Biogeosciences Discuss., doi:10.5194/bg-2017-47, 2017
Manuscript under review for journal Biogeosciences
Discussion started: 7 March 2017

Biogeosciences Discussions Open Access EGU

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

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Biogeosciences Discuss., doi:10.5194/bg-2017-47, 2017
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Abstract. Understanding physiological responses of organisms to warming and ocean acidification is the first step towards predicting the potential population, community and 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 from the same population, of the limpet *Cellana toreuma*. Heart rates (as a proxy for metabolic performance) and genes encoding heat-shock proteins were measured at different heat shock temperatures (26, 30, 34, 38 °C) in individuals acclimated under combinations of different pCO_2 (400 ppm, 1000 ppm) and temperature (20 °C, 24 °C) regimes. Analysis of heart rate showed significantly higher temperature coefficients (Q_{10} rates) for limpets at 20 °C than at 24 °C and lower post-acclimation thermal sensitivity of limpets at 400 ppm than at 1000 ppm. *hsp70* expression linearly increased with the increasing heat-shock temperatures, with the largest slope occurring in limpets under a future scenario (24 °C and 1000 ppm *p*CO2). These results suggested that limpets will have increased sensitivity and energy consumption under future conditions. Furthermore, the increased variation in physiological response under the future scenario indicated that some individuals were better to cope physiologically with these conditions. Therefore, while ocean acidification decreases the ability of many individuals to respond to thermal stress, physiological plasticity and variability seem to be crucial in allowing some intertidal animals to survive in a rapidly changing environment.

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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; Scheffers 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 (Firth and Williams, 2009; Dong et al., 2014). Indeed, it is increasingly being recognized that the interaction between global warming and ocean acidification may not only reduce an organism's resistance to environmental change (Munday et al., 2009), but 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 (Barry et al., 2011; Bellard et al., 2012; Parmesan and Yohe, 2003; Perry et al., 2005; Sunday et al., 2012), develop evolutionary adaptive changes (Hoffmann and Sgro, 2011; Sunday et al., 2014), 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

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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 climate change 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; Gibson et al., 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 (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). Therefore, we investigated the importance of physiological plasticity and variability 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 heat-shock proteins. This study provides novel information concerning the combined effects of increased temperature and pCO_2 on physiological plasticity in intertidal invertebrates, and is important

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76 in allowing predications of the ecological impacts of the future environmental changes.

77

78 **2 Material and Methods**

79 **2.1 Sample locality and study organism**

80 Xiamen (118°14′ E, 24°42′ N) is a representative location in China, which is in a region which is
81 experiencing some of the fastest rates of temperature rise and acidification (reduced pH) globally (Bao
82 and Ren, 2014). The sea surface temperature (SST) in Xiamen coastal area has risen a total of 1 °C since
83 1960, and is rising at a mean annual rate of 0.02 °C (Yan et al., 2016). The annual pH values of seawater
84 in Xiamen Bay have declined by 0.2 pH units from 1986 to 2012, a trend which is predicted to continue
85 based on simulations (Cai et al., 2016).

86 The limpet *C. toreuma* is a keystone species on rocky shores in the Western Pacific (Dong et al.,
87 2012) and occupies mid–low intertidal zones. This species is a gonochoric and broadcast spawner, whose
88 embryos develop into planktonic trocophore larvae and later into juvenile veligers before becoming fully
89 grown adults (Ruppert et al., 2004). As a common calcifier inhabiting coastal ecosystem, *C. toreuma*
90 plays an important ecological role, affecting the community structure of the associated biofilm. Therefore,
91 this species is a key organism for studying the relationship between physiological response to
92 temperature fluctuation and pH decline in highly variable intertidal zone, with great significance in
93 ecology.

