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

Jie Wang[1], Bayden D. Russell[2], Meng-wen Ding[1], Yun-wei Dong[1,3*]

[1]State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, 361000, China

[2]The Swire Institute of Marine Science and School of Biological Sciences, The University of Hong Kong, Hong Kong SAR, 999077, China

[3]Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Xiamen University, Xiamen 361102, China

*_Corresponding to_: Yun-wei Dong (dongyw@xmu.edu.cn)

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 *Cellana toreuma* from the same population in Xiamen. Limpets were collected from shaded mid-intertidal rock surfaces. They were acclimated under combinations of different pCO_2 concentrations (400 ppm and 1000 ppm, corresponding to pH 8.1 and 7.8) and temperatures (20 °C and 24 °C) in a short-term period (7 days), with the control condition (20 °C and 400 ppm) representing the average annual temperature and present-day pCO_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 pCO_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 (Helmuth et al., 2006; Hofmann and Todgham, 2010; Somero, 2012; Widdicombe and Spicer, 2008). Therefore, understanding the interaction of multiple environmental stressors on the physiological performance is crucial for predicting the consequences of environmental change on ecosystems (Deutsch et al., 2015). For example, salinity fluctuations coupled with high temperatures during emersion can have both sub-lethal physiological effects and lethal effects on intertidal molluscs (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). Yet, warming and ocean acidification are not unidirectional, but rather combined with rapid fluctuations on daily to seasonal and decadal time-scales. 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. Yet, organisms inhabiting variable environments, such as the intertidal zone, will be exposed to increasing extremes in temperature concomitant with increasing pCO_2, or ocean acidification (OA), in the future. While OA 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 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 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 molluscs (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); on the other hand, 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 temperate (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 trocophore 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, gazing on biofilm and being an important food source for other species (e.g. crabs, sea birds and sea stars). Therefore, this species is a key organism for studying the relationship between physiological response to thermal stress

and ocean acidification in 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 (SST) 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 pCO_2 concentrations (400 ppm and 1000 ppm) and temperatures (20 °C and 24 °C). We hypothesize that (1) limpets will show increased thermal sensitivity of metabolism and stress responses under elevated pCO_2 and temperatures; (2) short-term acclimation at high temperature and pCO_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 pCO_2 on stress response, energy consumption and physiological plasticity in intertidal invertebrates, potentially providing predications of the 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 on 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 firstly allowed to recover at 20 °C for 3 d with a tidal cycle of approximately 6 h immersion and 6 h emersion. These limpets were randomly allocated into one of four treatments and temporally acclimated in different pCO_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 d in climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which control both the *p*CO2 concentration and temperature under the same relative humidity and light intensity conditions. In each acclimation treatment, approximately 100 limpets were randomly allocated in ~ 30 containers (3 individuals in each container), to simulate field densities of ~ 1 limpet per 10 cm^2. Control conditions (20 °C, 400 ppm) represent the average annual temperature and ambient pCO_2 (~ 390 ppm) at the collection site, with high temperature (24 °C) and pCO_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 aerial exposure and 6 h seawater immersion. Seawater was pre-bubbled with air containing the corresponding pCO_2 concentrations in advance. pH was measured before and after the acclimation in seawater each time with PB-10 pH meter (Sartorius

148 Instruments, Germany) calibrated with National Institute of Standards and Technology standard pH
149 solutions (NIST, USA). Total dissolved inorganic carbon (DIC) was measured before and after the
150 acclimation in seawater each time using a dissolved inorganic carbon analyzer (As-C3, Apollo SciTech,
151 Colorado, USA), using a Li-Cor® non-dispersive infrared detector (Li-6252) with a precision of 0.1%
152 (Cai, 2003). Seawater carbonate chemistry parameters were estimated based on the measured values of
153 pH, DIC, temperature and salinity with the software CO2Calc v4.0.9 (Robbins et al., 2010). For CO2Calc
154 settings, the NBS scale was applied as the pH scale, and the CO_2 constant, the $KHSO_4$- constant and the
155 total Boron was set from Millero et al. (2006), Dickson et al. (1990) and Lee et al. (2010) respectively.
156 The information of the measured and calculated seawater chemistry parameters is summarized (Table 1).
157 After a 7-day acclimation period (crossed pCO_2 × Temperature treatments, above), the heat-shock
158 treatments were carried out to simulate the gradual temperature exposure of limpets in the field as
159 described in Denny et al. (2006) (Fig. A1). For each heat-shock treatment, 10 limpets were randomly
160 selected from each of four acclimation conditions (40 indiv. total) and transferred to artificial rocks (Fig.
161 A2), with individuals from LTLC and LTHC on one rock and individuals from HTLC and HTHC on
162 another rock. The artificial rocks were separately placed in 20 °C water baths and 24 °C water baths, and
163 heated at a rate of 6 °C per hour that simulated emersion in the natural condition at the collection site
164 (Han et al., 2013) to the designated temperatures (26, 30, 34 and 38 °C). After achieving the target
165 temperature, the temperature was maintained for the allotted time, and then decreased to the acclimation
166 temperature (20 or 24 °C) at a rate of 6 °C per hour, for a total exposure time of 7 h. Individuals from all
167 four acclimation conditions (n = 10 indiv. per treatment) were randomly selected, transferred to artificial
168 rocks and aerially exposed at 20 or 24 °C for 7 h, as non-heated control samples. After recovery at 20 or
169 24 °C seawater for 1 h, limpets were immediately collected and stored at -80 °C for gene expression

170 analysis.

171

172 **2.2 Cardiac performance measurement**

173 The cardiac performance of limpets was recorded during whole heating processes from the
174 acclimated temperature (20 or 24 °C) to the temperature where the heart stopped beating (n = 9-11 indiv.
175 per acclimation treatment). Each limpet was placed in a separate container during the measurement. The
176 containers were immersed in water baths, allowing the temperature in the container to be increased at a
177 rate of 6 °C per hour that simulated emersion in the natural environment. Heart rates were measured
178 using a non-invasive method (Chelazzi et al., 2001; Dong and Williams, 2011). The heartbeat was
179 detected by means of an infrared sensor fixed with Blue-Tac (Bostik, Staffordshire, UK) on the limpet
180 shell at a position above the heart. Variations in the light-dependent current produced by the heartbeat
181 were amplified, filtered and recorded using an infrared signal amplifier (AMP03, Newshift, Leiria,
182 Portugal) and Powerlab AD converter (4/30, ADInstruments, March-Hugstetten, Germany). Data were
183 viewed and analyzed using Lab Chart (version 7.0).

184 For determining the Arrhenius Breakpoint Temperature (ABT) of heart rate, discontinuities in the
185 slopes of heart rate with temperature were calculated from intersections of fitted 2-phase regressions
186 based on the minimum sum of squares using SigmaPlot 12.5 (SSPS Inc., Point Richmond, CA, USA) as
187 described by Giomi and Pörtner (2013).

188

2.3 Quantifying genes expression

Limpets were firstly 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 (RT) reactions were performed using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Shiga, Japan).

