



Ocean acidification increases the sensitivity of and variability in physiological responses of an intertidal limpet to thermal stress

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Abstract. Understanding physiological responses of organisms to warming and ocean acidification is the first step towards predicting the potential population- and community-level ecological impacts of these stressors. Increasingly, physiological plasticity is being recognized as important for organisms to adapt to the changing microclimates. Here, we evaluate the importance of physiological plasticity for coping with ocean acidification and elevated temperature, and its variability among individuals, of the intertidal limpet *Ce-lana toreuma* from the same population in Xiamen. Limpets were collected from shaded mid-intertidal rock surfaces. They were acclimated under combinations of different $p\text{CO}_2$ concentrations (400 and 1000 ppm, corresponding to a pH of 8.1 and 7.8) and temperatures (20 and 24 °C) in a short-term period (7 days), with the control conditions (20 °C and 400 ppm) representing the average annual temperature and present-day $p\text{CO}_2$ level at the collection site. Heart rates (as a proxy for metabolic performance) and expression of genes encoding inducible and constitutive heat-shock proteins (*hsp70* and *hsc70*) at different heat-shock temperatures (26, 30, 34, and 38 °C) were measured. Hsp70 and Hsc70 play important roles in protecting cells from heat stresses, but have different expression patterns, with Hsp70 significantly increased in expression during stress and Hsc70 constitutively expressed and only mildly induced during stress. Analysis of heart rate showed significantly higher temperature coefficients (Q_{10} rates) for limpets at 20 °C than at 24 °C and post-acclimation thermal sensitivity of limpets at

400 ppm was lower than at 1000 ppm. Expression of *hsp70* linearly increased with the increasing heat-shock temperatures, with the largest slope occurring in limpets acclimated under a future scenario (24 °C and 1000 ppm $p\text{CO}_2$). These results suggested that limpets showed increased sensitivity and stress response under future conditions. Furthermore, the increased variation in physiological response under the future scenario indicated that some individuals have higher physiological plasticity to cope with these conditions. While short-term acclimation to reduced pH seawater decreases the ability of partial individuals against thermal stress, physiological plasticity and variability seem to be crucial in allowing some intertidal animals to survive in a rapidly changing environment.

1 Introduction

Benthic organisms living in the intertidal zone will be exposed to increasingly variable and extreme environmental conditions, such as temperature, oxygen, and CO_2 , due to climatic change (IPCC, 2013; Kwiatkowski et al., 2016). These highly fluctuating environmental variables can significantly affect the physiological performance of coastal species (Hel-muth et al., 2006; Hofmann and Todgham, 2010; Somero, 2012; Widdicombe and Spicer, 2008). Therefore, understanding the interaction of multiple environmental stressors on physiological performance is crucial for predict-

ing the consequences of environmental change on ecosystems (Deutsch et al., 2015). For example, salinity fluctuations coupled with high temperatures during emersion can have both sublethal physiological effects and lethal effects on intertidal mollusks (Dong et al., 2014; Firth and Williams, 2009). Although ocean acidification can increase the growth of organisms in some cases (e.g., Gooding et al., 2009), there is increasing evidence that decreased pH exacerbates global warming, and interactions of ocean acidification and warming reduce resistance of an organism to environmental change (Munday et al., 2009) and subsequently affect population dynamics (Fabry et al., 2008; Hoegh-Guldberg et al., 2007; Kroeker et al., 2013; Rodolfo-Metalpa et al., 2011).

In the face of a changing environment, organisms can respond in three ways: exhibit shifts in distributional ranges (Parmesan and Yohe, 2003), develop adaptive changes (Hoffmann and Sgro, 2011), or perish (Fabricius et al., 2011). Prior to mortality or range shifts, environmental changes can often drive physiological adaptation or the evolution of phenotypic plasticity (Chevin et al., 2010; Sanford and Kelly, 2011). However, warming and ocean acidification are not unidirectional, but rather combined with rapid fluctuations on daily to seasonal and decadal timescales. Thus, the changing environment often does not provide clear signals to drive strong directional selection of traits, meaning that, usually, physiological plasticity is the more important factor in acclimation to changing environmental conditions (Hoffmann and Sgro, 2011; Pörtner et al., 2012; Somero et al., 2012). In a recent meta-analysis, Seebacher et al. (2015) demonstrated that acclimation to higher temperatures decreased the sensitivity to increased temperature in both freshwater and marine animals. While this response suggests that acclimation could reduce the impact of warming on organisms, the responses were only tested for shifts in mean temperature. Furthermore, organisms inhabiting variable environments, such as the intertidal zone, will be exposed to increasing extremes in temperature concomitant with increasing $p\text{CO}_2$, or ocean acidification, in the future. While ocean acidification has been suggested to increase the sensitivity of organisms to warming (Byrne and Przeslawski, 2013; Byrne, 2011; Kroeker et al., 2013), physiological plasticity and variation in responses may provide the basis for populations to survive.

Physiological variation, or plasticity, within a population is important for adapting to local microclimate and for evolution (Dong et al., 2017; Oleksiak et al., 2002; Prosser, 1955). For example, different color morphs of the gastropod *Littorina saxatilis* have enhanced physiological performance, which leads to increased survival under extreme conditions, indicating physiological differences may provide a selective advantage for those color morphs under an extremely fluctuating salinity and temperature regime in estuaries (Sokolova and Berger, 2000). For the limpet *Cellana toreuma*, highly variable expressions of genes related to stress responses and energy metabolism are important for surviving the harsh environment on subtropical rocky shores (Dong et al., 2014).

Heart rate, as a measure of cardiac activity, is a useful indicator for indicating physiological response to stress in mollusks (Dong and Williams, 2011; Xing et al., 2016). Animals exhibit a stable basal heart rate under conditions which are not thermally stressful, and heart rate increases and reaches a peak followed by a sudden decrease with temperature rising (Braby and Somero, 2006; Dong and Williams, 2011). The temperature at which a sharp discontinuity in slope occurs in an Arrhenius plot (i.e., Arrhenius breakpoint temperature, ABT) can represent the limit of metabolic functioning of animals (Nickerson et al., 1989; Somero, 2002). At the molecular level, expression of heat-shock proteins (HSPs) and *hsp* genes is induced above a certain temperature, reaches maximum, and finally ceases in response to heat shock (Han et al., 2013; Miller et al., 2009). Upregulation of HSPs and *hsp* genes is an energy-consuming mechanism for defense against thermal stress (Somero et al., 2016). As a commonly used biomarker, the Hsp70 multigenic family includes two proteins with divergent expression patterns (inducible Hsp70 and constitutive Hsc70). The inducible Hsp70 significantly increases in expression when animals are exposed to stressors and plays a role in maintaining protein stability (Feder and Hofmann, 1999); conversely, the constitutive Hsc70, which is transcribed continuously and may be mildly induced during stress, takes part in folding and repairing of denatured proteins (Dong et al., 2015) and plays a role in the formation of mitotic structures (Sconzo et al., 1999). Some studies have shown coordinated heart rate and expression of genes encoding to HSPs in response to elevated temperature (Han et al., 2013; Prusina et al., 2014). However, little is known about the patterns of heart rate and expression of *hsp* genes for coping with combined warming and ocean acidification.

