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*]

[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

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 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 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 at acidic 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 an organism's resistance 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 have three main options; shift their geographical distribution (Parmesan and Yohe, 2003), develop evolutionary 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 (HR), 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 HR under conditions which are not thermally stressful, and HR 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). Hsp70 significantly increases in expression when animals are exposed to stressors and plays a role in maintaining protein stability (Feder and Hofmann, 1999). Hsc70, which is constitutively expressed and may be mildly induced during stress, takes part in folding and repair of denatured proteins (Dong et al., 2015). Some studies have shown coordinated HR 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 ecosystem, *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, Xiamen (118°14′ E, 24°42′ N) is one of the hottest areas in China. 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 1986 to 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 increase their 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. This study 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 is to ensure 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, 20 °C + 400 ppm, as a control treatment; LTHC, 20 °C + 1000 ppm; HTLC, 24 °C + 400 ppm; HTHC, 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 filed 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 Instruments, Germany) calibrated with National Institute of Standards and Technology standard pH

145 solutions (NIST, USA). Total dissolved inorganic carbon (DIC) was measured before and after the
146 acclimation in seawater each time using a dissolved inorganic carbon analyzer (As-C3, Apollo SciTech,
147 Colorado, USA), using a Li-Cor® non-dispersive infrared detector (Li-6252) with a precision of 0.1%
148 (Cai, 2003). Seawater carbonate chemistry parameters were estimated based on the measured values of
149 pH, DIC, temperature and salinity with the software CO2Calc v4.0.9 (Robbins et al., 2010). For CO2Calc
150 settings, the NBS scale was applied as the pH scale, and the CO_2 constant, the $KHSO_4$- constant and the
151 total Boron was set from Millero et al. (2006), Dickson et al. (1990) and Lee et al. (2010) respectively.
152 The information of the measured and calculated seawater chemistry parameters is summarized (Table
153 A1).

154 After a 7-day acclimation period (crossed pCO_2 × Temperature treatments, above), the heat-shock
155 treatments were carried out to simulate the gradual temperature exposure of limpets in the filed as
156 described in Denny et al. (2006) (Fig. A1). For each heat-shock treatment, 10 limpets were randomly
157 selected from each of four acclimation conditions (40 indiv. total) and transferred to artificial rocks (Fig.
158 A2), with individuals from LTLC and LTHC on one rock and individuals from HTLC and HTHC on
159 another rock. The artificial rocks were separately placed in 20 °C water baths and 24 °C water baths, and
160 heated at a rate of 6 °C per hour that simulated emersion in the natural condition at the collection site
161 (Han et al., 2013) to the designated temperatures (26, 30, 34 and 38 °C). After achieving the target
162 temperature, the temperature was maintained for the allotted time, and then decreased to the acclimation
163 temperature (20 or 24 °C) at a rate of 6 °C per hour, for a total exposure time of 7 h. Individuals from all
164 four acclimation conditions (n = 10 indiv. per treatment) were randomly selected, transferred to artificial
165 rocks and aerially exposed at 20 or 24 °C for 7 h, as non-heated control samples. After recovery at 20 or
166 24 °C seawater for 1 h, limpets were immediately collected and stored at -80 °C for gene expression

167 analysis.

168

169 **2.2 Cardiac performance measurement**

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

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

185

186 **2.3 Quantifying genes expression**

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

193 The levels of mRNA of genes encoding two heats hock proteins, inducible heat-shock protein 70
194 (*hsp70*) and constitutive heat shock protein 70 (*hsc70*), were measured using real-time quantitative PCRs
195 in CFX96^{TM} Real-Time System (Bio-Rad Laboratories, Inc., Hercules CA, USA) followed the methods
196 described by Han et al. (2013) with specific primers (Table A2). For normalizing expression of genes,
197 we examined expression of *18S ribosomal RNA*, *β-actin*, *β-tubulin* genes, which typically have relatively
198 stable expression levels. The expression stability of these housekeeping genes was evaluated using the
199 GeNorm Algorithm (Primer Design, Ltd., Southampton University, Highfield Campus, Southampton
200 Hants, UK) as described by Etschmann et al. (2006). Based on the expression stability measures (M
201 values), all the three genes were selected as the reference genes for normalizing the level of expression
202 of stress-induced genes. All samples were measured in triplicates. Ct (dR) values were analyzed using
203 the CFX Manager^{TM} Software Version 3.0 (Bio-Rad). The expression of *hsp70* and *hsc70* was determined
204 relative to the value of *18S*, *β-actin* and *β-tublin* from a reference individual.
205

206 **2.4 Statistical analysis**

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

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

219 $$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \quad (1)$$

220 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
221 (Kelvin) (Fig. A3, modified from Seebacher et al. (2015)). The differences in Q_{10} among the four
222 acclimation conditions with different CO_2 concentrations (400 ppm vs. 1000 ppm) and temperatures
223 (20 °C vs. 24 °C) were analyzed using two-way ANOVA with Duncan's *post hoc* analysis using the SPSS
224 20.0 for Windows statistical package (IBM SPSS Statistics, Chicago, USA). Post-acclimation thermal
225 sensitivity of limpets in different CO_2 concentrations were calculated as described by Seebacher et al.
226 (2015). In each CO_2 concentration (400 ppm or 1000 ppm), the post-acclimation Q_{10} values were
227 calculated using the same equation as shown above, but R_2 was the average heart rate of the warm-

228 acclimated limpets at the acclimated temperature (T_2 = 24 °C), and R_1 was the average heart rate of cold-
229 acclimated limpets at T_1 = 20 °C (Fig. A3, modified from Seebacher et al. (2015)). It is worth noting that
230 post-acclimation thermal sensitivity should be considered with caution, as in the present study the
231 acclimation period (7 days) may not have been sufficient for full acclimation to altered conditions.