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95 **2.2 Limpet collection and experiment treatments**

96 The following experiments were conducted for the first time in July 2014 and the same experiment
97 was repeated in July 2016, which was to improve the quantity and quality of the data. Samples were
98 collected from Xiamen on a falling high tide, and were transported back State Key Laboratory of Marine
99 Environmental Science, Xiamen University, China within 2 h. Limpets were firstly allowed to recover at
100 20 °C for 3 d with a tidal cycle of approximately 6 h immersion and 6 h emersion. These limpets were
101 randomly allocated into four acclimation treatments (about 100 indiv. per acclimation treatment) and
102 acclimated for 7 d in different pCO_2 concentrations and temperatures (LTLC, 20 °C + 400 ppm, as a
103 control treatment; LTHC, 20 °C + 1000 ppm; HTLC, 24 °C + 400 ppm; HTHC, 24 °C + 1000 ppm) in
104 climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which can control the
105 *p*CO2 concentration. Control temperature (20 °C) and high temperature (24 °C), respectively, represent
106 the average annual temperature in the collection site and the average global increase (4 °C) predicted for
107 2100 by the Intergovernmental Panel on Climate Change (IPCC, 2007). Two pCO_2 levels, 400 ppm and
108 1000 ppm, represent the present-day situation and scenarios for 2100 respectively, as projected by IPCC
109 (2007).

110 Animals were kept in a simulated tidal cycle with 6 h aerial exposure and 6 h seawater immersion.
111 Seawater was pre-bubbled with air containing the corresponding pCO_2 concentrations in advance. pH
112 was measured before and after the acclimation in seawater each time with PB-10 pH meter (Sartorius
113 Instruments, Germany) calibrated with National Institute of Standards and Technology standard pH
114 solutions (NIST, USA). Total dissolved inorganic carbon (DIC) was measured before and after the
115 acclimation in seawater each time using a Li-Cor® non-dispersive infrared (NDIR) detector (Li-6252) by
116 a dissolved inorganic carbon analyzer (As-C3, Apollo SciTech, Colorado, USA) with a precision of 0.1%

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(Cai, 2003). Seawater carbonate chemistry parameters were estimated based on the measured values of pH, DIC, temperature and salinity with the software CO2Calc v4.0.9 (Robbins et al., 2010). For CO2Calc settings, the NBS scale was applied as the pH scale, and the CO_2 constant, the $KHSO_4$- constant and the total Boron was set from Millero et al. (2006), Dickson et al. (19990) and Lee et al. (2010) respectively. The information of the measured and calculated seawater chemistry parameters is summarized (Table A1).

After 7-day acclimation, individuals from all four acclimation conditions (n = 10 indiv. per acclimation treatment) were sampled and frozen at -80 °C as non-heated control samples. The remaining limpets were transferred to an artificial rock and heated at a rate of 6 °C per hour, to simulate a natural heating rate in summer during low tide in Xiamen Bay as described by Han et al. (2013), to designated temperatures (26, 30, 34 and 38 °C). The heat-shock treatments were carried out as described in Denny et al. (2006) (Fig. A1). After achieving the target temperature, the temperature was maintained 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 at 20 or 24 °C seawater for 1 h, limpets (n = 8-10 indiv. per heat shock temperature at each acclimation condition) were immediately collected and stored at -80 °C for gene expression quantification.

2.3 Cardiac performance measurement

The cardiac performance of limpets was recorded during whole heating processes from the acclimated temperature (20 or 24 °C) to the temperature where the heart stopped beating (n = 9-11 indiv. per acclimation treatment). Heart rates were measured using a non-invasive method (Chelazzi et al., 2001;

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Dong and Williams, 2011). The heartbeat was detected by means of an infrared sensor fixed with Blue-Tac (Bostik, Staffordshire, UK) on the limpet shell at a position above the heart. Variation in the light-dependent current produced by the heartbeat were amplified, filtered and recorded using an infrared signal amplifier (AMP03, Newshift, Leiria, Portugal) and Powerlab AD converter (4/30, ADInstruments, March-Hugstetten, Germany). Data were viewed and analyzed using Lab Chart (version 7.0).