The limpet *C. toreuma* is a keystone species on rocky shores in the western Pacific (Dong et al., 2012), occupying the mid-low intertidal zones (Morton and Morton, 1983). This species is a gonochoric and broadcast spawner, whose embryos develop into planktonic trochophore larvae and later into juvenile veligers before becoming fully grown adults (Ruppert et al., 2004). As a common calcifier inhabiting coastal ecosystems, *C. toreuma* plays an important ecological role in food chains (Ding et al., 2018), grazing on biofilm and being an important food source for other species (e.g., crabs, seabirds, and sea stars). Therefore, this species is a key organism for studying the relationship between physiological response to thermal stress and ocean acidification in a highly variable environment on the shore.

Under the impact of subtropical high-pressure systems, Xiamen (118°14'E, 24°42'N) is one of the hottest areas in China (Dong et al., 2017). The coastal seawater of this area is experiencing rapid temperature rise and acidification (Bao and Ren, 2014). The sea surface temperature in Xiamen coastal water has increased a total of 1 °C since 1960, and is rising at a mean annual rate of 0.02 °C (Yan et al., 2016). The annual pH values of seawater in Xiamen Bay have declined

by 0.2 pH units from 8.05 in 1986 to 7.85 in 2012, a trend which is predicted to continue based on simulations (Cai et al., 2016).

Here, we investigated the importance of physiological plasticity (based on the measurement of post-acclimation temperature sensitivity; see Seebacher et al., 2015) and variability (based on coefficient of variation) for *C. toreuma* to cope with ocean acidification and elevated temperatures by quantifying heart rates (as a proxy of metabolic performance) and expression of genes encoding inducible and constitutive heat-shock proteins (Hsp70 and Hsc70) after short-term acclimation in different $p\text{CO}_2$ concentrations (400 and 1000 ppm) and temperatures (20 and 24 °C). We hypothesize that (1) limpets will show increased thermal sensitivity of metabolism and stress responses under elevated $p\text{CO}_2$ and temperatures and (2) short-term acclimation at high temperature and $p\text{CO}_2$ will cause higher inter-individual physiological variation. Our study, by measuring both heart rate and heat-shock protein gene expression, provides novel information concerning the combined effects of increased temperature and $p\text{CO}_2$ on stress response, energy consumption, and physiological plasticity in intertidal invertebrates, potentially providing predications of ecological impacts of the future environmental changes.

2 Material and methods

2.1 Limpet collection and experiment treatments

Samples were collected from shaded rock surfaces at mid-tidal level in Xiamen during a falling high tide in July (in situ temperature: 30.8 ± 0.8 °C). The sampling ensured that all limpets have similar thermal history, given the possible impacts from microclimate (Dong et al., 2017; Lathlean and Seuront, 2014). They were transported to the State Key Laboratory of Marine Environmental Science, Xiamen University, China, within 2 h. Limpets were first allowed to recover at 20 °C for 3 days with a tidal cycle of approximately 6 h of immersion and 6 h of emersion. These limpets were randomly allocated into one of four treatments and temporally acclimated in different $p\text{CO}_2$ concentrations and temperatures (LTLC, low temperature and low CO_2 , 20 °C + 400 ppm, as a control treatment; LTHC, low temperature and high CO_2 , 20 °C + 1000 ppm; HTLC, high temperature and low CO_2 , 24 °C + 400 ppm; HTHC, high temperature and high CO_2 , 24 °C + 1000 ppm) for 7 days in climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which control both the $p\text{CO}_2$ concentration and temperature under the same relative humidity and light intensity conditions. In each acclimation treatment, approximately 100 limpets were randomly allocated into ~30 containers (three individuals in each container) to simulate field densities of ~1 limpet per 10 cm². Control conditions (20 °C, 400 ppm) represent the average annual temperature

and ambient $p\text{CO}_2$ (~390 ppm) at the collection site, with high temperature (24 °C) and $p\text{CO}_2$ (1000 ppm) representing the average global increase (4 °C, 600 ppm) predicted for 2100 by the Intergovernmental Panel on Climate Change (IPCC, 2007).

Animals were kept in a simulated tidal cycle with 6 h of aerial exposure and 6 h of seawater immersion. Seawater was pre-bubbled with air containing the corresponding $p\text{CO}_2$ concentrations in advance. The pH was measured before and after the acclimation in seawater each time with a PB-10 pH meter (Sartorius Instruments, Germany) calibrated with National Institute of Standards and Technology standard pH solutions (NIST, USA). Total dissolved inorganic carbon was measured before and after the acclimation in seawater each time using a dissolved inorganic carbon analyzer (As-C3, Apollo SciTech, Colorado, USA), using a Li-Cor[®] nondispersive infrared detector (Li-6252) with a precision of 0.1 % (Cai, 2003). Seawater carbonate chemistry parameters were estimated based on the measured values of pH, dissolved inorganic carbon, temperature, and salinity with the software CO2Calc v4.0.9 (Robbins et al., 2010). For CO2Calc settings, the NIST scale was applied as the pH scale, and the CO_2 constant, the KHSO_4 -constant and the total boron were set from Millero et al. (2006), Dickson et al. (1990), and Lee et al. (2010), respectively. The information of the measured and calculated seawater chemistry parameters is summarized (Table 1).