The levels of mRNA of genes encoding two heats hock proteins, inducible heat-shock protein 70 (*hsp70*) and constitutive heat shock protein 70 (*hsc70*), were measured using real-time quantitative PCRs in CFX96™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules CA, USA) followed the methods described by Han et al. (2013) with specific primers (Table A1). For normalizing expression of genes, we examined expression of *18S ribosomal RNA*, *β-actin*, *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 the 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-tublin* from a reference individual.

209 **2.4 Statistical analysis**

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

216 Thermal sensitivity is the change in a physiological rate function reacting to a rapid change in
217 environmental temperature within the same acclimation set temperature (Fig. A3, modified from
218 Seebacher et al. (2015)). In the present study, thermal sensitivity was determined in the temperature
219 coefficient (Q_{10}) values of heart rate. Q_{10} was calculated using heart-rate data from the temperature at
220 which the experiment started (T_1 = 24 °C) to the temperature to which temperature increased 10 °C (T_2
221 = 33 °C) with Eq. (1):

222 $$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \quad (1)$$

223 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
224 (Kelvin) (Fig. A3, modified from Seebacher et al. (2015)). The differences in Q_{10} among the four
225 acclimation conditions with different CO_2 concentrations (400 ppm vs. 1000 ppm) and temperatures
226 (20 °C vs. 24 °C) were analyzed using two-way ANOVA with Duncan's *post hoc* analysis using the SPSS
227 20.0 for Windows statistical package (IBM SPSS Statistics, Chicago, USA). Post-acclimation thermal
228 sensitivity of limpets in different CO_2 concentrations were calculated as described by Seebacher et al.
229 (2015). In each CO_2 concentration (400 ppm or 1000 ppm), the post-acclimation Q_{10} values were
230 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 °C), and R_1 was the average heart rate of cold-acclimated limpets at T_1 = 20 °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 ~ 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 ± SD: LTLC, 38.9 ± 2.9 °C; HTLC, 38.2 ± 1.8 °C; LTHC, 40.0 ± 3.3 °C; HTHC, 37.7 ± 2.3 °C) (Fig. A4). Temperature (Two-way ANOVA, $F_{1, 35}$ = 3.375, P = 0.075) and

252 pCO_2 (Two-way ANOVA, $F_{1, 35} = 0.118$, $P = 0.733$) both had non-significant effects on ABTs, and there
253 was a non-significant interaction between temperature and pCO_2 (Two-way ANOVA, $F_{1, 35} = 0.908$, $P =$
254 0.347) (Table A3; Fig. A4).

255 Temperature coefficients (Q_{10} rates) were higher for limpets acclimated at 20 °C than at 24 °C (Two-
256 way ANOVA, $F_{1, 35} = 5.878$, $P = 0.02$), but there was no significant difference for acclimation to different
257 pCO_2 concentrations (Two-way ANOVA, $F_{1, 35} = 1.332$, $P > 0.05$) and for the interaction between
258 temperature and pCO_2 (Two-way ANOVA, $F_{1, 35} = 0.1135$, $P > 0.05$) (Table A3; Fig. 2). The post-
259 acclimation thermal sensitivity of limpets acclimated at low CO_2 (2.12) was lower than that of limpets at
260 high CO_2 (2.95) (Fig. 2).

261 The coefficients of variations (CV) of ABT in the four different acclimation conditions were
262 different (Table 2). After low temperature and high CO_2 acclimation (LTHC, 8.22%), CV of ABT was
263 higher than those in the other three conditions (LTLC, 7.34% and HTLC, 4.48%, HTHC, 6.08%). CV of
264 Q_{10} under LTHC condition was the highest in all the four acclimation conditions (Table 2).

265

266 **3.2 Gene expression**

267 Levels of *hsp70* mRNA (log-transformed) linearly increased with the increasing heat-shock
268 temperatures (Fig. 3). ANCOVA analysis showed that the slopes of the linear regressions were
269 significantly different among different acclimation conditions ($F_{4, 189} = 42.62$, $P < 0.001$), and the slope
270 of HTHC condition was higher than those of the other three acclimation conditions. Thus, the rate of
271 increase in production of *hsp70* mRNA in response to warming was greater at the elevated CO_2
272 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 °C 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%) ≈ HCLT (80.12%) > LCLT (56.20%) (Table 2).

4 Discussion

Short-term acclimation at elevated temperature and pCO_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 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 CO_2 elevated the sensitivity of heat shock responses to thermal stress. The

295 expression of inducible *hsp70* mRNA steadily increased from 20°C to 38°C for individuals across all
296 experimental treatments. However, rates of upregulation of *hsp70* mRNA in limpets acclimated at high
297 temperature and high CO_2 (HTHC) were significantly higher than those of limpets acclimated at the other
298 three acclimation conditions. As a molecular chaperon, Hsp70 protein plays crucial roles in maintaining
299 protein stability with the expense of a large amount of energy (Feder and Hofmann, 1999; Tomanek and
300 Sanford, 2003). By comparing the expression patterns of Hsp70 of different *Chlorostoma* species
301 (formerly *Tegula*) that have distinct vertical distribution, Tomanek and Somero (1999, 2000) found that
302 there existed interspecific difference in the frequency of the induction of Hsp70 synthesis and
303 interspecific divergence of the time-course of Hsp70 synthesis. These studies from genus *Chlorostoma*
304 suggested that species that live higher in the intertidal zone spend more energy for proteostasis and restore
305 proteostasis to cope with a second consecutive day of high temperatures (Somero et al., 2016). Usually,
306 the expression of Hsp70 of less thermal-tolerant species is more sensitive to increases in temperature
307 (limpet *Lottia*, Dong et al., 2008; snail *Chlorostoma*, Tomanek, 2002), and the rapid upregulation of
308 *hsp70* mRNA in limpets exposed to future conditions potentially represents a high sensitivity of limpets
309 to thermal stress in the face of ocean acidification. Due to the expensive energy consumption during the
310 synthesis and function of *hsp70*, the more rapid upregulation of *hsp70* mRNA in these limpets also
311 indicates more energy was allocated into cellular homeostasis, which then can affect the growth and
312 reproduction of limpets.

313 The expression patterns of constitutive *hsc70* mRNA were different among limpets acclimated at the
314 four acclimation conditions. Hsc70 is constitutively expressed and is a molecular chaperone involved in
315 the *in vivo* folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain
316 similar promoter regions, there are differential expressions to a given stimulus between them (Hansen et

317 al., 1991). Some studies showed that thermal stress could significantly induce the up-regulation of both
318 *hsc70* gene and Hsc70 protein in the killifish *Fundulus heteroclitus* (Fangue et al., 2006), the shrimp
319 *Penaeus monodon* (Chuang et al., 2007), and the coral *Veretillum cynomorium* (Teixeira et al., 2013). In
320 the present study, for limpets acclimated under HTLC and LTHC (i.e. only temperature or CO_2 condition
321 changed in comparison with the LTLC treatment), there was significant upregulation of *hsc*70 mRNA
322 when the heat shock temperatures were beyond 30 °C. However, the expression of *hsc70* mRNA showed
323 no significant difference among different heat-shock temperatures under predicated future environmental
324 conditions (HTHC: 24 °C and 1000 ppm). These results indicate that the upregulation of *hsc70* mRNA
325 in response to heat shock represents an increasing capability for coping with the enhanced protein
326 denaturation and more energy allocated into the somatic maintenance after being exposed to either
327 warming or high CO_2 environment. The insignificant upregulation of *hsc70* in response to thermal stress
328 indicates that limpets acclimated under HTHC may employ a "preparative defense" strategy (Dong et al.,
329 2008) to maintain high constitutive levels of *hsc70* as a mechanism to copy with unpredictable heat stress.
330 However, the absence of significant upregulation of *hsc70* mRNA in limpets acclimated to future
331 conditions (warming and elevated CO_2) might also be attributed to the very high variation of gene
332 expression at 38°C (CV, 90.36 %). In the context of future conditions, multiple environmental stressors
333 can induce diverse physiological responses among different individuals, which might be an evolutionary
334 adaptation to the harsh environment on the shore.

