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 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 and 38 °C) in individuals temporally acclimated (7 d) under combinations of different pCO_2 concentrations (400 ppm and 1000 ppm) and temperatures (20 °C and 24 °C). Analysis of heart rate showed significantly higher temperature coefficients (Q_{10} rates) for limpets at 20 °C than at 24 °C and post-acclimation thermal sensitivity of limpets at 400 ppm was lower than at 1000 ppm. Expression of *hsp70* linearly increased with the increasing heat-shock temperatures, with the largest slope occurring in limpets acclimated under a future scenario (24 °C and 1000 ppm *p*CO2). 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 were better to cope physiologically 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 (Firth and Williams, 2009; Dong et al., 2014). Although ocean acidification can increase the growth of organisms in some cases (e.g. Gooding et al., 2009), increasing evidence showed that rising ocean acidity exacerbates global warming, reduces an organism's resistance to environmental change (Munday et al., 2009), and subsequently affects 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

54 environmental conditions (Hoffmann and Sgro, 2011; Pörtner et al., 2012; Somero et al., 2012). In a
55 recent meta-analysis, Seebacher et al. (2015) demonstrated that acclimation to higher temperatures
56 decreased the sensitivity to climate change in both freshwater and marine animals. While this response
57 suggests that acclimation could reduce the impact of warming on organisms, the responses were only
58 tested for shifts in mean temperature. Yet, organisms inhabiting variable environments, such as the
59 intertidal zone, will be exposed to increasing extremes in temperature concomitant with increasing pCO_2,
60 or ocean acidification (OA), in the future. While OA has been suggested to increase the sensitivity of
61 organisms to warming (Byrne and Przeslawski, 2013; Gibson et al., 2011; Kroeker et al., 2013),
62 physiological plasticity and variation in responses may provide the basis for populations to survive.
63 Physiological variation, or plasticity, within population is important for adapting to local
64 microclimate and for evolution (Dong et al., 2017; Oleksiak et al., 2002; Prosser, 1955). For example,
65 different color morphs of the gastropod *Littorina saxatilis* have enhanced physiological performance
66 which leads to increased survival under extreme conditions, indicating physiological differences may
67 provide a selective advantage for those color morphs under extremely fluctuating salinity and
68 temperature regime in estuaries (Sokolova and Berger, 2000). For the limpet *Cellana toreuma*, highly
69 variable expressions of genes related to stress responses and energy metabolism are important for
70 surviving the harsh environment on subtropical rocky shores (Dong et al., 2014).
71 The limpet *C. toreuma* is a keystone species on rocky shores in the Western Pacific (Dong et al.,
72 2012) and occupies mid–low intertidal zones (Morton and Morton 1983). This species is a gonochoric
73 and broadcast spawner, whose embryos develop into planktonic trocophore larvae and later into juvenile
74 veligers before becoming fully grown adults (Ruppert et al., 2004). As a common calcifier inhabiting
75 coastal ecosystem, *C. toreuma* plays an important ecological role in affecting the community structure

of the associated biofilm. 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 area has risen 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 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 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 physiological plasticity in intertidal invertebrates, and is important in allowing 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 Xiamen, and were transported back 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 four acclimation 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 can control the *p*CO2 concentration. There were about 100 indiv. per acclimation treatment, and the density was ~ one limpet per 10 cm^2 in all acclimation treatments. This density was similar to that when we collected the samples. Control temperature (20 °C) and high temperature (24 °C), respectively, represent the average annual temperature in the collection site and the average global increase (4 °C) predicted for 2100 by the Intergovernmental Panel on Climate Change (IPCC, 2007). Two pCO_2 levels, 400 ppm and 1000 ppm, represent the present-day situation and scenarios for 2100 respectively, as projected by 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 solutions (NIST, USA). Total dissolved inorganic carbon (DIC) was measured before and after the acclimation in seawater each time using a Li-Cor® non-dispersive infrared (NDIR) detector (Li-6252) by a dissolved inorganic carbon analyzer (As-C3, Apollo SciTech, Colorado, USA) with a precision of 0.1%

(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 short-term acclimation, individuals from all four acclimation conditions (n = 10 indiv. per acclimation treatment) were randomly sampled and frozen at -80 °C as non-heated control samples. In each acclimation treatment, 40 limpets were randomly selected and were transferred to an artificial rock (see Fig. A1). The rock was heated at a rate of 6 °C per hour (a natural heating rate, Han et al., 2013) to the designated temperatures (26, 30, 34 and 38 °C). The heat-shock treatments were carried out as described in Denny et al. (2006) (Fig. A2). 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.