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

2.4 Quantifying genes expression

Limpets were firstly taken out from – 80 °C; 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^{TM} Real-Time System (Bio-Rad Laboratories, Inc., Hercules CA, USA) followed the methods described by Han et al. (2013) with specific primers (Table A2). For normalizing expression of genes,

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159 we examined expression of *18S ribosomal RNA*, *β-actin*, *β-tubulin* genes, which typically have relatively
160 stable expression levels. The expression stability of these housekeeping genes was evaluated using the
161 GeNorm Algorithm (Primer Design, Ltd., Southampton University, Highfield Campus, Southampton
162 Hants, UK) as described by Etschmann et al. (2006). Based on the expression stability measures (M
163 values), all the three genes were selected as the reference genes for normalizing the level of expression
164 of stress-induced genes. All samples were measured in triplicates. Ct (dR) values were analyzed using
165 the CFX Manager™ Software Version 3.0 (Bio-Rad). The expression of *hsp70* and *hsc70* was determined
166 relative to the value of *18S*, *β-actin* and *β-tublin* from a reference individual.
167

168 **2.5 Statistical analysis**

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

175 Thermal sensitivity stands for the change in a physiological rate function reacting to a rapid change
176 in environmental temperature within the same acclimation set temperature (Fig. A2, modified from
177 Seebacher et al. (2015)). In the present study, thermal sensitivity is seen in the temperature coefficient
178 (Q_{10}) values of heart rate. Q_{10} was calculated using heart-rate data from the temperature at which the
179 experiment started (T_1 = 24 °C) to the temperature to which temperature increased 10 °C (T_2 = 33 °C)

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with Eq. (1):

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

where R is the heart rate (R_1 and R_2 are the heart rate at T_1 and T_2 respectively), and T is the temperature (Kelvin) (Fig. A2, modified from Seebacher et al. (2015)). The differences in Q_{10} among the four acclimation conditions with different CO_2 concentrations (400 ppm vs. 1000 ppm) and temperatures (20 °C vs. 24 °C) were analyzed using two-way ANOVA with Duncan's *post hoc* analysis using the SPSS 20.0 for Windows statistical package (IBM SPSS Statistics, Chicago, USA). Post-acclimation thermal sensitivity of limpets in different CO_2 concentrations were calculated as described by Seebacher et al. (2015). In each CO_2 concentration (400 ppm or 1000 ppm), the post-acclimation Q_{10} values were calculated using the same equation as shown above, but R_2 was the heart rate of the warm-acclimated limpets at the acclimated temperature (T_2 = 24 °C), and R_1 was the heart rate of cold-acclimated limpets at T_1 = 20 °C (Fig. A2, modified from Seebacher et al. (2015)).

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).

3 Results

3.1 Cardiac performance

The maximal heart rate was ~ 30 % higher in limpets acclimated to control conditions (20 °C, 400

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ppm) than the other treatments (Fig. 1 and Table A3) indicating reduced metabolic performance under high temperatures and pCO_2 conditions. The ABTs of limpets ranged from 34.5 °C to 44.2 °C and showed a trend to be reduced for HT treatments, but did not differ statistically (Fig. A3; Two-way ANOVA, $P > 0.05$).

Temperature coefficients (Q_{10} rates) were higher for limpets acclimated at 20 °C than at 24 °C (Fig. 2, Two-way ANOVA, $P = 0.02$), but there was no significant difference for acclimation to different pCO_2 concentrations ($P > 0.05$). The post-acclimation thermal sensitivity of limpets acclimated at low CO_2 (2.12) was lower than limpets at high CO_2 (2.95), indicating that the latter are more metabolically sensitive to temperature.

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 ($F = 42.62$, $P < 0.001$), 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

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(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

Ocean acidification and thermal stress are inherently linked to rising atmospheric pCO_2 and will be manifested in combination in the future (Bijma et al., 2013; Connelll and Russell, 2009; Hale et al., 2011; Walther et al., 2009). Despite this certainty and the likelihood that ocean acidification will affect the physiological plasticity to thermal stress (Pörtner et al., 2010), there is currently limited information on how this may manifest in populations of organisms which inhabit stressful environments (Dupont and Thorndyke, 2009; Dupont and Pörtner, 2013). Here, we show that the thermal sensitivity of limpets acclimated to current atmospheric CO_2 (~ 400 ppm) is lower than that of limpets acclimated to 1000 ppm (2.12 vs. 2.95, respectively). Post-acclimation thermal sensitivity represents the extent to which ectothermic animals can acclimate to longer-term increases in temperature (several days to weeks) (Seebacher et al., 2015). Thus, 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

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to an extreme thermal environment. For example, the operative temperatures, from 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.