After a 7-day acclimation period (crossed $p\text{CO}_2 \times$ temperature treatments, above), the heat-shock treatments were carried out to simulate the gradual temperature exposure of limpets in the field as described in Denny et al. (2006) (Fig. A1). For each heat-shock treatment, 10 limpets were randomly selected from each of four acclimation conditions (40 individual total) and transferred to artificial rocks (Fig. A2), with individuals from LTLC and LTHC on one rock and individuals from HTLC and HTHC on another rock. The artificial rocks were separately placed in 20 °C water baths and 24 °C water baths and heated at a rate of 6 °C h⁻¹ that simulated emersion in the natural condition at the collection site (Han et al., 2013) to the designated temperatures (26, 30, 34, and 38 °C). After achieving the target temperature, the temperature was maintained for the allotted time, and then decreased to the acclimation temperature (20 or 24 °C) at a rate of 6 °C h⁻¹, for a total exposure time of 7 h. Individuals from all four acclimation conditions ($n = 10$ individuals per treatment) were randomly selected, transferred to artificial rocks, and aurally exposed at 20 or 24 °C for 7 h, as non-heated control samples. After recovery at 20 or 24 °C seawater for 1 h, limpets were immediately collected and stored at -80 °C for gene expression analysis.

2.2 Cardiac performance measurement

The cardiac performance of limpets was recorded during whole heating processes from the acclimated temperature (20

Table 1. Measured and calculated seawater carbonate chemistry variables of each acclimation treatment during the experimental period*.

	20 °C & 400 ppm	24 °C & 400 ppm	20 °C & 1000 ppm	24 °C & 1000 ppm
Temperature (°C)	20.94 ± 0.88	24.84 ± 0.87	20.59 ± 0.91	25.01 ± 0.67
Salinity (psu)	27.89 ± 0.88	27.96 ± 0.75	28.18 ± 0.75	27.79 ± 0.58
A_T (μmol kg ⁻¹)	2082.70 ± 191.28	2083.016 ± 190.58	2081.19 ± 165.93	2083.29 ± 163.58
DIC_T (μmol kg ⁻¹)	1910.57 ± 174.42	1910.57 ± 174.42	1992.76 ± 157.22	1992.15 ± 149.76
pCO_2 (μatm)	562.18 ± 83.20	561.81 ± 83.04	1008.66 ± 113.41	992.36 ± 47.04
pH (NIST)	8.05 ± 0.05	8.05 ± 0.05	7.82 ± 0.04	7.83 ± 0.04
CO_3^{2-} (μmol kg ⁻¹)	130.50 ± 21.25	130.64 ± 20.85	81.64 ± 11.76	83.42 ± 11.95
Ω_{cal}	3.31 ± 0.55	3.32 ± 0.54	2.07 ± 0.30	2.12 ± 0.30

* Seawater temperature, salinity, pH, and total dissolved inorganic carbon (DIC_T) were monitored every 6 h. Total alkalinity (A_T), pCO_2 , CO_3^{2-} , and Ω_{cal} were calculated using CO2SYS software. Results were pooled and averaged over sampling times. Values are given as mean ± SD.

or 24 °C) to the temperature at which the heart stopped beating ($n = 9$ –11 individuals per acclimation treatment). Each limpet was placed in a separate container during the measurement. The containers were immersed in water baths, allowing the temperature in the container to be increased at a rate of 6 °C h⁻¹ that simulated emersion in the natural environment. Heart rates were measured using a noninvasive method (Chelazzi et al., 2001; Dong and Williams, 2011). The heartbeat was detected by means of an infrared sensor fixed with Blu Tack (Bostik, Staffordshire, UK) on the limpet shell at a position above the heart. Variations in the light-dependent current produced by the heartbeat were amplified, filtered, and recorded using an infrared signal amplifier (AMP03, Newshift, Leiria, Portugal) and Powerlab AD converter (4/30, ADInstruments, March-Hugstetten, Germany). Data were viewed and analyzed using Lab Chart (version 7.0).

For determining the ABT of heart rate, discontinuities in the slopes of heart rate with temperature were calculated from intersections of fitted two-phase regressions based on the minimum sum of squares using SigmaPlot 12.5 (SSPS Inc., Point Richmond, CA, USA) as described by Giomi and Pörtner (2013).

2.3 Quantifying gene expression

Limpets were first taken out from – 80 °C; the foot muscle was cut off immediately using RNA-free scissors (180 °C before using); the muscle (~ 50 mg) was cut into pieces in a 1.5 mL EP tube containing RNA lysis buffer provided by Eastep reagent kit (Promega, USA); total RNA was isolated using Eastep reagent kit (Promega, USA). The first strand of cDNA was synthesized using total RNA as a template. Reverse transcriptase reactions were performed using a PrimeScript reverse transcriptase reagent kit with gDNA Eraser (Takara, Shiga, Japan).

The levels of mRNA of genes encoding two heat-shock proteins, inducible heat-shock protein 70 (*hsp70*) and constitutive heat-shock protein 70 (*hsc70*), were measured us-

ing real-time quantitative polymerase chain reactions in a CFX96™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules CA, USA) following the methods described by Han et al. (2013) with specific primers (Table A1). For normalizing expression of genes, we examined expression of the *18S ribosomal RNA*, *β-actin*, and *β-tubulin* genes, which typically have relatively stable expression levels. The expression stability of these housekeeping genes was evaluated using the geNorm algorithm (Primer Design, Ltd., Southampton University, Highfield Campus, Southampton Hants, UK) as described by Etschmann et al. (2006). Based on the expression stability measures (M values), all three genes were selected as the reference genes for normalizing the level of expression of stress-induced genes. All samples were measured in triplicates. Ct (dR) values were analyzed using the CFX Manager™ software version 3.0 (Bio-Rad). The expression of *hsp70* and *hsc70* was determined relative to the value of *18S*, *β-actin*, and *β-tubulin* from a reference individual.

2.4 Statistical analysis

The general additive mixed model (GAMM) was used to compare thermal sensitivities of heart rate among limpets acclimated at different temperatures (20 or 24 °C) and CO_2 concentrations (400 or 1000 ppm). Analyses were conducted with the mgcv (Wood, 2004) and nlme (Pinheiro et al., 2013) libraries in R Version 3.0 (R Core Team, 2014). The generalized additive model (GAM), describing heart rate as a function of temperature, was used to test for how heart rates of limpets from each treatment deviated from those of limpets from control conditions (20 °C, 400 ppm) (Angilletta et al., 2013).