2.2 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). Each limpet was placed in a separate container during the measurement. Heart

rates were measured using a non-invasive method (Chelazzi et al., 2001; 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).

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

2.4 Statistical analysis

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

Thermal sensitivity stands for the change in a physiological rate function reacting to a rapid change in environmental temperature within the same acclimation set temperature (Fig. A2, modified from Seebacher et al. (2015)). In the present study, thermal sensitivity is seen in the temperature coefficient (Q_{10}) values of heart rate. Q_{10} was calculated using heart-rate data from the temperature at which the

181 experiment started (T_1 = 24 °C) to the temperature to which temperature increased 10 °C (T_2 = 33 °C)
182 with Eq. (1):
183 $$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \quad (1)$$
184 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
185 (Kelvin) (Fig. A2, modified from Seebacher et al. (2015)). The differences in Q_{10} among the four
186 acclimation conditions with different CO_2 concentrations (400 ppm vs. 1000 ppm) and temperatures
187 (20 °C vs. 24 °C) were analyzed using two-way ANOVA with Duncan's *post hoc* analysis using the SPSS
188 20.0 for Windows statistical package (IBM SPSS Statistics, Chicago, USA). Post-acclimation thermal
189 sensitivity of limpets in different CO_2 concentrations were calculated as described by Seebacher et al.
190 (2015). In each CO_2 concentration (400 ppm or 1000 ppm), the post-acclimation Q_{10} values were
191 calculated using the same equation as shown above, but R_2 was the heart rate of the warm-acclimated
192 limpets at the acclimated temperature (T_2 = 24 °C), and R_1 was the heart rate of cold-acclimated limpets
193 at T_1 = 20 °C (Fig. A3, modified from Seebacher et al. (2015)).
194 The differences in levels of *hsp70* and *hsc70* among different heat shock temperatures within a same
195 acclimation condition were analyzed using one-way ANOVA with Duncan's *post hoc* analysis. The
196 relationships between heat shock temperature and log-transformed gene expression (*hsp70* and *hsc70*)
197 were fitted using linear regressions and the differences in slopes of the linear regressions were analyzed
198 using Analysis of Covariance (ANCOVA).
199 The coefficient of variation (CV) of ABT, Q_{10} and *hsc70* mRNA expression at 38 °C were
200 calculated for each acclimation condition. The CV is the variance in a sample divided by the mean of
201 that sample, providing a method to compare the variation within a sample relative to the mean. It is
202 generally accepted that higher CV demonstrates that there is greater variation among individuals within

one treatment than another (Reed et al., 2002).

3 Results

3.1 Cardiac performance

The maximal heart rate was ~ 30 % higher in limpets acclimated to control conditions (20 °C, 400 ppm) than the other treatments (Fig. 1 and Table 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 (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), 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 ($F_{4, 189} = 42.62$, $P < 0.001$), 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 against thermal stress. 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 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). By comparing the expression patterns of *Hsp70* of different *Chlorostoma* species (formerly *Tegula*) that have distinct vertical distribution, Tomanek and Somero (1999, 2000) found that there existed interspecific difference in the frequency of the induction of *Hsp70* synthesis and interspecific divergence of the time-course of *Hsp70* synthesis. These studies from genus *Chlorostoma* suggested that species that live higher in the intertidal cost more energy for proteostasis and restore proteostasis to cope with a second consecutive day of high temperatures (Semero et al., 2016). Usually, the expression of *Hsp70* of less thermal-tolerant species is more sensitive to increases in temperature (limpet *Lottia*, Dong et al., 2008; snail *Chlorostoma*, Tomanek, 2002), and the rapid upregulation of *hsp70* mRNA in limpets