Increased temperature and CO_2 increase the sensitivity of heat shock responses to thermal stress. The expression of *hsp70* mRNA steadily increased from 20°C to 38°C for individuals across all experimental treatments. However, rates of upregulation of *hsp70* mRNA in limpets acclimated at high temperature and high CO_2 (HTHC) were significantly higher than those of limpets acclimated at the other three acclimation conditions. As a molecular chaperon, *Hsp70* plays crucial roles in maintaining protein stability with the expense of a large amount of energy (Feder and Hofmann, 1999; Tomanek and Sanford, 2003). Usually, the expression of *hsp70* of less thermal-tolerant species is more sensitive to increases in temperature (Dong et al., 2008; Tomanek, 2002), and the rapid upregulation of *hsp70* mRNA in limpets exposed to future conditions potentially represents a high sensitivity of limpets to thermal stress in the face of ocean acidification. Due to the expensive energy consumption during the synthesis and function of *hsp70*, the more rapid upregulation of *hsp70* mRNA in these limpets also indicates more energy was allocated into cellular homeostasis, which then can affect the limpet's growth and reproduction. This change in the metabolic partitioning in individuals could ultimately lead to a decline in fitness and population-level responses.

The expression patterns of *hsc70* mRNA were different among limpets at the four acclimation conditions. *Hsc70* is constitutively expressed and is a molecular chaperone involved in the *in vivo* folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain similar promoter regions, there are differential expressions to a given stimulus between them (Hansen et al., 1991). In the

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present study, the expression of *hsc70* mRNA showed no significant difference among different heat-shock temperatures under predicated future environmental conditions (24 °C and 1000 ppm). If only one environmental factor changed (i.e., temperature or CO_2), however, there was significant upregulation of *hsc70* mRNA when the heat shock temperatures were beyond 30 °C. These results indicate that expression of *hsc70* mRNA is relatively constitutive. That is, 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 for weeks. However, the absence of significant upregulation of *hsc70* mRNA in limpets acclimated to future conditions (warming and elevated CO_2) might 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

288 selective pressure, these individuals would form the basis for future generations while less plastic
289 individuals would be removed from populations.

290 In conclusion, the resilience of intertidal limpets to thermal stress is weakened after exposure to
291 predicted future conditions. Yet, the combination of elevated temperature and CO_2 concentration
292 prompted divergence of physiological and molecular responses. These results suggest that while
293 organisms may be able to protect themselves from the damaging effects of thermal stress in the short-
294 term, changes to multiple environmental conditions may drive population-level responses through
295 physiological responses (e.g. Giomi et al., 2016). Further, the increased variation in responses, and the
296 observation that some individuals were more capable to physiologically cope with the conditions, may
297 be associated with intergenerational adaptation, but this speculation needs further evidence. As the
298 "weaker" individuals are lost, the offspring in the next generation will be better physiologically adapted
299 to warming under high-CO_2 conditions. Therefore, while elevated CO_2 and the associated ocean
300 acidification decrease the ability of many individuals to respond to thermal stress, it appears that
301 physiological plasticity and variability could be adaptive mechanisms in at least some populations of
302 intertidal organisms.

303

304 **Authors' contributions**

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

308

309 **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.

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

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

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

[2]After acclimated at different CO_2 and temperature for one week, limpets (n = 8-10) from each acclimation treatment were randomly selected and heat shocked at designated temperatures. Levels of *hsc70* mRNA at 38 °C in different acclimation treatments were used for calculating coefficients of variation.