335 Variation and plasticity in both physiological and molecular responses to thermal stress are not only
336 important for coping with future environmental changes but also underpin evolutionary and adaptive
337 changes through selective pressures (Franks and Hoffmann, 2012). In the present study, the coefficients
338 of variation in physiological responses of limpets acclimated to simulated future conditions, including

ABT, Q_{10} and *hsc70* mRNA, were higher than those in the other three acclimation conditions. Crucially, this means that a subset of individuals in our experimental population might be more physiologically pre-adapted to cope with heat shock. Once acclimated to 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 interpreted 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 upregulation of *hsp70* and *hsc70* expression in *C. toreuma* under gradual exposure. Similar expression patterns have been also 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 (upregulation 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 d). Yet, the combination of elevated temperature and CO_2 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-CO_2 conditions. Therefore, while elevated CO_2 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.

Authors' contributions

B.D.R and Y.-W.D. designed experiments. W.J. and M.-W.D. conducted experiments. Y.-W.D., B.D.R, W.J. and M.-W.D. performed analyses. The manuscript was co-written by Y.-W.D., W.J. and M.-W.D.,

and revised by B.D.R.

Competing interests

The authors declare no conflict of interests.

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References

Angilletta, M. J., Zelic, M. H., Adrian, G. J., Hurliman, A. M., and Smith, C. D.: Heat tolerance during embryonic development has not diverged among populations of a widespread species (*Sceloporus undulatus*), Conserv. Physiol., 1, cot018, 2013.

Bao, B. and Ren, G. Y.: Climatological characteristics and long-term change of SST over the marginal seas of China, Cont. Shelf Res., 77, 96-106, 2014.

Braby, C. E. and Somero, G. N.: Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*), J. Exp. Biol., 209, 2554-2566, 2006.

Byrne, M.: Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean, Oceanogr. Mar. Biol. Annu. Rev., 49, 1-42, 2011.

Byrne, M. and Przeslawski, R.: Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories, Integr. Comp. Biol., 53, 582-596, 2013.

Cai, W. J.: Riverine inorganic carbon flux and rate of biological uptake in the Mississippi River plume,

Geophys. Res. Lett., 30, 2003.

Cai, M., Liu, Y., Chen, K., Huang, D., and Yang, S.: Quantitative analysis of anthropogenic influences on coastal water–A new perspective, Ecol. Indic., 67, 673-683, 2016.

Chelazzi, G., De Pirro, M., and Williams, G. A.: Cardiac responses to abiotic factors in two tropical limpets, occurring at different levels of the shore, Mar. Biol., 139, 1079-1085, 2001.

Chevin, L. M., Lande, R., and Mace, G. M.: Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory, PLoS Biol., 8, e1000357, 2010.

Chuang, K. H., Ho, S. H., and Song, Y. L.: Cloning and expression analysis of heat shock cognate 70 gene promoter in tiger shrimp (*Penaeus monodon*), Gene, 405, 10-18, 2007.

Denny, M. W., Miller, L. P., and Harley, C. D.: Thermal stress on intertidal limpets: long-term hindcasts and lethal limits, J. Exp. Biol., 209, 2420-2431, 2006.

Deutsch, C., Ferrel, A., Seibel, B., Portner, H. O., and Huey, R. B.: Climate change tightens a metabolic constraint on marine habitats, Science, 348, 1132-1135, 2015.

Dickson, A. G.: Standard potential of the reaction: AgCl(s) + H2(g) = Ag(s) + HCl(aq), and the standard acidity constant of the ion HSO4−in synthetic sea water from 273.15 to 318.15 K, J. Chem. Thermodyn., 22, 113–127, 1990.

Dong, Y. W., Han, G. D., Ganmanee, M., and Wang, J.: Latitudinal variability of physiological responses to heat stress of the intertidal limpet *Cellana toreuma* along the Asian coast, Mar. Ecol. Progr. Ser., 529, 107-119, 2015.

Dong, Y. W., Han, G. D., and Huang, X. W.: Stress modulation of cellular metabolic sensors: interaction of stress from temperature and rainfall on the intertidal limpet *Cellana toreuma*, Mol. Ecol., 23, 4541-4554, 2014.

Dong, Y. W., Li, X. X., Choi, F. M., Williams, G. A., Somero, G. N., and Helmuth, B.: Untangling the roles of microclimate, behaviour and physiological polymorphism in governing vulnerability of intertidal snails to heat stress, Proc. R. Soc. B., 284, 2017.

Dong, Y. W., Miller, L. P., Sanders, J. G., and Somero, G. N.: Heat-shock protein 70 (Hsp70) expression in four limpets of the genus *Lottia*: interspecific variation in constitutive and inducible synthesis correlates with in situ exposure to heat stress, Biol. Bull., 215, 173-181, 2008.

Dong, Y. W., Wang, H. S., Han, G. D., Ke, C. H., Zhan, X., Nakano, T., and Williams, G. A.: The impact