Thermal sensitivity is the change in a physiological rate function reacting to a rapid change in environmental temperature within the same acclimation set temperature (Fig. A3, modified from Seebacher et al., 2015). In the present study, thermal sensitivity was determined in the temperature coefficient (Q_{10}) values of heart rate. Q_{10} was calculated using heart rate data from the temperature at which the experiment

started ($T_1 = 24^\circ\text{C}$) to the temperature to which temperature increased, 10°C ($T_2 = 33^\circ\text{C}$), with Eq. (1):

$$Q_{10} = \left(\frac{R_2}{R_1} \right)^{\frac{10}{T_2 - T_1}}, \quad (1)$$

where R is the heart rate (R_1 and R_2 are the heart rate at T_1 and T_2 , respectively), and T is the temperature (Kelvin) (Fig. A3, modified from Seebacher et al., 2015). The differences in Q_{10} among the four acclimation conditions with different CO_2 concentrations (400 ppm vs. 1000 ppm) and temperatures (20°C vs. 24°C) were analyzed using two-way ANOVA with Duncan's post hoc analysis using the SPSS 20.0 for Windows statistical package (IBM SPSS Statistics, Chicago, USA). Post-acclimation thermal sensitivity of limpets in different CO_2 concentrations was calculated as described by Seebacher et al. (2015). In each CO_2 concentration (400 or 1000 ppm), the post-acclimation Q_{10} values were calculated using the same equation as shown above, but R_2 was the average heart rate of the warm-acclimated limpets at the acclimated temperature ($T_2 = 24^\circ\text{C}$), and R_1 was the average heart rate of cold-acclimated limpets at $T_1 = 20^\circ\text{C}$ (Fig. A3, modified from Seebacher et al., 2015). It is worth noting that post-acclimation thermal sensitivity should be considered with caution, as in the present study the acclimation period (7 days) may not have been sufficient for full acclimation to altered conditions.

The differences in levels of *hsp70* and *hsc70* among different heat-shock temperatures within a same acclimation condition were analyzed using one-way ANOVA with Duncan's post hoc analysis. The relationships between heat-shock temperature and log-transformed gene expression (*hsp70* and *hsc70*) were fitted using linear regressions and the differences in slopes of the linear regressions were analyzed using analysis of covariance (ANCOVA).

The coefficient of variation (CV) of ABT, Q_{10} , and *hsc70* mRNA expression at 38°C were calculated for each acclimation condition. The CV is the variance in a sample divided by the mean of that sample, providing a method to compare the variation within a sample relative to the mean. It is generally accepted that higher CV demonstrates that there is greater variation among individuals within one treatment than another (Reed et al., 2002).

3 Results

3.1 Cardiac performance

The maximal heart rate was $\sim 30\%$ higher in limpets acclimated to control conditions (20°C , 400 ppm) than the other treatments (Fig. 1 and Table A2). The ABTs of limpets showed a trend to be reduced for high-temperature treatments (mean \pm SD: LTLC, $38.9 \pm 2.9^\circ\text{C}$; HTLC, $38.2 \pm 1.8^\circ\text{C}$; LTHC, $40.0 \pm 3.3^\circ\text{C}$; HTHC, $37.7 \pm 2.3^\circ\text{C}$) (Fig. A4). Temperature (two-way ANOVA, $F_{1,35} = 3.375$, $P = 0.075$) and

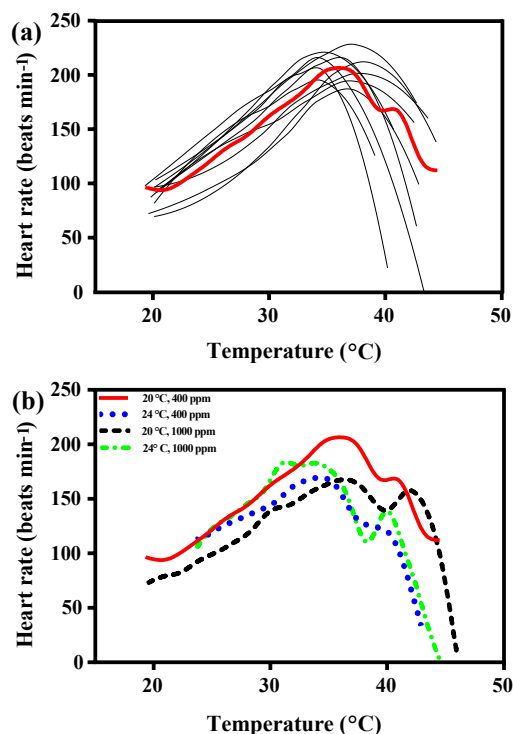


Figure 1. (a) Heart rates of all limpets acclimated to 20°C and 400 ppm, presented as an example of heart rate calculation for limpets in all treatments. The black lines correspond to smoothed fits (using the loess algorithm) of heart rates for each of the individual limpets. The red line represents the most likely general additive mixed model (GAMM) to depict the trajectory of heart rates for limpets with increasing temperature; (b) GAMM lines of limpets acclimated at the different experimental temperature and CO_2 conditions.

$p\text{CO}_2$ (two-way ANOVA, $F_{1,35} = 0.118$, $P = 0.733$) both had non-significant effects on ABTs, and there was a non-significant interaction between temperature and $p\text{CO}_2$ (two-way ANOVA, $F_{1,35} = 0.908$, $P = 0.347$) (Table A3; Fig. A4).

Temperature coefficients (Q_{10} rates) were higher for limpets acclimated at 20°C than at 24°C (two-way ANOVA, $F_{1,35} = 5.878$, $P = 0.02$), but there was no significant difference for acclimation to different $p\text{CO}_2$ concentrations (two-way ANOVA, $F_{1,35} = 1.332$, $P > 0.05$) and for the interaction between temperature and $p\text{CO}_2$ (two-way ANOVA, $F_{1,35} = 0.1135$, $P > 0.05$) (Table A3; Fig. 2). The post-acclimation thermal sensitivity of limpets acclimated at low CO_2 (2.12) was lower than that of limpets at high CO_2 (2.95) (Fig. 2).

The CVs of ABT in the four different acclimation conditions were different (Table 2). After low temperature and high CO_2 acclimation (LTHC, 8.22 %), the CV of ABT was higher than that in the other three conditions (LTLC, 7.34 %; HTLC, 4.48 %; HTHC, 6.08 %). The CV of Q_{10} under LTHC

Table 2. Coefficients of variation (%) of Arrhenius breakpoint temperature (ABT), temperature coefficients (Q_{10}), and *hsc70* mRNA expression at 38 °C^{1,2}.