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

237 The coefficient of variation (CV) of ABT, Q_{10} and *hsc70* mRNA expression at 38 °C were
238 calculated for each acclimation condition. The CV is the variance in a sample divided by the mean of
239 that sample, providing a method to compare the variation within a sample relative to the mean. It is
240 generally accepted that higher CV demonstrates that there is greater variation among individuals within
241 one treatment than another (Reed et al., 2002).
242

243 ## 3 Results

244 ### 3.1 Cardiac performance

245 The maximal heart rate was ~ 30 % higher in limpets acclimated to control conditions (20 °C, 400
246 ppm) than the other treatments (Fig. 1 and Table A3). The ABTs of limpets showed a trend to be reduced
247 for HT treatments (mean ± SD: LTLC, 38.9 ± 2.9 °C; HTLC, 38.2 ± 1.8 °C; LTHC, 40.0 ± 3.3 °C; HTHC,
248 37.7 ± 2.3 °C) (Fig. A4). Temperature (Two-way ANOVA, $F_{1, 35}$ = 3.375, P = 0.075) and pCO_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 pCO_2 (Two-way ANOVA, $F_{1, 35}$ = 0.908, P = 0.347) (Table A4; 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 pCO_2 concentrations (Two-way ANOVA, $F_{1, 35}$ = 1.332, P > 0.05) and for the interaction between temperature and pCO_2 (Two-way ANOVA, $F_{1, 35}$ = 0.1135, P > 0.05) (Table A4; Fig. 2). The post-acclimation thermal sensitivity of limpets acclimated at low CO_2 (2.12) was lower than limpets at high CO_2 (2.95) (Fig. 2).

The coefficients of variations (CV) of ABT in the four different acclimation conditions were different (Table 1). After low temperature and high CO_2 acclimation (LTHC, 8.22%), CV of ABT was higher than those in the other three conditions (LTLC, 7.34% and HTLC, 4.48%, HTHC, 6.08%). After acclimated at LTHC, CV of Q_{10} was the highest in all the four acclimation conditions (Table 1).

3.2 Gene expression

Levels of *hsp70* mRNA (log-transformed) linearly increased with the increasing heat-shock temperatures (Fig. 3). ANCOVA analysis 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 limpets was higher than those 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 CO_2 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 1).

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

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

310 The expression patterns of constitutive *hsc70* mRNA were different among limpets acclimated at the
311 four acclimation conditions. Hsc70 is constitutively expressed and is a molecular chaperone involved in
312 the *in vivo* folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain
313 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 up-regulation of both *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 CO_2 condition changed in comparison with the LTLC treatment), there was significant upregulation of *hsc*70 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 high CO_2 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 copy with unpredictable heat stress. However, the absence of significant upregulation of *hsc70* mRNA in limpets acclimated to future conditions (warming and elevated CO_2) might also be attributed to the very high variation of gene expression 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 change 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 in 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 combing 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 organism's response 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.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (41276126, 41476115), Nature Science funds for Distinguished Young Scholars of Fujian Province, China (2011J06017), Program for New Century Excellent Talents of Ministry of Education, China, and the State Key laboratory of Marine Environmental Science visiting fellowship to B.D.R.