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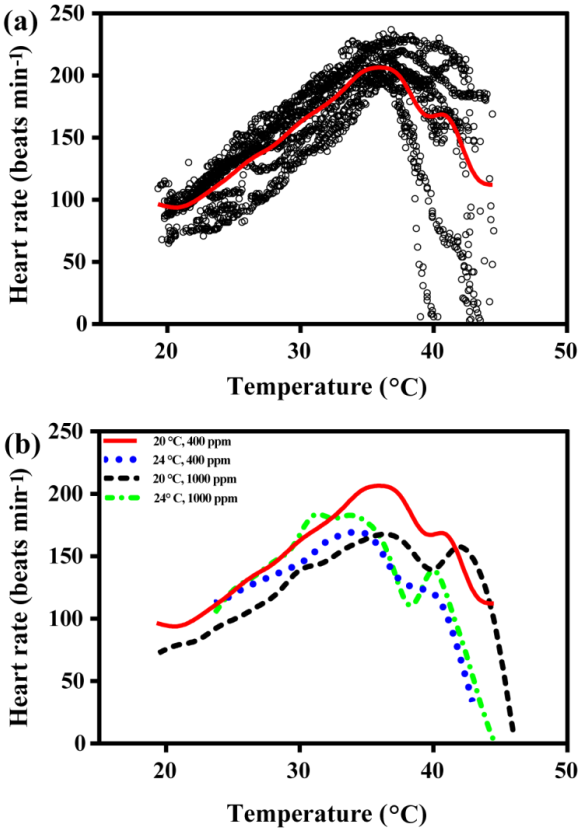

Figure 1. (a) Heart rates of all limpets acclimated to 24 °C and 400ppm, presented as an example of HR calculation for limpets in all treatments. The red line represents the most likely general additive mixed model (GAMM) to depict the trajectory of hearts rate for limpets with increasing temperature; (b) GAMM lines of limpets acclimated at the different experimental temperature and CO_2 conditions.

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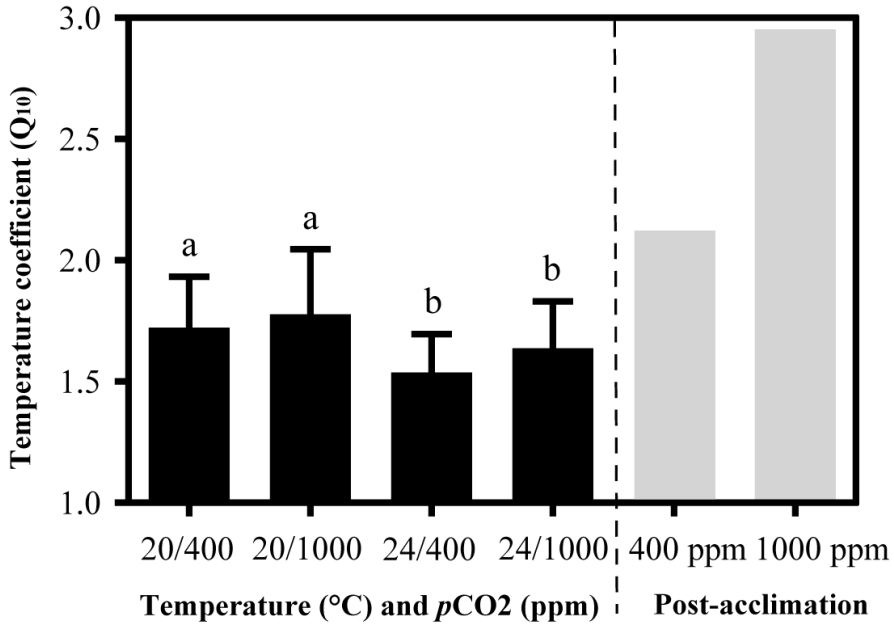

Figure 2. Temperature coefficients (Q10) of limpets acclimated at different temperatures (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). The temperature coefficient (Q10) values were calculated for all limpets using heart rate data from 24 to 33°C. Post-acclimation temperature sensitivity was calculated between individuals acclimated at 20 and 24°C (grey bars; *sensu* Seebacher et al., 2015) for each CO_2 concentration, where higher thermal sensitivity indicates less acclimation to thermal stress. Different letters represent significant differences in the Q_{10} among different acclimation treatments.