Temperature	CO ₂	ABT	Q_{10}	<i>hsc70</i> mRNA
20	400	7.34	10.23	56.20
	1000	8.22	15.08	80.44
24	400	4.48	10.08	80.12
	1000	6.08	11.82	90.36

¹ Temperature coefficients (Q_{10}) were calculated using heart rate from 24 to 33 °C. ² After acclimation at different CO₂ and temperature for 1 week, limpets ($n = 8–10$) from each acclimation treatment were randomly selected and heat shocked at designated temperatures. Levels of *hsc70* mRNA at 38 °C in different acclimation treatments were used for calculating coefficients of variation.

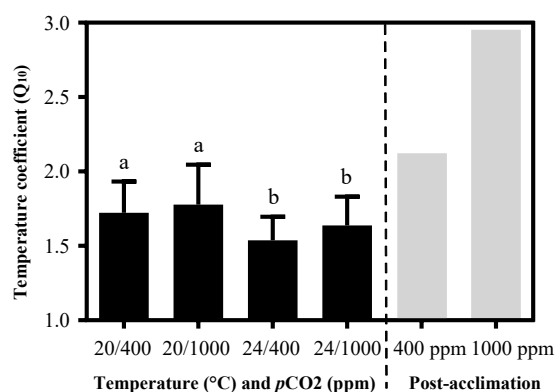


Figure 2. Temperature coefficients (Q_{10}) of limpets acclimated at different temperatures (20 or 24 °C) and CO₂ concentrations (400 or 1000 ppm). The temperature coefficient (Q_{10}) values were calculated for all limpets using heart rate data from 24 to 33 °C. Post-acclimation temperature sensitivity was calculated among individuals acclimated at 20 and 24 °C (grey bars; sensu Seebacher et al., 2015) for each CO₂ concentration, where higher thermal sensitivity indicates less acclimation to thermal stress. The calculation of post-acclimation Q_{10} is carried out for the mean response of all individuals as the same individuals are not used at each acclimation temperature. Therefore, it is not possible to calculate an estimate of variation or error for post-acclimation Q_{10} . Different letters represent significant differences in the Q_{10} among different acclimation treatments.

conditions was the highest in all four acclimation conditions (Table 2).

3.2 Gene expression

Levels of *hsp70* mRNA (log transformed) linearly increased with the increasing heat-shock temperatures (Fig. 3). ANCOVA showed that the slopes of the linear regressions were significantly different among different acclimation conditions ($F_{4,189} = 42.62$, $P < 0.001$), and the slope of HTHC conditions was higher than that of the other three acclimation conditions. Thus, the rate of increase in production of *hsp70*

mRNA in response to warming was greater at the elevated CO₂ concentration.

The responses of *hsc70* mRNA to heat shock were divergent among the four acclimation conditions (Fig. 4). For HTHC limpets, there were no significant differences among different heat-shock temperatures ($F_{4,42} = 2.11$, $P = 0.096$). For LTLC, LTHC, and HTLC limpets, levels of *hsc70* mRNA after being heat-shocked at 38 °C were higher than corresponding levels of *hsc70* mRNA at 20 or 24 °C (Duncan's post hoc analysis, $F_{4,42} = 4.389$, $P = 0.005$; $F_{4,44} = 8.521$, $P < 0.0001$; $F_{4,42} = 5.713$, $P = 0.001$). The coefficients of variation of *hsc* mRNA after heat shock of 38 °C were different among different acclimation conditions: HTHC (90.36 %) > LTHC (80.44 %) \approx HCLT (80.12 %) > LCLT (56.20 %) (Table 2).

4 Discussion

Short-term acclimation at elevated temperature and $p\text{CO}_2$ can increase physiological sensitivity of limpets to thermal stress. The higher thermal sensitivity of limpets acclimated to 1000 ppm indicates that the resilience of limpets to thermal stress associated with warming will be compromised under future ocean acidification. This prediction is contrary to the general thought that intertidal ectotherms, such as limpets and other gastropods, will demonstrate high tolerance to thermal stress because they are adapted to an extreme thermal environment. For example, the operative temperatures, which *C. toreuma* suffers under in the field, frequently exceed 40 °C in summer along Asian coastlines and the limpet can survive at temperatures in excess of 45 °C (Dong et al., 2015). Our data show, however, that ocean acidification will lead to increased sensitivity to changes to future thermal regimes, indicating a synergistic negative effect. The change in the metabolic partitioning in individuals could ultimately lead to a decline in fitness and population-level responses in the future.

Increased temperature and CO₂ elevated the sensitivity of heat-shock responses to thermal stress. The expression of inducible *hsp70* mRNA steadily increased from 20 to 38 °C for individuals across all experimental treatments. However, rates of upregulation of *hsp70* mRNA in limpets acclimated at high temperature and high CO₂ (HTHC) were significantly higher than that of limpets acclimated at the other three acclimation conditions. As a molecular chaperone, Hsp70 protein plays crucial roles in maintaining protein stability with the expense of a large amount of energy (Feder and Hofmann, 1999; Tomanek and Sanford, 2003). By comparing the expression patterns of Hsp70 of different *Chlorostoma* species (formerly *Tegula*) that have distinct vertical distributions, Tomanek and Somero (1999, 2000) found that there were interspecific differences in the frequency of the induction of Hsp70 synthesis and interspecific divergence of the time course of Hsp70 synthesis. These studies from the genus