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,

405 Geophys. Res. Lett., 30, 2003.
406 Cai, M., Liu, Y., Chen, K., Huang, D., and Yang, S.: Quantitative analysis of anthropogenic influences
407 on coastal water–A new perspective, Ecol. Indic., 67, 673-683, 2016.
408 Chelazzi, G., De Pirro, M., and Williams, G. A.: Cardiac responses to abiotic factors in two tropical
409 limpets, occurring at different levels of the shore, Mar. Biol., 139, 1079-1085, 2001.
410 Chevin, L. M., Lande, R., and Mace, G. M.: Adaptation, plasticity, and extinction in a changing
411 environment: towards a predictive theory, PLoS Biol., 8, e1000357, 2010.
412 Chuang, K. H., Ho, S. H., and Song, Y. L.: Cloning and expression analysis of heat shock cognate 70
413 gene promoter in tiger shrimp (*Penaeus monodon*), Gene, 405, 10-18, 2007.
414 Denny, M. W., Miller, L. P., and Harley, C. D.: Thermal stress on intertidal limpets: long-term hindcasts
415 and lethal limits, J. Exp. Biol., 209, 2420-2431, 2006.
416 Deutsch, C., Ferrel, A., Seibel, B., Portner, H. O., and Huey, R. B.: Climate change tightens a metabolic
417 constraint on marine habitats, Science, 348, 1132-1135, 2015.
418 Dickson, A. G.: Standard potential of the reaction: AgCl(s) + H2(g) = Ag(s) + HCl(aq), and the standard
419 acidity constant of the ion HSO4−in synthetic sea water from 273.15 to 318.15 K, J. Chem.
420 Thermodyn., 22, 113–127, 1990.
421 Dong, Y. W., Han, G. D., Ganmanee, M., and Wang, J.: Latitudinal variability of physiological responses
422 to heat stress of the intertidal limpet *Cellana toreuma* along the Asian coast, Mar. Ecol. Progr. Ser.,
423 529, 107-119, 2015.
424 Dong, Y. W., Han, G. D., and Huang, X. W.: Stress modulation of cellular metabolic sensors: interaction
425 of stress from temperature and rainfall on the intertidal limpet *Cellana toreuma*, Mol. Ecol., 23, 4541-
426 4554, 2014.
427 Dong, Y. W., Li, X. X., Choi, F. M., Williams, G. A., Somero, G. N., and Helmuth, B.: Untangling the
428 roles of microclimate, behaviour and physiological polymorphism in governing vulnerability of
429 intertidal snails to heat stress, Proc. R. Soc. B., 284, 2017.
430 Dong, Y. W., Miller, L. P., Sanders, J. G., and Somero, G. N.: Heat-shock protein 70 (Hsp70) expression
431 in four limpets of the genus *Lottia*: interspecific variation in constitutive and inducible synthesis
432 correlates with in situ exposure to heat stress, Biol. Bull., 215, 173-181, 2008.
433 Dong, Y. W., Wang, H. S., Han, G. D., Ke, C. H., Zhan, X., Nakano, T., and Williams, G. A.: The impact

of Yangtze River discharge, ocean currents and historical events on the biogeographic pattern of *Cellana toreuma* along the China coast, PLoS One 7, e36178, 2012.

Dong, Y. W. and Williams, G. A.: Variations in cardiac performance and heat shock protein expression to thermal stress in two differently zoned limpets on a tropical rocky shore, Mar. Biol., 158, 1223-1231, 2011.

Etschmann, B., Wilcken, B., Stoevesand, K., von der Schulenburg, A., and Sterner-Kock, A.: Selection of reference genes for quantitative real-time PCR analysis in canine mammary tumors using the GeNorm algorithm, Vet. Pathol., 43, 934-942, 2006.

Fabricius, K. E., Langdon, C., Uthicke, S., Humphrey, C., Noonan, S., De'ath, G., Okazaki, R., Muehllehner, N., Glas, M. S., and Lough, J. M.: Losers and winners in coral reefs acclimatized to elevated carbon dioxide concentrations, Nat. Clim. Change., 1, 165-169, 2011.

Fabry, V. J., Seibel, B. A., Feely, R. A., and Orr, J. C.: Impacts of ocean acidification on marine fauna and ecosystem processes, ICES J. Mar. Sci., 65, 414-432, 2008.

Fangue, N. A., Hofmeister, M., and Schulte, P. M.: Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*, J. Exp. Biol., 209, 2859-2872, 2006.

Feder, M. E. and Hofmann, G. E.: Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology, Annu. Rev. Physiol., 61, 243-282, 1999.

Firth, L. B. and Williams, G. A.: The influence of multiple environmental stressors on the limpet *Cellana toreuma* during the summer monsoon season in Hong Kong, J. Exp. Mar. Biol. Ecol., 375, 70-75, 2009.

Franks, S. J. and Hoffmann, A. A.: Genetics of climate change adaptation, Annu. Rev. Genet., 46, 185-208, 2012.

Giomi, F., Mandaglio, C., Ganmanee, M., Han, G. D., Dong, Y. W., Williams, G. A., and Sara, G.: The importance of thermal history: costs and benefits of heat exposure in a tropical, rocky shore oyster, J. Exp. Biol., 219, 686-694, 2016.

Giomi, F. and Poertner, H. O.: A role for haemolymph oxygen capacity in heat tolerance of eurythermal crabs, Front. Physiol., 4, 110, 2013.

Gooding, R. A., Harley, C. D., and Tang, E.: Elevated water temperature and carbon dioxide 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