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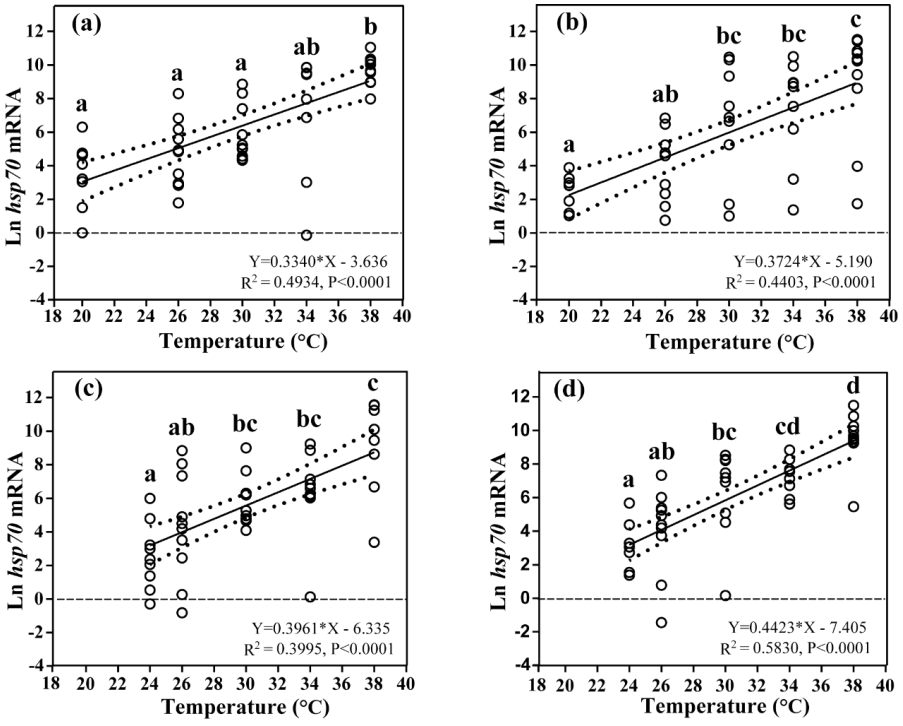

Figure 3. Effects of heat-shock temperature on the expression of *hsp70* mRNA in limpets acclimated at (a) 20°C and 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between heat-shock temperature and log-transformed gene expression of *hsp70* was fitted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

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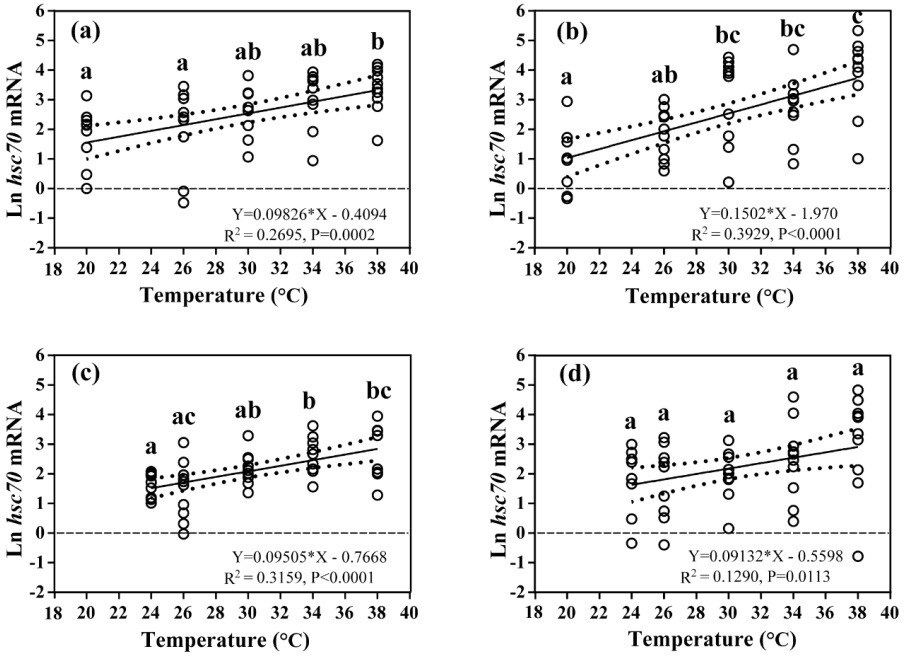

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520

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

522 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

525 among different heat-shock temperatures.