437 of Yangtze River discharge, ocean currents and historical events on the biogeographic pattern of
438 *Cellana toreuma* along the China coast, PLoS One, 7, e36178, 2012.
439 Dong, Y. W. and Williams, G. A.: Variations in cardiac performance and heat shock protein expression
440 to thermal stress in two differently zoned limpets on a tropical rocky shore, Mar. Biol., 158, 1223-
441 1231, 2011.
442 Etschmann, B., Wilcken, B., Stoevesand, K., von der Schulenburg, A., and Sterner-Kock, A.: Selection
443 of reference genes for quantitative real-time PCR analysis in canine mammary tumors using the
444 GeNorm algorithm, Vet. Pathol., 43, 934-942, 2006.
445 Fabricius, K. E., Langdon, C., Uthicke, S., Humphrey, C., Noonan, S., De'ath, G., Okazaki, R.,
446 Muehllehner, N., Glas, M. S., and Lough, J. M.: Losers and winners in coral reefs acclimatized to
447 elevated carbon dioxide concentrations, Nat. Clim. Change., 1, 165-169, 2011.
448 Fabry, V. J., Seibel, B. A., Feely, R. A., and Orr, J. C.: Impacts of ocean acidification on marine fauna
449 and ecosystem processes, ICES J. Mar. Sci., 65, 414-432, 2008.
450 Fangue, N. A., Hofmeister, M., and Schulte, P. M.: Intraspecific variation in thermal tolerance and heat
451 shock protein gene expression in common killifish, *Fundulus heteroclitus*, J. Exp. Biol., 209, 2859-
452 2872, 2006.
453 Feder, M. E. and Hofmann, G. E.: Heat-shock proteins, molecular chaperones, and the stress response:
454 evolutionary and ecological physiology, Annu. Rev. Physiol., 61, 243-282, 1999.
455 Firth, L. B. and Williams, G. A.: The influence of multiple environmental stressors on the limpet *Cellana*
456 *toreuma* during the summer monsoon season in Hong Kong, J. Exp. Mar. Biol. Ecol., 375, 70-75, 2009.
457 Franks, S. J. and Hoffmann, A. A.: Genetics of climate change adaptation, Annu. Rev. Genet., 46, 185-
458 208, 2012.
459 Giomi, F., Mandaglio, C., Ganmanee, M., Han, G. D., Dong, Y. W., Williams, G. A., and Sara, G.: The
460 importance of thermal history: costs and benefits of heat exposure in a tropical, rocky shore oyster, J.
461 Exp. Biol., 219, 686-694, 2016.
462 Giomi, F. and Poertner, H. O.: A role for haemolymph oxygen capacity in heat tolerance of eurythermal
463 crabs, Front. Physiol., 4, 110, 2013.
464 Gooding, R. A., Harley, C. D., and Tang, E.: Elevated water temperature and carbon dioxide
465 concentration increase the growth of a keystone echinoderm, P. Natl, Acad. Sci., 106, 9316-9321, 2009.

Han, G. D., Zhang, S., Marshall, D. J., Ke, C. H., and Dong, Y. W. Metabolic energy sensors (AMPK and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal limpet: linking energetic allocation with environmental temperature during aerial emersion, J. Exp. Biol., 216, 3273-3282, 2013.

Hansen, L. K., Houchins, J. P., and O'Leary, J. J.: Differential regulation of HSC70, HSP70, HSP90 alpha, and HSP90 beta mRNA expression by mitogen activation and heat shock in human lymphocytes, Exp. Cell. Res., 192, 587-596, 1991.

Helmuth, B., Mieszkowska, N., Moore, P., and Hawkins, S. J.: Living on the edge of two changing worlds: forecasting the responses of rocky intertidal ecosystems to climate change, Annu. Rev. Ecol. Evol. Syst., 37, 373-404, 2006.

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Harvell, C. D., Sale, P. F., Edwards, A. J., Caldeira, K., Knowlton, N., Eakin, C. M., Iglesias-Prieto, R., Muthiga, N., Bradbury, R. H., Dubi, A., and Hatziolos, M. E.: Coral reefs under rapid climate change and ocean acidification, Science, 318, 1737-1742, 2007.

Hoffmann, A. A. and Sgro, C. M.: Climate change and evolutionary adaptation, Nature, 470, 479-485, 2011.

Hofmann, G. E. and Todgham, A. E.: Living in the now: physiological mechanisms to tolerate a rapidly changing environment, Annu. Rev. Physiol., 72, 127-145, 2010.

IPCC: Climate change 2007: The physical science basis, in: Working group I contribution to the fourth assessment report of the IPCC, edited by Solomon, S., Cambridge Uni. Press, New York, 2007.

IPCC: Climate change 2013: The physical science basis, in: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Clilmate Change, edited by: Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex, V., and Midgley, P. M., Cambridge Univ. Press, New York, 2013.

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, G. M., and Gattuso, J. P.: Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming, Glob. Chang. Biol., 19, 1884-1896, 2013.

Kwiatkowski, L., Gaylord, B., Hill, T., Hosfelt, J., Kroeker, K. J., Nebuchina, Y., Ninokawa, A., Russell, A. D., Rivest, E. B., and Sesboüé, M.: Nighttime dissolution in a temperate coastal ocean ecosystem

increases under acidification, Sci. Rep., 6, 2016.

Lathlean, J. and Seuront, L.: Infrared thermography in marine ecology: methods, previous applications and future challenges, Mar. Ecol. Progr. Ser., 514, 263-277, 2014.

Lee, K., Kim, T. W., Byrne, R. H., Millero, F. J., Feely, R. A., and Liu, Y. M.: The universal ratio of boron to chlorinity for the North Pacific and North Atlantic oceans, Geochim. Cosmochim. Ac., 74, 1801-1811, 2010.

Millero, F. J., Graham, T. B., Huang, F., Bustos-Serrano, H., and Pierrot, D.: Dissociation constants of carbonic acid in seawater as a function of salinity and temperature, Mar. Chem., 100, 80-94, 2006.

Morton, B. S. and Morton, J. E.: The seashore ecology of Hong Kong, Hong Kong University Press, Hong Kong, 1983.

Munday, P. L., Crawley, N. E., and Nilsson, G. E.: Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes, Mar. Ecol. Prog. Ser., 388, 235-242, 2009.

Nickerson, D. M., Facey, D. E., and Grossman, G. D.: Estimating physiological thresholds with continuous two-phase regression, Physiol. Zool., 62, 866-887, 1989.

Oleksiak, M. F., Churchill, G. A., and Crawford, D. L.: Variation in gene expression within and among natural populations, Nat. Genet., 32, 261-266, 2002.

Parmesan, C. and Yohe, G.: A globally coherent fingerprint of climate change impacts across natural systems, Nature, 421, 37-42, 2003.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., and Maintainer, R.: Package ‘nlme’, 2013.

Pörtner, H. O.: Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes, Mar. Ecol. Progr. Ser., 470, 273-290, 2012.

Prosser, C. L.: Physiological Variation in Animals, Biol. Rev., 30, 229-261, 1955.

Prusina, I., Sarà, G., De Pirro, M., Dong, Y. W., Han, G. D., Glamuzina, B., and Williams, G. A.: Variations in physiological responses to thermal stress in congeneric limpets in the Mediterranean Sea, J. Exp. Mar. Biol. Ecol., 456, 34-40, 2014.

Reed, G. F., Lynn, F., and Meade, B. D.: Use of coefficient of variation in assessing variability of quantitative assays, Clin. Diagn. Lab. Immun., 9, 1235-1239, 2002.

Robbins, L. L., Hansen, M. E., Kleypas, J. A., and Meylan, S. C.: CO2calc: A user-friendly seawater

carbon calculator for Windows, Mac OS X, and iOS (iPhone), US Geological Survey, 2010.

Rodolfo-Metalpa, R., Houlbreque, F., Tambutte, E., Boisson, F., Baggini, C., Patti, F. P., Jeffree, R., Fine, M., Foggo, A., Gattuso, J. P., and Hall-Spencer, J. M.: Coral and mollusc resistance to ocean acidification adversely affected by warming, Nat. Clim. Change, 1, 308-312, 2011.

Ruppert, E. E., Fox, R. D., Ruppert, R. S., Fox, R. S., and Barnes, R. D.: Invertebrate zoology: a functional evolutionary approach, No. 592 RUPi, 2004.