492 increases under acidification, Sci. Rep., 6, 2016.
493 Lathlean, J. and Seuront, L.: Infrared thermography in marine ecology: methods, previous applications
494 and future challenges, Mar. Ecol. Progr. Ser., 514, 263-277, 2014.
495 Lee, K., Kim, T. W., Byrne, R. H., Millero, F. J., Feely, R. A., and Liu, Y. M.: The universal ratio of boron
496 to chlorinity for the North Pacific and North Atlantic oceans, Geochim Cosmochim. Ac., 74, 1801-
497 1811, 2010.
498 Millero, F. J., Graham, T. B., Huang, F., Bustos-Serrano, H., and Pierrot, D.: Dissociation constants of
499 carbonic acid in seawater as a function of salinity and temperature, Mar. Chem., 100, 80-94, 2006.
500 Morton, B. S. and Morton, J. E.: The seashore ecology of Hong Kong, Hong Kong University Press,
501 Hong Kong, 1983.
502 Munday, P. L., Crawley, N. E., and Nilsson, G. E.: Interacting effects of elevated temperature and ocean
503 acidification on the aerobic performance of coral reef fishes, Mar. Ecol. Prog. Ser., 388, 235-242, 2009.
504 Nickerson, D. M., Facey, D. E., and Grossman, G. D.: Estimating physiological thresholds with
505 continuous two-phase regression, Physiol. Zool., 62, 866-887, 1989.
506 Oleksiak, M. F., Churchill, G. A., and Crawford, D. L.: Variation in gene expression within and among
507 natural populations, Nat. Genet., 32, 261-266, 2002.
508 Parmesan, C. and Yohe, G.: A globally coherent fingerprint of climate change impacts across natural
509 systems, Nature, 421, 37-42, 2003.
510 Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., and Maintainer, R.:
511 Package ‘nlme’, 2013.
512 Pörtner, H. O.: Integrating climate-related stressor effects on marine organisms: unifying principles
513 linking molecule to ecosystem-level changes, Mar. Ecol. Progr. Ser., 470, 273-290, 2012.
514 Prosser, C. L.: Physiological Variation in Animals, Biol. Rev., 30, 229-261, 1955.
515 Prusina, I., Sarà, G., De Pirro, M., Dong, Y. W., Han, G. D., Glamuzina, B., and Williams, G. A.:
516 Variations in physiological responses to thermal stress in congeneric limpets in the Mediterranean Sea.
517 J. Exp. Mar. Biol. Ecol., 456, 34-40, 2014.
518 Reed, G. F., Lynn, F., and Meade, B. D.: Use of coefficient of variation in assessing variability of
519 quantitative assays, Clin. Diagn. Lab. Immun., 9, 1235-1239, 2002.
520 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.

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 experimental field test in two congeneric intertidal gastropods (Genus: *Tegula*), Biol. Bull., 205, 276-284, 2003.

Tomanek, L. and Somero, G. N.: Evolutionary and acclimation-induced variation in the heat-shock

responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography, J. Exp. Biol., 202, 2925-2936, 1999.

Tomanek, L. and Somero, G. N.: Time course and magnitude of synthesis of heat-shock proteins in congeneric marine snails (genus *Tegula*) from different tidal heights, Physiol. Biochem. Zool., 73, 249-256, 2000.

Widdicombe, S. and Spicer, J. I.: Predicting the impact of ocean acidification on benthic biodiversity: what can animal physiology tell us?, J. Exp. Mar. Biol. Ecol., 366, 187-197, 2008.

Williams, S. E., Shoo, L. P., Isaac, J. L., Hoffmann, A. A., and Langham, G.: Towards an integrated framework for assessing the vulnerability of species to climate change, PLoS Biol., 6, 2621-2626, 2008.

Wood, S.: mgcv: GAMs with GCV smoothness estimation and GAMMs by REML/PQL, R package version 1, 2004.

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.: Cardiac performance: a thermal tolerance indicator in scallops, Mar. Biol., 163, 244, 2016.

Yan, X. H., Cai, R. S., and Bai, Y. S.: Long-term change of the marine environment and plankton in the Xiamen Sea under the influence of climate change and human sewage, Toxico. Enviro. Chem., 98, 669-678, 2016.

568 **Table 1.** Coefficients of variation (%) of Arrhenius break temperature (ABT), temperature coefficients (Q_{10}) and

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

570

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

571 [1]Temperature coefficients (Q_{10}) were calculated using heart rate from 24 to 33 °C

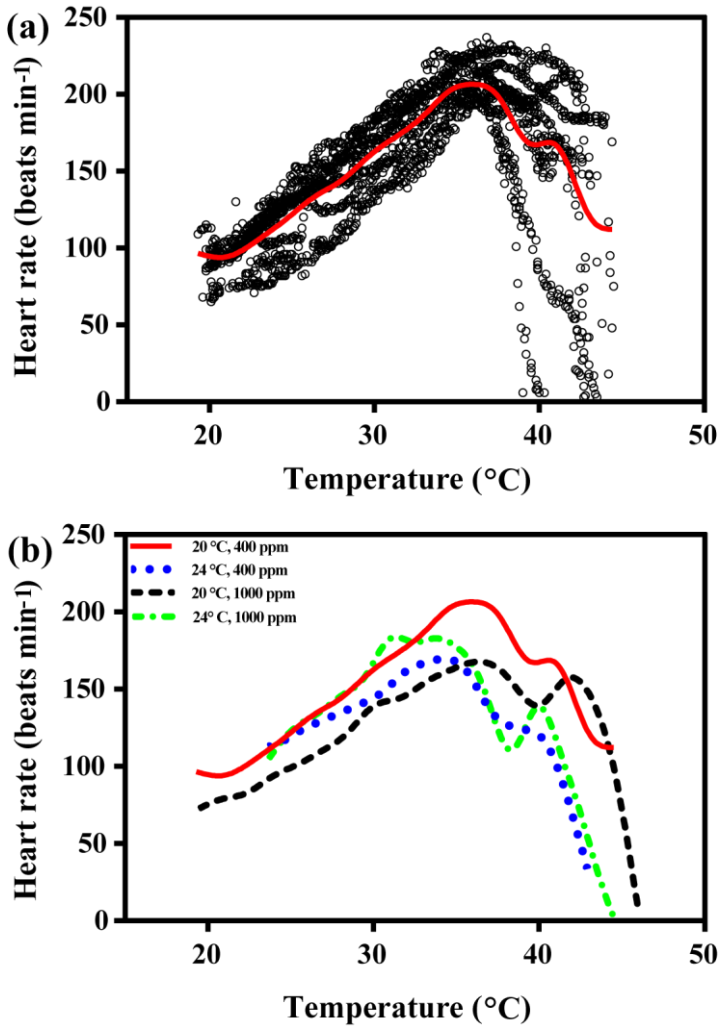
572 [2]After acclimated at different CO_2 and temperature for one week, limpets (n = 8-10) from each acclimation treatment