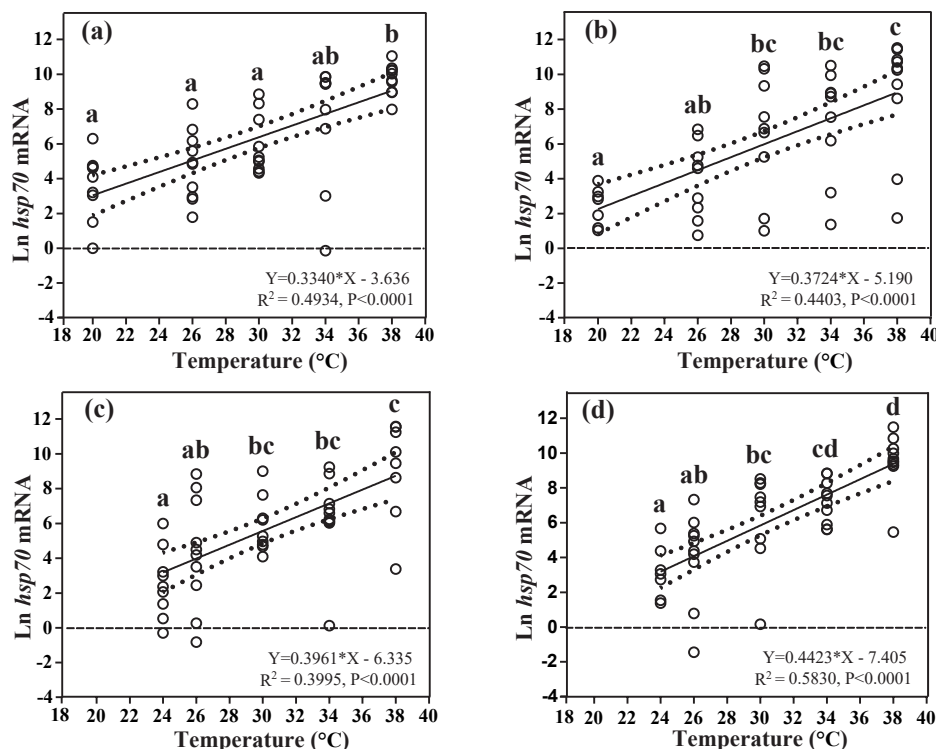


Figure 3. Effects of heat-shock temperature on the expression of *hsp70* mRNA in limpets acclimated at (a) 20 °C and 400 ppm, (b) 20 °C and 1000 ppm, (c) 24 °C and 400 ppm, and (d) 24 °C and 1000 ppm. The relationship between heat-shock temperature and log-transformed gene expression of *hsp70* was fitted using linear regressions with 95 % confidence intervals (dashed lines). Different letters represent significant differences in the level of *hsp70* mRNA among different heat-shock temperatures.

Chlorostoma suggested that species that live higher in the intertidal zone spend more energy for proteostasis and restoring proteostasis to cope with a second consecutive day of high temperatures (Somero et al., 2016). Usually, the expression of Hsp70 of less thermal-tolerant species is more sensitive to increases in temperature (limpet *Lottia*, Dong et al., 2008; snail *Chlorostoma*, Tomanek, 2002), and the rapid upregulation of *hsp70* mRNA in limpets exposed to future conditions potentially represents a high sensitivity of limpets to thermal stress in the face of ocean acidification. Due to the expensive energy consumption during the synthesis and function of *hsp70*, the more rapid upregulation of *hsp70* mRNA in these limpets also indicates more energy was allocated into cellular homeostasis, which then can affect the growth and reproduction of limpets.

The expression patterns of constitutive *hsc70* mRNA were different among limpets acclimated at the four acclimation conditions. Hsc70 is constitutively expressed and is a molecular chaperone involved in the in vivo folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain similar promoter regions, there are differential expressions to a given stimulus between them (Hansen et al., 1991). Some studies showed that thermal stress could significantly induce the upregulation of both the *hsc70* gene and

Hsc70 protein in the killifish *Fundulus heteroclitus* (Fangue et al., 2006), the shrimp *Penaeus monodon* (Chuang et al., 2007), and the coral *Veretillum cynomorium* (Teixeira et al., 2013). In the present study, for limpets acclimated under HTLC and LTHC (i.e., only temperature or CO₂ condition changed in comparison with the LTLC treatment), there was significant upregulation of *hsc70* mRNA when the heat-shock temperatures were beyond 30 °C. However, the expression of *hsc70* mRNA showed no significant difference among different heat-shock temperatures under predicated future environmental conditions (HTHC: 24 °C and 1000 ppm). These results indicate that the upregulation of *hsc70* mRNA in response to heat shock represents an increasing capability for coping with the enhanced protein denaturation and more energy allocated into the somatic maintenance after being exposed to either warming or a high-CO₂ environment. The insignificant upregulation of *hsc70* in response to thermal stress indicates that limpets acclimated under HTHC may employ a “preparative defense” strategy (Dong et al., 2008) to maintain high constitutive levels of *hsc70* as a mechanism to cope with unpredictable heat stress. However, the absence of significant upregulation of *hsc70* mRNA in limpets acclimated to future conditions (warming and elevated CO₂) might also be attributed to the very high variation in gene ex-

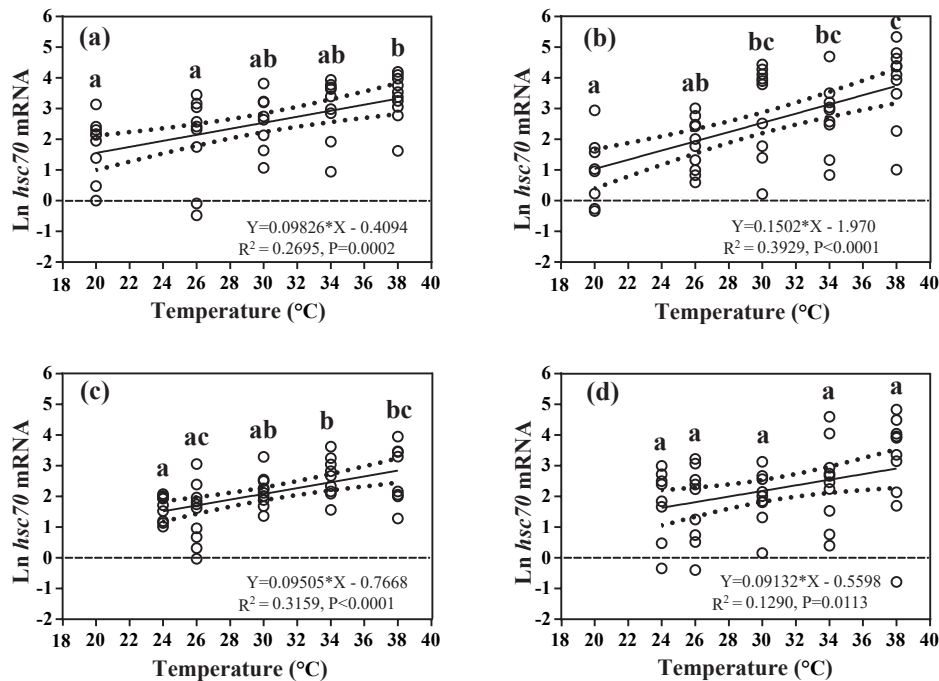


Figure 4. Effects of heat-shock temperature on the expression of *hsc70* mRNA in limpets acclimated at (a) 20 °C and 400 ppm, (b) 20 °C and 1000 ppm, (c) 24 °C and 400 ppm, and (d) 24 °C and 1000 ppm. The relationship between heat-shock temperature and log-transformed gene expression of *hsc70* was fitted using linear regressions with 95 % confidence intervals (dashed lines). Different letters represent significant differences in the level of *hsc70* mRNA among different heat-shock temperatures.

pression at 38 °C (CV, 90.36 %). In the context of future conditions, multiple environmental stressors can induce diverse physiological responses among different individuals, which might be an evolutionary adaptation to the harsh environment on the shore.