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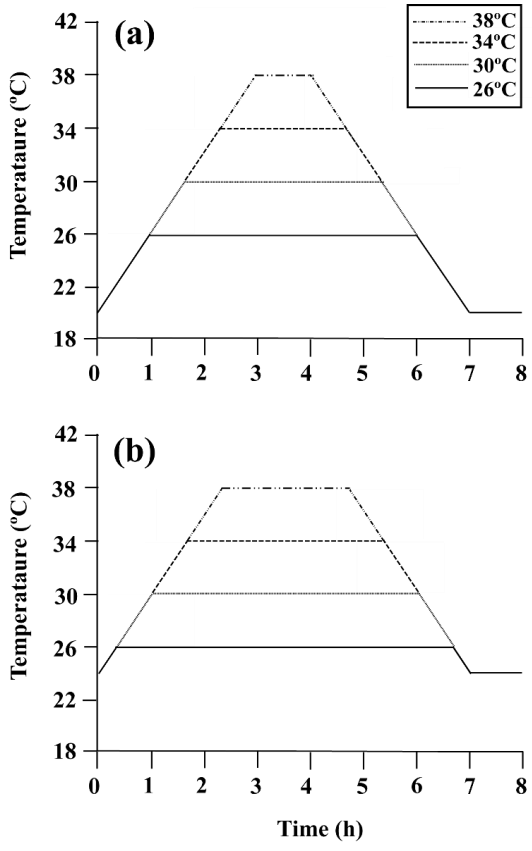
527 **Appendix:**



528

529 **Figure A1.** Diagram of the heating protocol for (a) limpets acclimated at 20 °C and (b) limpets acclimated at 24 °C.

530 Limpets were heated at a rate of 6°C per hour from acclimation temperatures (20 or 24 °C) to designated temperatures

531 (26, 30, 34 and 38 °C) for simulating a natural heating rate in summer. After achieving the target temperature, the

532 temperature was held at the designated level for the allotted time, and then decreased to acclimated temperatures (20

533 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,

534 limpets (n = 8-10) in each treatment were immediately collected and stored at -80 °C for gene expression

535 measurement.

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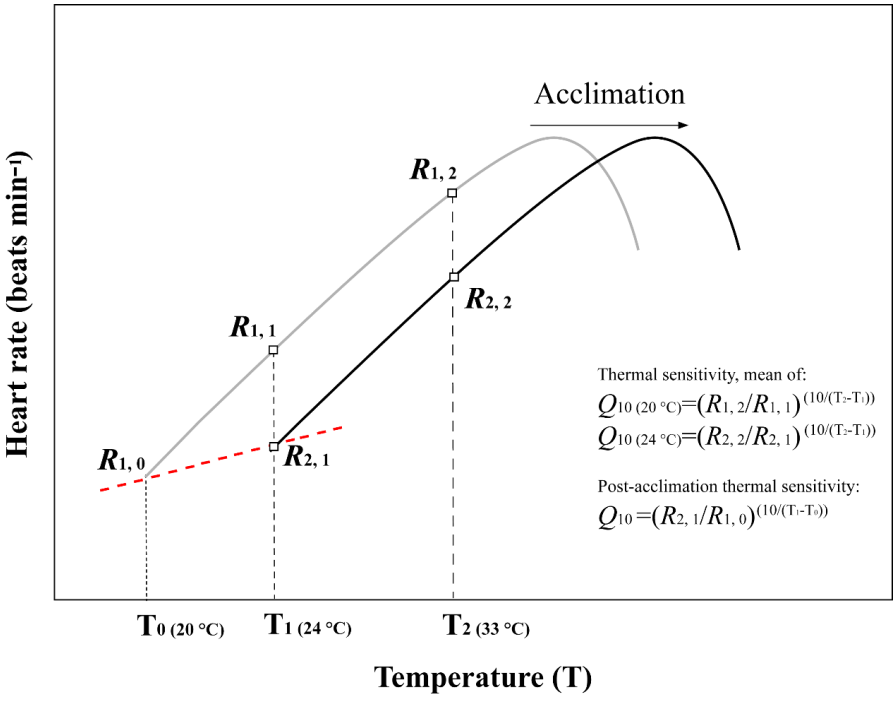