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

574 acclimation treatments were used for calculating coefficients of variation.

575

576



577

578

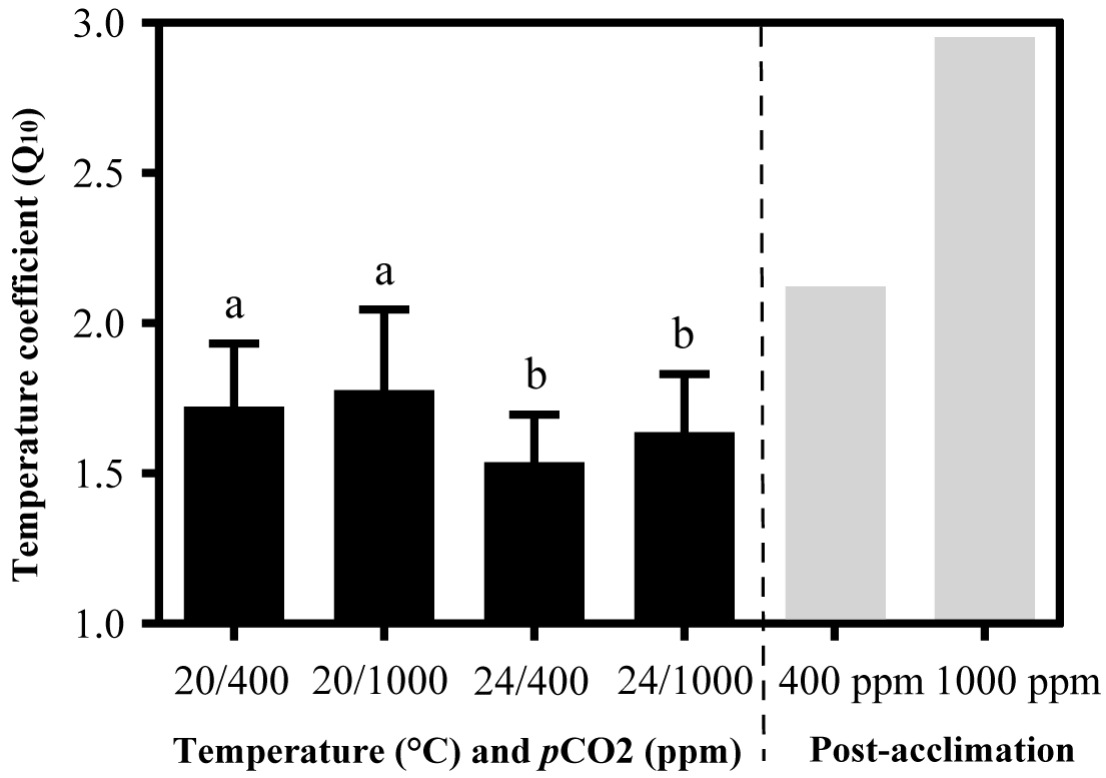
579 **Figure 1.** (a) Heart rates of all limpets acclimated to 20 °C and 400ppm, presented as an example of HR calculation

580 for limpets in all treatments. The red line represents the most likely general additive mixed model (GAMM) to depict

581 the trajectory of hearts rate for limpets with increasing temperature; (b) GAMM lines of limpets acclimated at the

582 different experimental temperature and CO_2 conditions.