Sanford, E. and Kelly, M. W.: Local adaptation in marine invertebrates, Ann. Rev. Mar. Sci., 3, 509-535, 2011.

Sconzo, G., Palla, F., Agueli, C., Spinelli, G., Giudice, G., Cascino, D., and Geraci, F.: Constitutive hsp70 is essential to mitosis during early cleavage of *Paracentrotus lividus* embryos: the blockage of constitutive hsp70 impairs mitosis, Biochem. Bioph. Res. Co., 260, 143-149, 1999.

Seebacher, F., White, C. R., and Franklin, C. E.: Physiological plasticity increases resilience of ectothermic animals to climate change, Nat. Clim. Change, 5, 61-66, 2015.

Sokolova, I. M. and Berger, V. J.: Physiological variation related to shell colour polymorphism in White Sea *Littorina saxatilis*, J. Exp. Mar. Biol. Ecol., 245, 1-23, 2000.

Somero, G. N.: Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living, Integr. Comp. Biol., 42, 780-789, 2002.

Somero, G. N.: The physiology of global change: linking patterns to mechanisms, Annu. Rev. Mar. Sci., 4, 39-61, 2012.

Somero, G. N., Lockwood, B. L., and Tomanek, L.: Biochemical adaptation: response to environmental challenges, from life's origins to the Anthropocene, Sinauer Associates, 2016.

Team, R. C.: R: A language and environment for statistical computing, Vienna, Austria: R Foundation for Statistical Computing, 2014.

Teixeira, T., Diniz, M., Calado, R., and Rosa, R.: Coral physiological adaptations to air exposure: heat shock and oxidative stress responses in *Veretillum cynomorium*, J. Exp. Mar. Biol. Ecol., 439, 35-41, 2013.

Tomanek, L.: The heat-shock response: its variation, regulation and ecological importance in intertidal gastropods (genus *Tegula*), Integr. Comp. Biol., 42, 797-807, 2002.

Tomanek, L. and Sanford, E.: Heat-shock protein 70 (Hsp70) as a biochemical stress indicator: An

553 experimental field test in two congeneric intertidal gastropods (Genus: *Tegula*), Biol. Bull., 205, 276-
554 284, 2003.
555 Tomanek, L. and Somero, G. N.: Evolutionary and acclimation-induced variation in the heat-shock
556 responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for
557 limits of thermotolerance and biogeography, J. Exp. Biol., 202, 2925-2936, 1999.
558 Tomanek, L. and Somero, G. N.: Time course and magnitude of synthesis of heat-shock proteins in
559 congeneric marine snails (genus *Tegula*) from different tidal heights, Physiol. Biochem. Zool., 73, 249-
560 256, 2000.
561 Widdicombe, S. and Spicer, J. I.: Predicting the impact of ocean acidification on benthic biodiversity:
562 what can animal physiology tell us?, J. Exp. Mar. Biol. Ecol., 366, 187-197, 2008.
563 Williams, S. E., Shoo, L. P., Isaac, J. L., Hoffmann, A. A., and Langham, G.: Towards an integrated
564 framework for assessing the vulnerability of species to climate change, PLoS Biol., 6, 2621-2626,
565 2008.
566 Wood, S.: mgcv: GAMs with GCV smoothness estimation and GAMMs by REML/PQL, R package
567 version 1, 2004.
568 Xing, Q., Li, Y. P., Guo, H. B., Yu, Q., Huang, X. T., Wang, S., Hu, X. L., Zhang, L. L., and Bao, Z. M.:
569 Cardiac performance: a thermal tolerance indicator in scallops, Mar. Biol., 163, 244, 2016.
570 Yan, X. H., Cai, R. S., and Bai, Y. S.: Long-term change of the marine environment and plankton in the
571 Xiamen Sea under the influence of climate change and human sewage, Toxico. Enviro. Chem., 98,
572 669-678, 2016.
573

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

575 experimental period[1]

576

	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)	2082.70±191.28	2083.016±190.58	2081.19±165.93	2083.29±163.58
C_T (μmol/kg)	1910.57±174.42	1910.57±174.42	1992.76±157.22	1992.15±149.76
pCO_2 (μtam)	562.18±83.20	561.81±83.04	1008.66±113.41	992.36±47.04
pH (NBS scale)	8.05±0.05	8.05±0.05	7.82±0.04	7.83±0.04
CO_3^{2-} (μmol/kg)	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

577 [1]Seawater temperature, salinity, pH and total dissolved inorganic carbon (C_T) were monitored every 6 h. Total

578 alkalinity (A_T), pCO_2, CO_3^{2-} and Ω_{cal} were calculated using CO2SYS software. Results were pooled and averaged

579 over sampling times. Values are given as mean ± SD.

580

581 **Table 2.** Coefficients of variation (%) of Arrhenius Breakpoint Temperature (ABT), temperature coefficients (Q_{10})

582 and *hsc70* mRNA expression at 38 °C[1, 2]

583

Temperature	CO_2	ABT	Q_{10}	*hsc70* 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

584 [1]Temperature coefficients (Q_{10}) were calculated using heart rate from 24 to 33 °C.

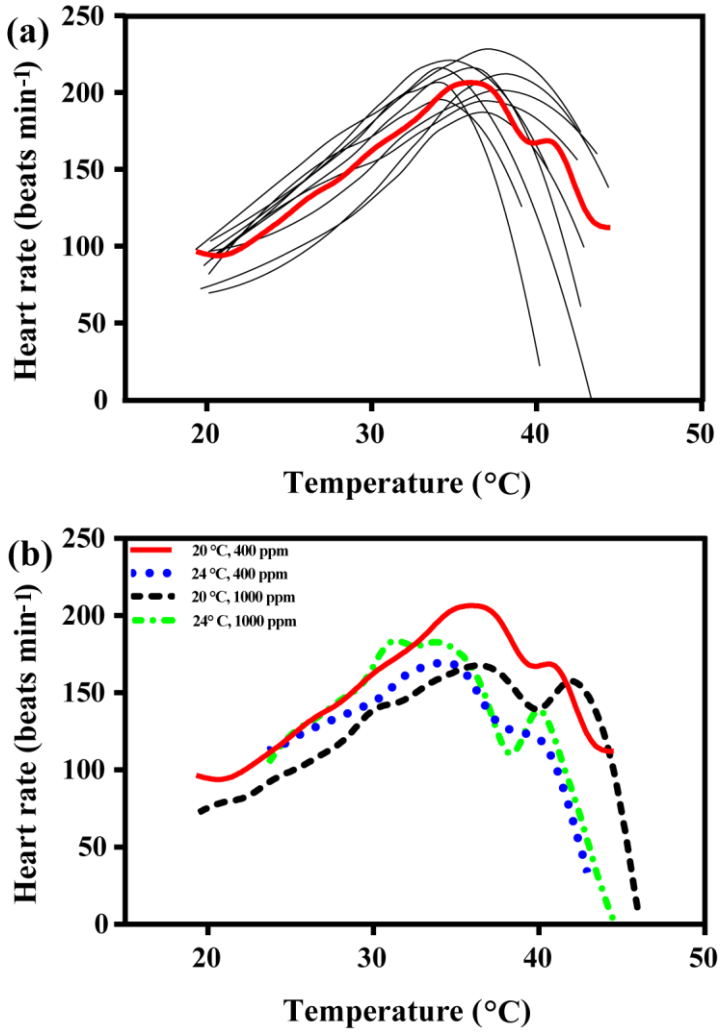
585 [2]After acclimated at different CO_2 and temperature for one week, limpets (n = 8-10) from each acclimation treatment

586 were randomly selected and heat shocked at designated temperatures. Levels of *hsc70* mRNA at 38 °C in different

587 acclimation treatments were used for calculating coefficients of variation.