Figure A2. Schematic diagram of temperature coefficients (Q_{10}) and post-acclimation Q_{10} calculations. This figure was modified from Seebacher et al. (2015). Black line and grey line showed the heart rate of limpets at the warm-acclimated temperature (24 °C) and the cold-acclimated temperature (20 °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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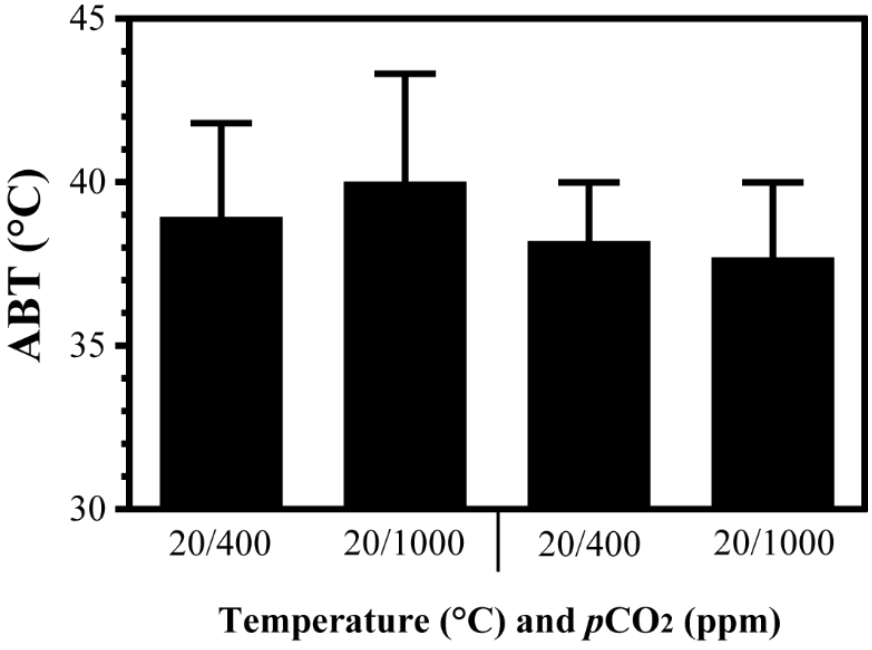

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548 **Figure A3.** Arrhenius break point temperature of heart rate (ABT) of limpets acclimated at different temperatures

549 (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). After acclimation in different conditions, limpets were

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

551 rates were recorded and ABTs were calculated.

552

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

555 experimental period[1]

556

	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 (umol/kg)	2082.70±191.28	2083.016±190.58	2081.19±165.93	2083.29±163.58
C_T (umol/kg)	1910.57±174.42	1910.57±174.42	1992.76±157.22	1992.15±149.76
pCO_2 (utam)	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-} (umol/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

557 [1]Seawater temperature, salinity, pH and total dissolved inorganic carbon (C_T) were monitored every 6 h. Total

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

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

560

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

562

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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565 **Table A3.** Inferential statistics for the most likely general additive mixed models (GAMM) of heart rate during
566 continuous warming of limpet *Cellana toreuma* acclimated at different temperatures (20 and 24 °C) and pCO_2 (400
567 and 1000 ppm)[1]
568

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**

569 [1]The generalized additive model describes heart rate as a function of temperature, or *f*(T), instead of using a fixed
570 parameter to describe the effect of temperature. Additional functions were included to describe how heart rates of *C.*
571 *toreuma* from each treatment deviated from those of *C. toreuma* from 20 °C and 400 ppm.
572