583



584

585

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

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

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

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

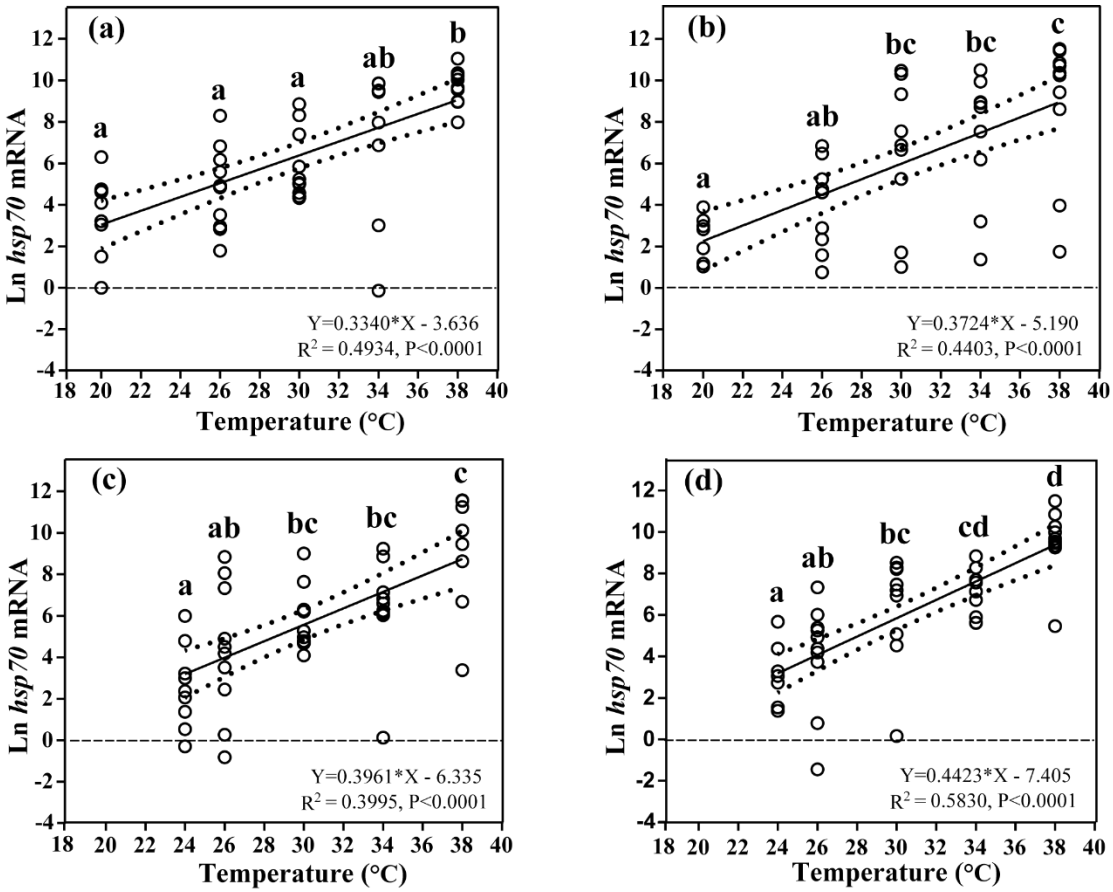
590 sensitivity indicates less acclimation to thermal stress. The calculation of post-acclimation Q_{10} is done for the mean

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

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

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

594



595

596

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

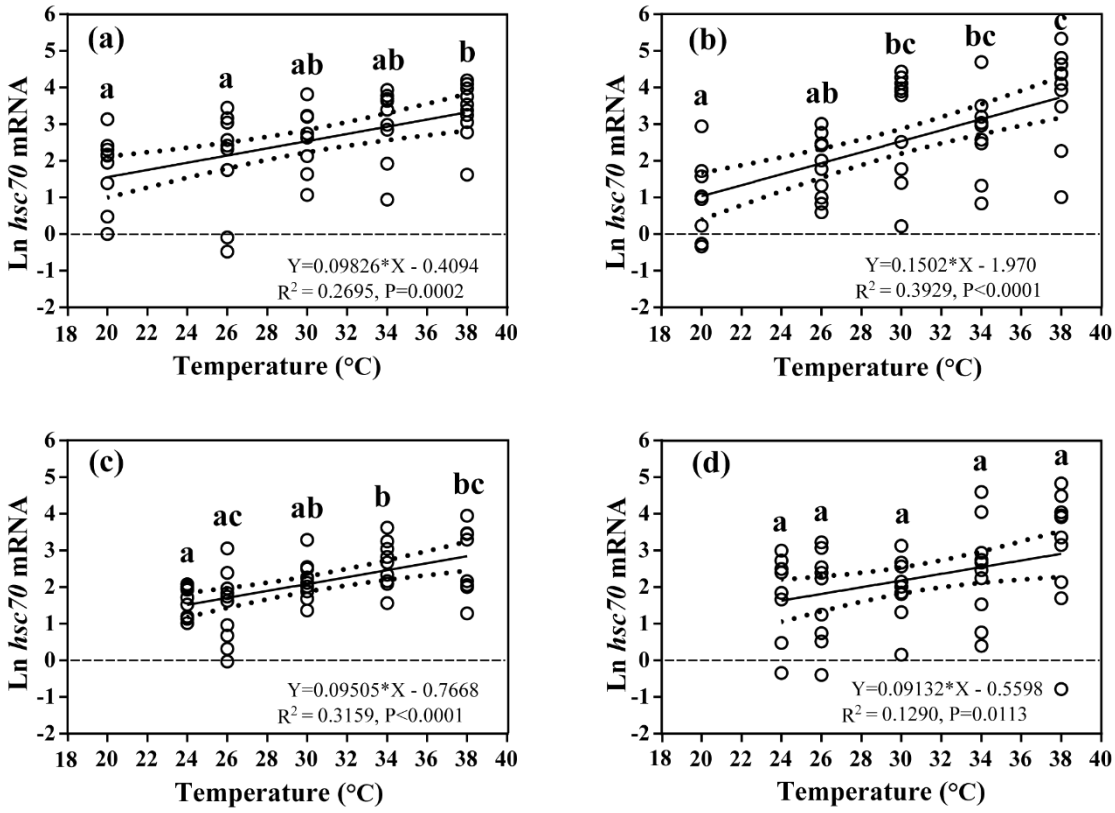
598 and 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

601 among different heat-shock temperatures.