Variation and plasticity in both physiological and molecular responses to thermal stress are not only important for coping with future environmental changes but also underpin evolutionary and adaptive changes through selective pressures (Franks and Hoffmann, 2012). In the present study, the coefficients of variation in physiological responses of limpets acclimated to simulated future conditions, including ABT, Q_{10} , and *hsc70* mRNA, were higher than that in the other three acclimation conditions. Crucially, this means that a subset of individuals in our experimental population might be more physiologically preadapted to cope with heat shock. Once acclimated to a future climate change scenario (warming and ocean acidification), this variation in physiological performance increased, indicating that in a harsher environment the physiological plasticity of some individuals allows them to modify their physiological tolerance limits and increase chances for survival and reproduction (Williams et al., 2008). Under high selective pressure, these individuals would form the basis for future generations while less-plastic individuals would be removed from populations. However, differences among the coefficients of variation need to be in-

terpreted with caution, as multiple factors can cause this type of variation, including the variable environmental history of individuals despite a 7-day acclimation, competition among individuals during the acclimation period, or the sample size (around 10 limpets per treatment).

Intertidal limpets may experience two sorts of stressful temperature exposures in the field, abrupt or gradual exposure (Denny et al., 2006). The present study showed the up-regulation of *hsp70* and *hsc70* expression in *C. toreuma* under gradual exposure. Similar expression patterns have also been observed in *Hsp70* under gradual thermal exposure in other intertidal limpets (Dong et al., 2008; Miller et al., 2009). Importantly, the gradual experimental change in thermal environment used here mimics conditions that most intertidal species experience in the field and is important for predicting how animals will resolve prolonged aerial exposure during low tide. Conversely, experimentally simulating abrupt thermal change helps us understand physiological responses to some extreme conditions, such as heat wave (up-regulation of *hsp70* in intertidal limpets; Prusina et al., 2014). Therefore, future work combining both abrupt and gradual exposure may offer insight into how intertidal species respond to climate change and extreme weather events in the future. Further, since our findings are based on static experimental conditions, the results should be treated with caution when we predict the response of an organism to future

climate change in the highly variable natural environment. Therefore, future studies with long-term acclimation, larger sample size, and variable treatment conditions are recommended in order to validate our findings.

In conclusion, the resilience of intertidal limpets to thermal stress is weakened after exposure to predicted future conditions for a short-term acclimation period (7 days). However, the combination of elevated temperature and CO₂ concentration prompted divergence of physiological and molecular responses. These results suggest that while organisms may be able to protect themselves from the damaging effects of thermal stress in the short term, changes to multiple environmental conditions may drive population-level responses through physiological responses (e.g., Giomi et al., 2016). Further, the increased variation in responses, and the observation that some individuals were more capable to physiologically cope with the conditions, may be associated with intergenerational adaptation, but this speculation needs further evidence. As the “weaker” individuals are lost, the offspring in the next generation will be better physiologically adapted to warming under high-CO₂ conditions. Therefore, while elevated CO₂ and the associated ocean acidification decrease the ability of many individuals to respond to thermal stress, it appears that physiological plasticity and variability could be adaptive mechanisms in at least some populations of intertidal organisms. Our research underlines the importance of physiological plasticity and variability for coastal species coping with warming and ocean acidification.

Data availability. All data associated with this paper can be found at the designated Dryad depository (<https://doi.org/10.5061/dryad.7s3m38n>; Wang et al., 2018).

Appendix A

Table A1. Functions and primers of selected genes of the *Cellana* limpet.

Gene name	Gene symbol	Function	Primers (5'-3')
Heat-shock cognate 71 kDa protein	<i>hsc70</i>	molecular chaperone	F: CCTGAATGTGTCCGCTGTG R: TTCCTGTCTTCCTCGCTGAT
Heat-shock protein 70	<i>hsp70</i>	molecular chaperone	F: CAACACCTTCACGACTTA R: CCACAGCAGATACATTCA
Beta-actin	<i>β-actin</i>	reference gene	F: AGGTATTGCCGACAGAATG R: TTGGAAGGTGGACAGAGA
Tubulin beta chain	<i>β-tubulin</i>	reference gene	F: AGGTGCTGAATTGGTAGAC R: TTGCTGATGAGGAGAGTTC
18S ribosomal RNA	<i>18s</i>	reference gene	F: ATAGCCTATATCGGAGTT R: ATGGATACATCAAGGTTAT

Table A2. Inferential statistics for the most likely general additive mixed models (GAMMs) of heart rate during continuous warming of the limpet *Cellana toreuma* acclimated at different temperatures (20 and 24 °C) and $p\text{CO}_2$ levels (400 and 1000 ppm)*.

Effect	d.f.	<i>F</i>	<i>P</i> value
$f(T)$ for <i>C. toreuma</i> from 20 °C and 400 ppm	18.46	191.2	<0.001
Deviation from $f(T)$ for <i>C. toreuma</i> from 20 °C and 1000 ppm	17.2	25.018	<0.001
Deviation from $f(T)$ for <i>C. toreuma</i> from 24 °C and 400 ppm	16.157	65.328	<0.001
Deviation from $f(T)$ for <i>C. toreuma</i> from 24 °C and 1000 ppm	20.194	41.634	<0.001
$f(T)$ for <i>C. toreuma</i> from 20 °C and 1000 ppm	18.75	135	<0.001
Deviation from $f(T)$ for <i>C. toreuma</i> from 24 °C and 400 ppm	10.502	42.441	<0.001
Deviation from $f(T)$ for <i>C. toreuma</i> from 24 °C and 1000 ppm	19.753	40.229	<0.001
$f(T)$ for <i>C. toreuma</i> from 24 °C and 400 ppm	13.3	35.58	<0.001
Deviation from $f(T)$ for <i>C. toreuma</i> from 24 °C and 1000 ppm	13.337	6.364	<0.001
$f(T)$ for <i>C. toreuma</i> from 24 °C and 1000 ppm	18.35	52.54	<0.001

* The generalized additive model describes heart rate as a function of temperature, or $f(T)$, instead of using a fixed parameter to describe the effect of temperature. Additional functions were included to describe how heart rates of *C. toreuma* from each treatment deviated from those of *C. toreuma* from 20 °C and 400 ppm.