588

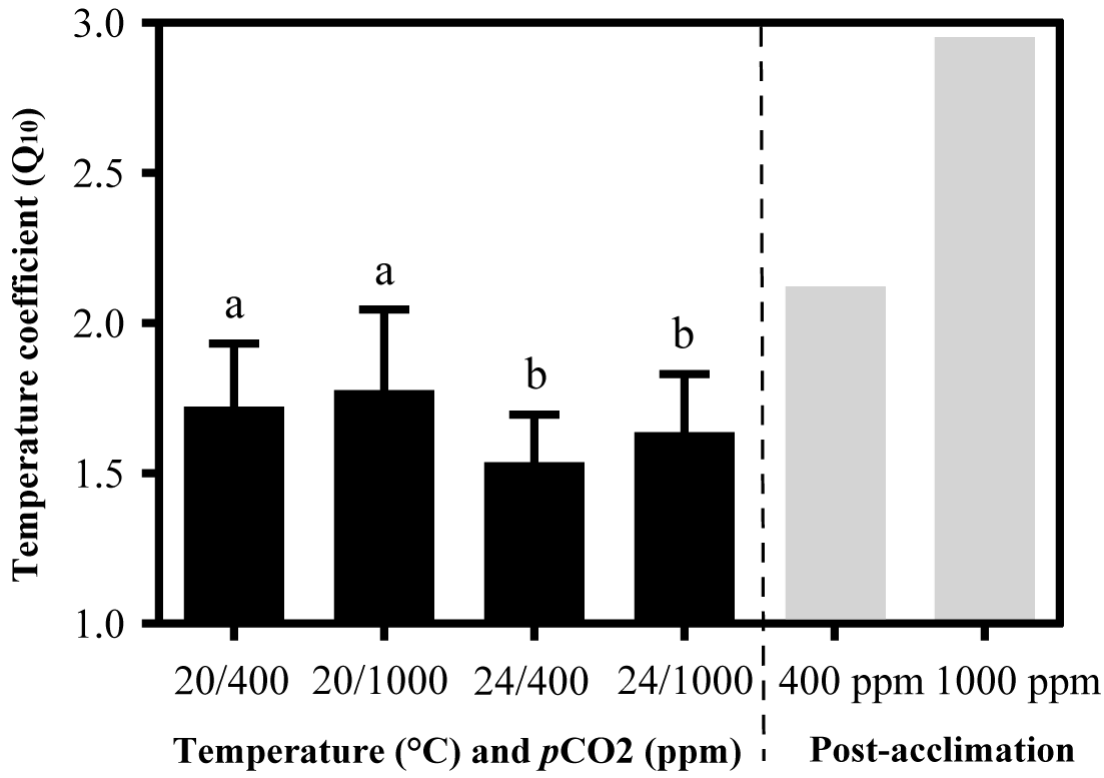
589



590
591

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

597



598

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600 **Figure 2.** Temperature coefficients (Q_{10}) of limpets acclimated at different temperatures (20 or 24 °C) and CO_2

601 concentrations (400 or 1000 ppm). The temperature coefficient (Q_{10}) values were calculated for all limpets using

602 heart rate data from 24 to 33°C. Post-acclimation temperature sensitivity was calculated between individuals

603 acclimated at 20 and 24°C (grey bars; *sensu* Seebacher et al., 2015) for each CO_2 concentration, where higher thermal

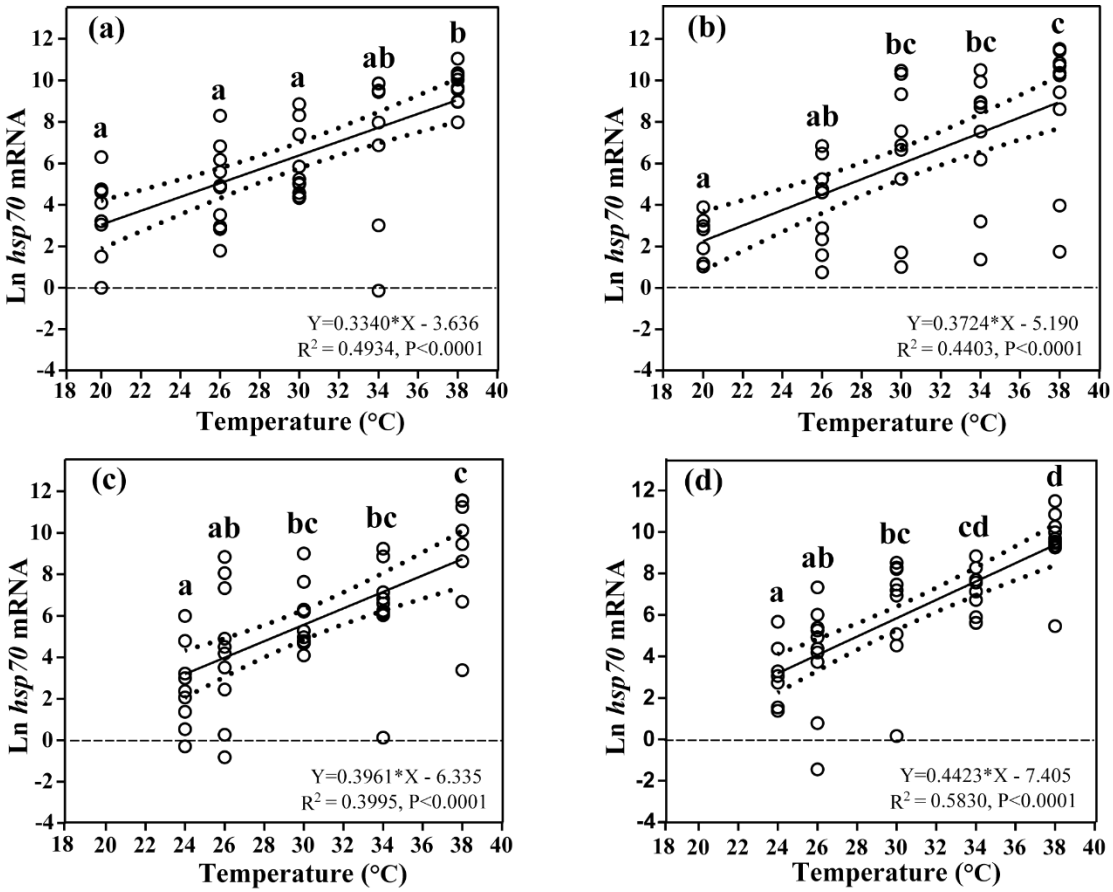
604 sensitivity indicates less acclimation to thermal stress. The calculation of post-acclimation Q_{10} is done for the mean

605 response of all individuals as the same individual are not used at each acclimation temperature. Therefore, it is not

606 possible to calculate an estimate of variation or error for post-acclimation Q_{10}. Different letters represent significant

607 differences in the Q_{10} among different acclimation treatments.