602



603

604

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

606 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

609 among different heat-shock temperatures.

610

Appendix:

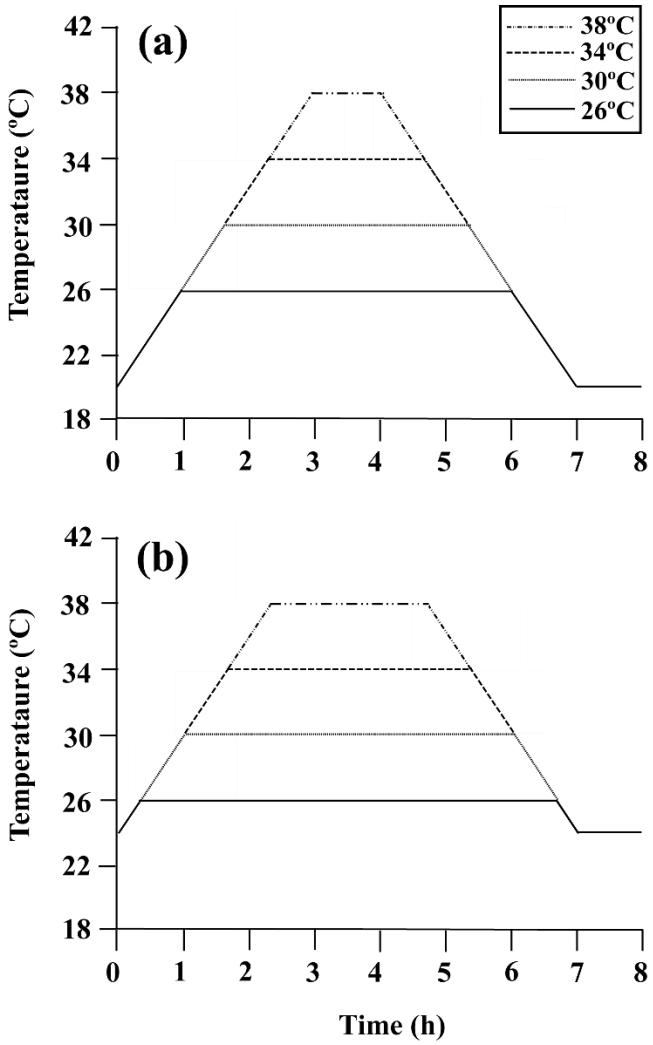


Figure A1. Diagram of the heating protocol for (a) limpets acclimated at 20 °C and (b) limpets acclimated at 24 °C. Limpets were heated at a rate of 6°C per hour from acclimation temperatures (20 or 24 °C) to designated temperatures (26, 30, 34 and 38 °C) for simulating a natural heating rate in summer.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