Table A3. Two-way ANOVA to investigate the effects of temperature (20 and 24 °C) and $p\text{CO}_2$ (400 and 1000 ppm) on Arrhenius breakpoint temperature (ABT) of heart rate and temperature coefficients (Q_{10}) on *Cellana toreuma*.

Source of variation	DF	SS	MS	F	P
Two-way ANOVA for ABT					
Temperature	1	22.580	22.580	3.375	0.075
$p\text{CO}_2$	1	0.790	0.790	0.118	0.733
Temperature \times $p\text{CO}_2$	1	6.076	6.076	0.908	0.347
Residual	35	234.200	6.692		
Two-way ANOVA for Q_{10}					
Temperature	1	0.257	0.257	5.878	0.021
$p\text{CO}_2$	1	0.058	0.058	1.332	0.256
Temperature \times $p\text{CO}_2$	1	0.005	0.005	0.1135	0.738
Residual	35	1.527	0.0436		

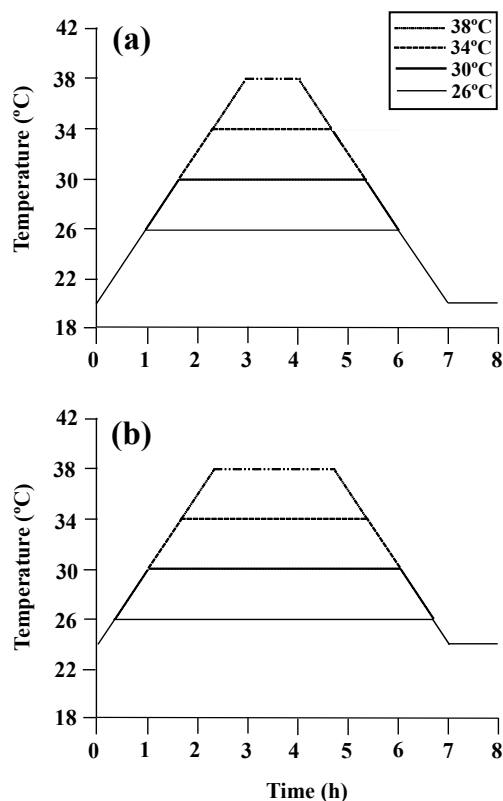


Figure A1. Diagram of the heating protocol for (a) limpets acclimated at 20 °C and (b) limpets acclimated at 24 °C. Limpets were heated at a rate of 6 °C h⁻¹ from acclimation temperatures (20 or 24 °C) to designated temperatures (26, 30, 34, and 38 °C) for simulating a natural heating rate in summer. After achieving the target temperature, the temperature was held at the designated level for the allotted time and then decreased to acclimated temperatures (20 or 24 °C) at a rate of 6 °C h⁻¹, for a total exposure time of 7 h. After recovery in 20 or 24 °C seawater for 1 h, limpets ($n = 8$ –10) in each treatment were immediately collected and stored at –80 °C for gene expression measurement.



Figure A2. The photo of artificial rock (60 cm length \times 30 cm width). Limpets were placed on artificial rock and heated to the designated temperature.

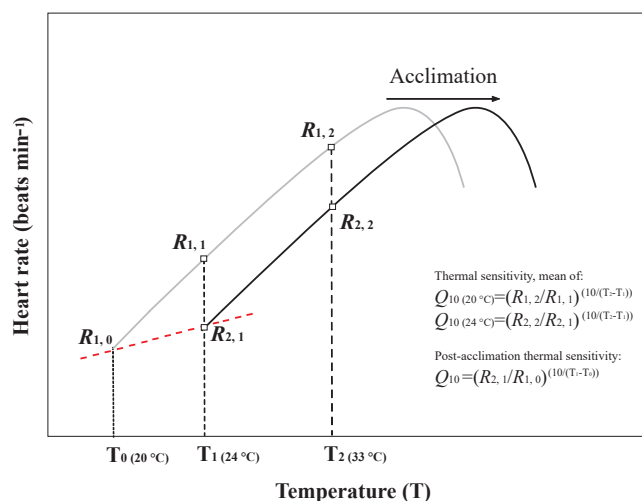


Figure A3. Schematic diagram of temperature coefficients (Q_{10}) and post-acclimation Q_{10} calculations. This figure was modified from Seebacher et al. (2015). The black line and grey line showed the heart rate of limpets from the warm-acclimated temperature (24 °C) and the cold-acclimated temperature (20 °C), respectively. Q_{10} values for thermal sensitivities were calculated from data for limpets kept at an acclimation treatment in which heart rate was measured at two different temperatures. Q_{10} value for post-acclimation thermal sensitivities was calculated across two temperature acclimation conditions under the same $p\text{CO}_2$ condition.

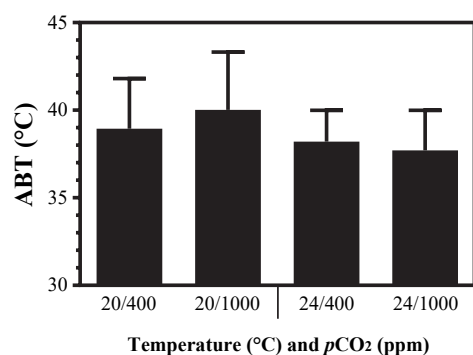


Figure A4. Arrhenius breakpoint temperature (ABT) of heart rate of limpets acclimated at different temperatures (20 or 24 °C) and CO_2 concentrations (400 or 1000 ppm). After acclimation in different conditions, limpets were heated continuously from acclimation temperatures until the heart stopped beating. During the heating process, heart rates were recorded and ABTs were calculated.

Author contributions. BDR and YWD designed the experiments. WJ and MWD conducted the experiments. YWD, BDR, WJ, and MWD performed analyses. The paper was co-written by YWD, WJ, and MWD and revised by BDR.

Competing interests. The authors declare that they have no conflict of interest.

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