608



609

610

611 **Figure 3.** Effects of heat-shock temperature on the expression of *hsp70* mRNA in limpets acclimated at (a) 20°C

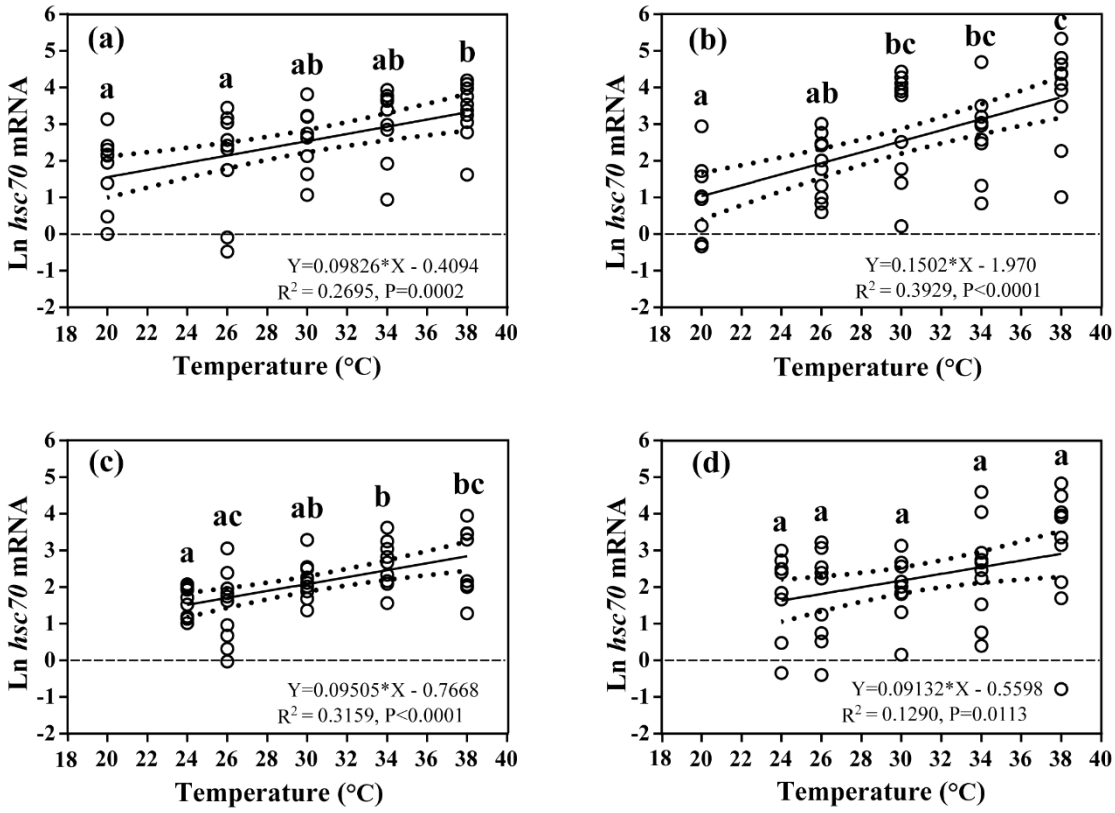
612 and 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

613 heat-shock temperature and log-transformed gene expression of *hsp70* was fitted using linear regressions with 95%

614 confidence intervals (dashed lines). Different letters represent significant differences in the level of *hsp70* mRNA

615 among different heat-shock temperatures.

616



617

618

619 **Figure 4.** Effects of heat-shock temperature on the expression of *hsc70* mRNA in limpets acclimated at (a) 20°C and

620 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

621 heat-shock temperature and log-transformed gene expression of *hsc70* was fitted using linear regressions with 95%

622 confidence intervals (dahs lines). Different letters represent significant differences in the level of *hsc70* mRNA

623 among different heat-shock temperatures.

624

Appendix:

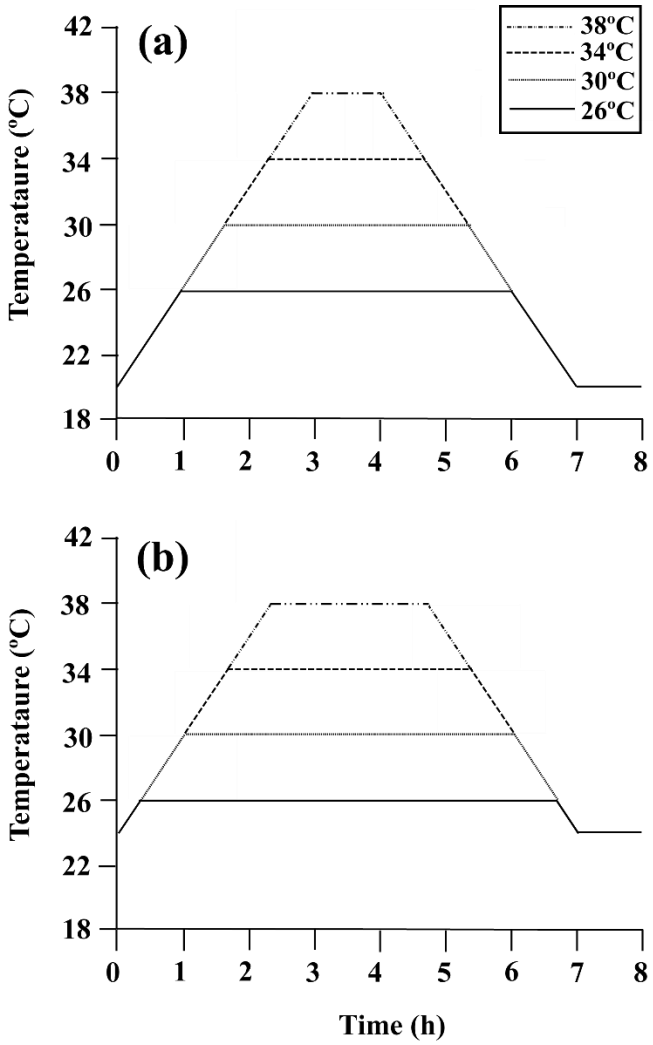


Figure A1. Diagram of the heating protocol for (a) limpets acclimated at 20 °C and (b) limpets acclimated at 24 °C. Limpets were heated at a rate of 6°C per hour from acclimation temperatures (20 or 24 °C) to designated temperatures (26, 30, 34 and 38 °C) for simulating a natural heating rate in summer. 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 per hour, 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 × 30 cm width). Limpets were placed on artificial rock and heated to the designated temperate.

