersed *versus* immersed limpets. The capacity of *L. digitalis* to maintain cellular protein integrity in response to temperature increases under emersion and immersion clearly differs. However, whether the magnitude of protein damage is greater under emersed conditions or whether emersed limpets have adaptive anticipatory mechanisms and a greater capacity to upregulate cellular defense mechanisms under emersion requires further investigation.

Thermal safety margins: linking physiology to the field

Thermal safety margins, defined as the window between an animal's optimal performance temperature (T_{opt}) and the environmental temperature (T_e), are often calculated for physiological measures of organismal fitness in nature as they can be used to assess how close animals are currently living to their limits, as well as whether there is the physiological capacity to acclimatized to further increases in environmental temperature (Deutsch et al., 2008; Denny and Dowd, 2012). Cardiac performance curves suggest that T_{opt} of *L. digitalis* is ~30°C. Intertidal rock temperatures at Fort Ross, CA, USA, where *L. digitalis* were collected, exceeded 30°C on numerous occasions. In fact, intertidal rock temperatures exceed the final BPT of cardiac function measured for emersed limpets repeatedly throughout the summer. Although limpet body temperatures in the field were not measured in the current study, biophysical models suggest that substrate temperature is the primary factor determining limpet body temperature (Denny and Harley, 2006; Miller et al., 2009; Chaperon and Seuront, 2011). Negative thermal margin events, where T_e exceeds T_{opt} , may elicit additional bioenergetic costs, whereby energy is re-directed towards buffering thermal stress, leaving less

energy available for reproduction and survival, resulting in an overall decrease in organismal performance. Negative thermal safety margins have been shown in the sprinting speed of basking and non-basking tropical forest lizards *Anolis cristatellus* and *A. gundlachi*, such that slight increases in environmental temperature will exceed their T_{opt} and compromise performance (Huey et al., 2009). Stillman has also shown in porcelain crab congeners that species found in the upper intertidal zone have little to no capacity for acclimation of CT_{max} in comparison to the lower intertidal crabs (Stillman, 2003). The stochastic and episodic nature of high temperature exposures in the intertidal zone may make it difficult for intertidal organisms to acclimatize to temperature extremes in nature. For example, the capacity and energy required to upregulate a cellular stress response following one severe heat stress to protect against another of similar magnitude may not be beneficial if that second severe heat stress is not predictable in timing or occurs after the cellular response has occurred and returned to baseline. Negative thermal safety margins recorded in intertidal organisms may be evidence of this lack of acclimatization.

Concluding remarks

In summary, the thermal physiology and upper thermal limits of cardiac performance of *L. digitalis* differ under emersion and immersion. Through comparisons of cardiac performance and metabolic parameters, this study provides insight into additional physiological and biochemical mechanisms that are recruited to enable limpets to live in a variable intertidal environment that is often aerially exposed to rapid increases in temperature. The findings of this study provide preliminary evidence of anticipatory mechanisms associated with aerial exposure that may play a role in maintaining high temperature tolerance in this species. More research is warranted to explore the multiple breaks in heart performance, as well as the temperature insensitivity of metabolic rate, to better understand both the mechanisms and adaptive significance of these potential energy conservation strategies in intertidal species. Furthermore, in the current study limpets were acclimated to constant conditions for 14 days before experimentation and it would be important to understand how tidal entrainment (i.e. daily aerial exposure) integrates to modulate thermal physiology during emersion. By pairing ecological temperature data with physiological performance assessments, it is evident that *L. digitalis* is currently living close to its upper temperature tolerance limits, with multiple days in summer exceeding their upper thermal limits of cardiac performance.

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AUTHOR CONTRIBUTIONS

Both authors contributed significantly to the conception, design and execution of the study, interpretation of the findings as well as drafting and revising of the article.

COMPETING INTERESTS

No competing interests declared.

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