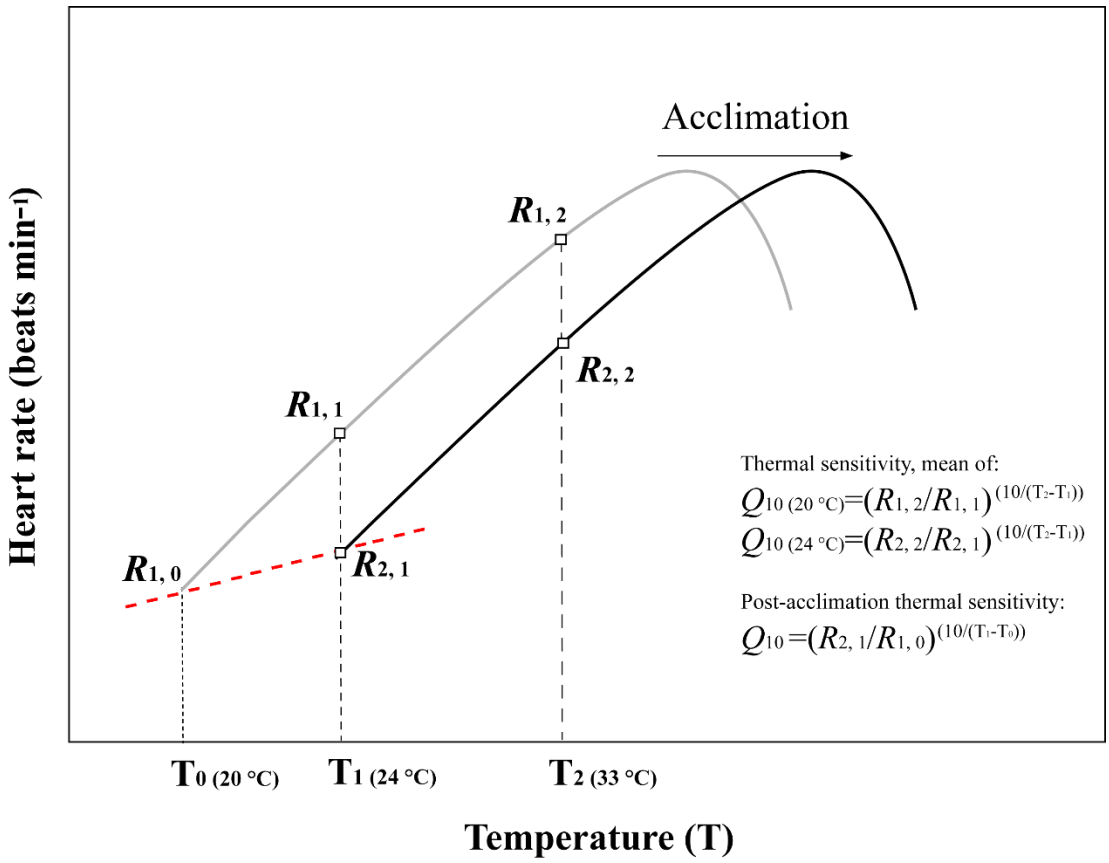
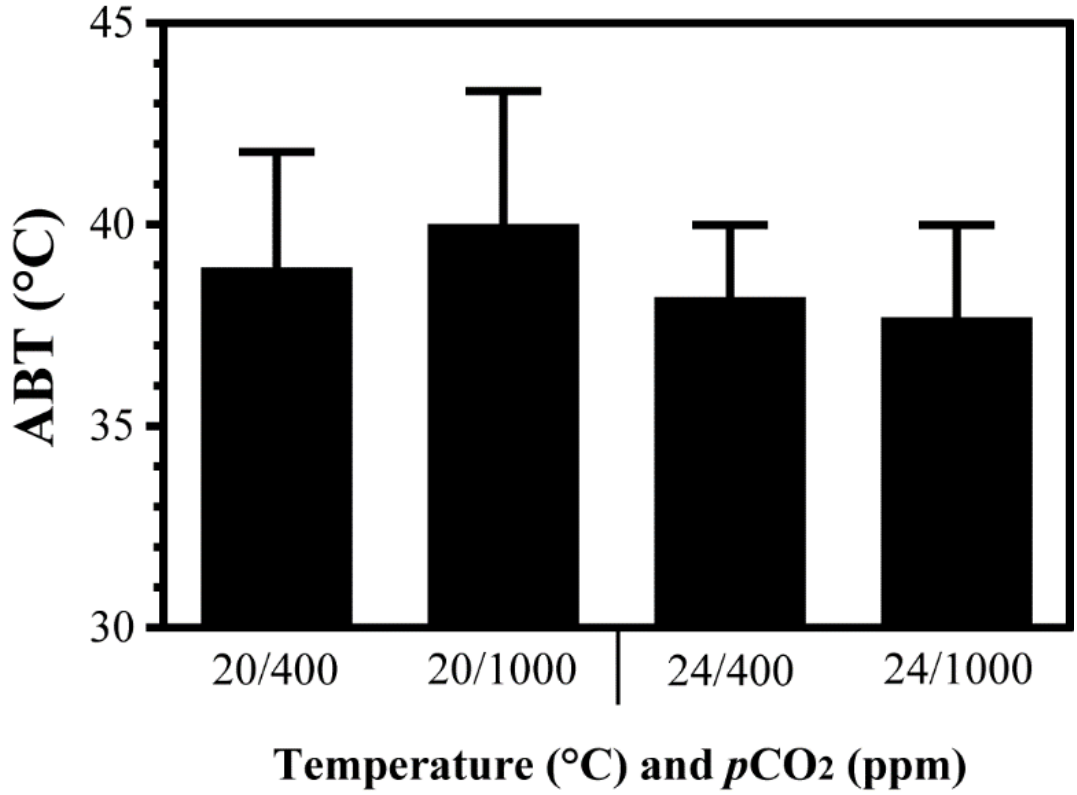


Figure A3. Schematic diagram of temperature coefficients (Q_{10}) and post-acclimation Q_{10} calculations. This figure was modified from Seebacher et al. (2015). Black line and grey line showed the heart rate of limpets from the warm-acclimated temperature (24 °C) and the cold-acclimated temperature (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.



636

637

638 **Figure A4.** Arrhenius breakpoint temperature of heart rate (ABT) of limpets acclimated at different temperatures

639 (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). After acclimation in different conditions, limpets were

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

641 rates were recorded and ABTs were calculated.

642

643

644 **Table A1.** Measured and calculated seawater carbonate chemistry variables of each acclimation treatment during the

645 experimental period[1]

646

	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 (‰)	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

647 [1]Seawater temperature, salinity, pH and total dissolved inorganic carbon (C_T) were monitored every 6 h. Total

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

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

650

651 **Table A2.** Functions and primers of selected genes of *Cellana* limpet

652

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

653
654

655 **Table A3.** Inferential statistics for the most likely general additive mixed models (GAMM) of heart rate during
656 continuous warming of limpet *Cellana toreuma* acclimated at different temperatures (20 and 24 °C) and pCO_2 (400
657 and 1000 ppm)[1]
658

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**

659 [1]The generalized additive model describes heart rate as a function of temperature, or *f*(T), instead of using a fixed
660 parameter to describe the effect of temperature. Additional functions were included to describe how heart rates of *C.*
661 *toreuma* from each treatment deviated from those of *C. toreuma* from 20 °C and 400 ppm.
662

Table A4. 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 of heart rate (ABT) 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		