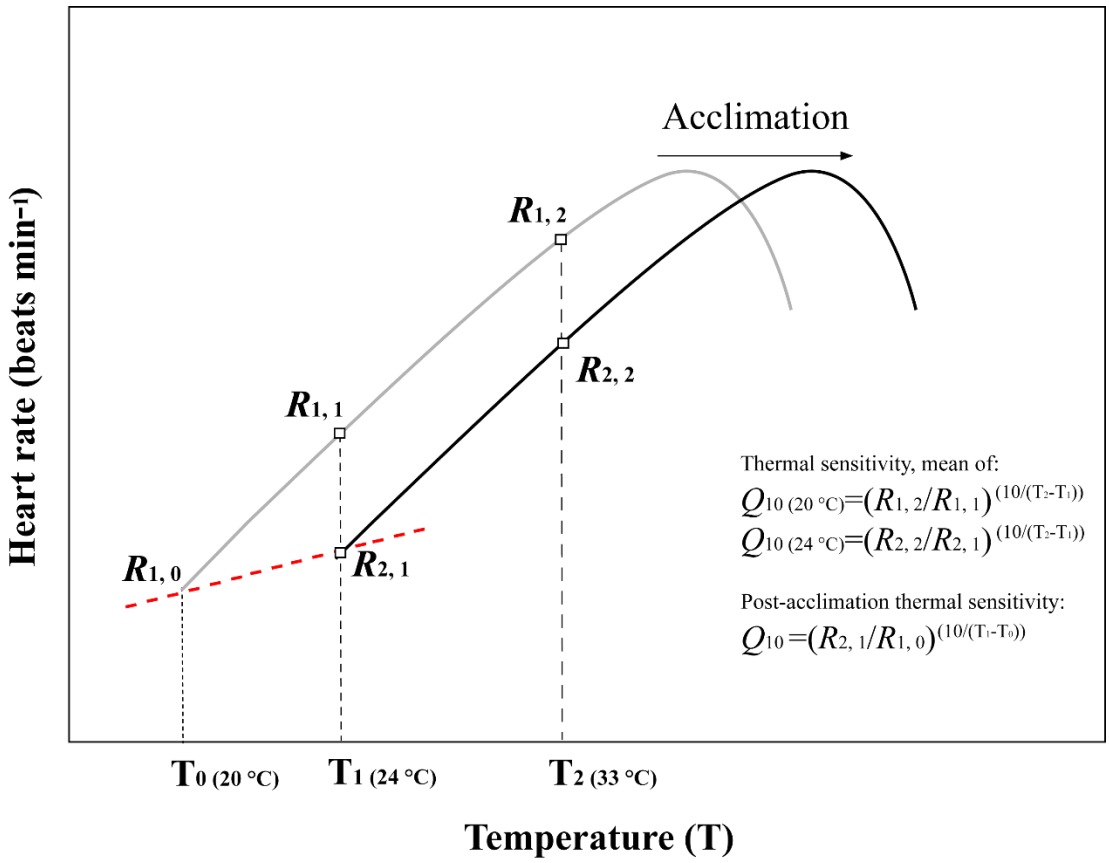
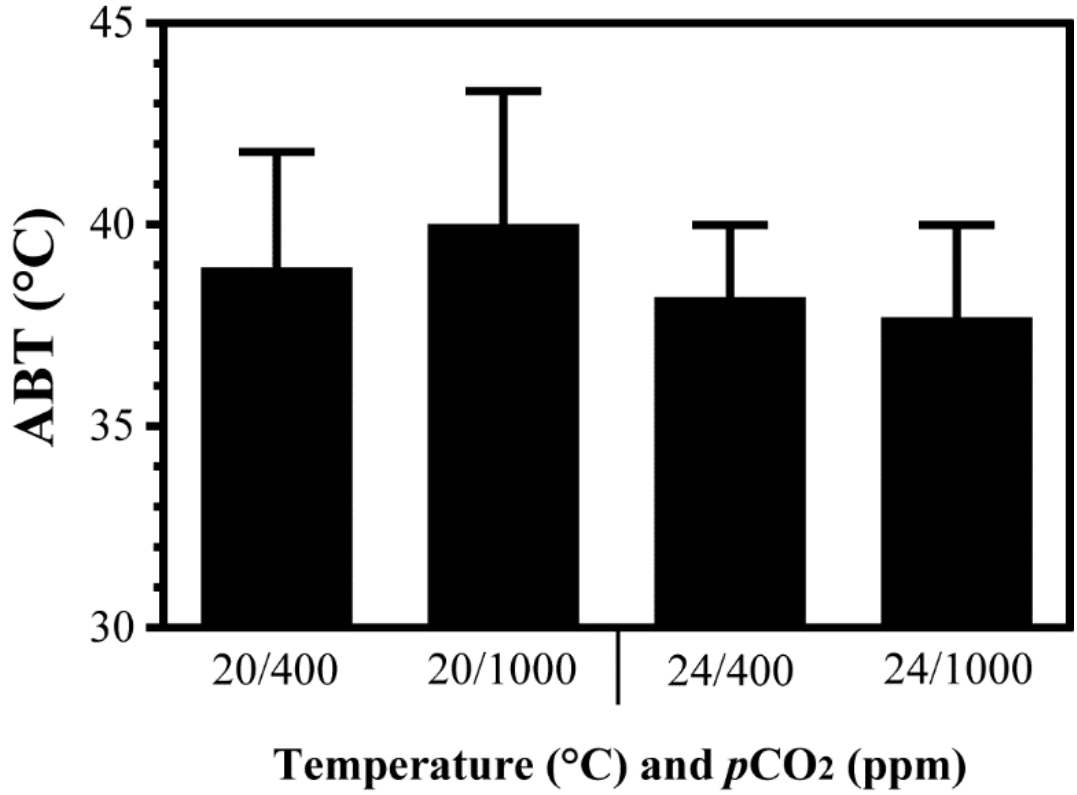


Figure A3. Schematic diagram of temperature coefficients (Q_{10}) and post-acclimation Q_{10} calculations. This figure was modified from Seebacher et al. (2015). Black line and grey line showed the heart rate of limpets from the warm-acclimated temperature (24 °C) and the cold-acclimated temperature (20 °C), respectively. Q_{10} values for thermal sensitivities were calculated from data for limpets kept at an acclimation treatment in which heart rate were measured at two different temperatures. Q_{10} value for post-acclimation thermal sensitivities was calculated across two temperature acclimation conditions under the same pCO_2 condition.



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652 **Figure A4.** Arrhenius Breakpoint Temperature (ABT) of heart rate of limpets acclimated at different temperatures

653 (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). After acclimation in different conditions, limpets were

654 heated continuously from acclimation temperatures to the heart stopped beating. During the heating process, heart

655 rates were recorded and ABTs were calculated.

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

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

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Table A2. Inferential statistics for the most likely general additive mixed models (GAMM) of heart rate during continuous warming of limpet *Cellana toreuma* acclimated at different temperatures (20 and 24 °C) and pCO_2 (400 and 1000 ppm)[1]

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

[1]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 °C and 24 °C) and pCO_2 (400 ppm 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*CO2	1	0.790	0.790	0.118	0.733
Temperature × *p*CO2	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*CO2	1	0.058	0.058	1.332	0.256
Temperature × *p*CO2	1	0.005	0.005	0.1135	0.738
Residual	35	1.527	0.0436		