exposed to future conditions potentially represents a high sensitivity of limpets to thermal stress in the face of ocean acidification. Due to the expensive energy consumption during the synthesis and function of *hsp70*, the more rapid upregulation of *hsp70* mRNA in these limpets also indicates more energy was allocated into cellular homeostasis, which then can affect the 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 acclimated at the four acclimation conditions. *Hsc70* is constitutively expressed and is a molecular chaperone involved in the *in vivo* folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain similar promoter regions, there are differential expressions to a given stimulus between them (Hansen et al., 1991). Some studies showed that thermal stress could significantly induce the 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.,

289 2008) to maintain high constitutive levels of *hsc70* as a mechanism to copy with unpredictable heat stress.
290 However, the absence of significant upregulation of *hsc70* mRNA in limpets acclimated to future
291 conditions (warming and elevated CO_2) might also be attributed to the very high variation of gene
292 expression at 38°C (CV, 90.36 %). In the context of future conditions, multiple environmental stressors
293 can induce diverse physiological responses among different individuals, which might be an evolutionary
294 adaptation to the harsh environment on the shore.

295 Variation and plasticity in both physiological and molecular responses to thermal stress are not only
296 important for coping with future environmental change but also underpin evolutionary and adaptive
297 changes through selective pressures (Franks and Hoffmann, 2012). In the present study, the coefficients
298 of variation in physiological responses of limpets acclimated in simulated future conditions, including
299 ABT, Q_{10} and *hsc70* mRNA, were higher than those in the other three acclimation conditions. Crucially,
300 this means that a subset of individuals in our experimental population might be more physiologically
301 pre-adapted to cope with heat shock. Once acclimated to future climate change scenario (warming and
302 ocean acidification), this variation in physiological performance increased, indicating that in a harsher
303 environment the physiological plasticity of some individuals allows them to modify their physiological
304 tolerance limits and increase chances for survival and reproduction (Williams et al., 2008). Under high
305 selective pressure, these individuals would form the basis for future generations while less plastic
306 individuals would be removed from populations. However, the results about the coefficients of variation
307 need to be interpreted with caution, as the sample size (around 10 limpets per treatment) in the present
308 study may affect the prediction accuracy.

309 In conclusion, the resilience of intertidal limpets to thermal stress is weakened after exposure to
310 predicted future conditions for a short-term acclimation period (7 d). Yet, the combination of elevated

311 temperature and CO_2 concentration prompted divergence of physiological and molecular responses.
312 These results suggest that while organisms may be able to protect themselves from the damaging effects
313 of thermal stress in the short-term, changes to multiple environmental conditions may drive population-
314 level responses through physiological responses (e.g. Giomi et al., 2016). Further, the increased variation
315 in responses, and the observation that some individuals were more capable to physiologically cope with
316 the conditions, may be associated with intergenerational adaptation, but this speculation needs further
317 evidence. As the "weaker" individuals are lost, the offspring in the next generation will be better
318 physiologically adapted to warming under high-CO_2 conditions. Therefore, while elevated CO_2 and the
319 associated ocean acidification decrease the ability of many individuals to respond to thermal stress, it
320 appears that physiological plasticity and variability could be adaptive mechanisms in at least some
321 populations of intertidal organisms. Our research underlined the importance of physiological plasticity
322 and variability for coastal species coping with warming and ocean acidification. However, the present
323 study has only examined the physiological responses of limpets to heat stress after short-term acclimation.
324 Future studies with long-term acclimation and a larger sample size are therefore recommended in order
325 to validate our findings.
326
327 **Authors' contributions**
328 B.D.R and Y.-W.D. designed experiments. W.J. and M.-W.D. conducted experiments. Y.-W.D., B.D.R,
329 W.J. and M.-W.D. performed analyses. The manuscript was co-written by Y.-W.D., W.J. and M.-W.D.,
330 and revised by B.D.R.
331
332 **Competing interests**

The authors declare no conflict of interests.

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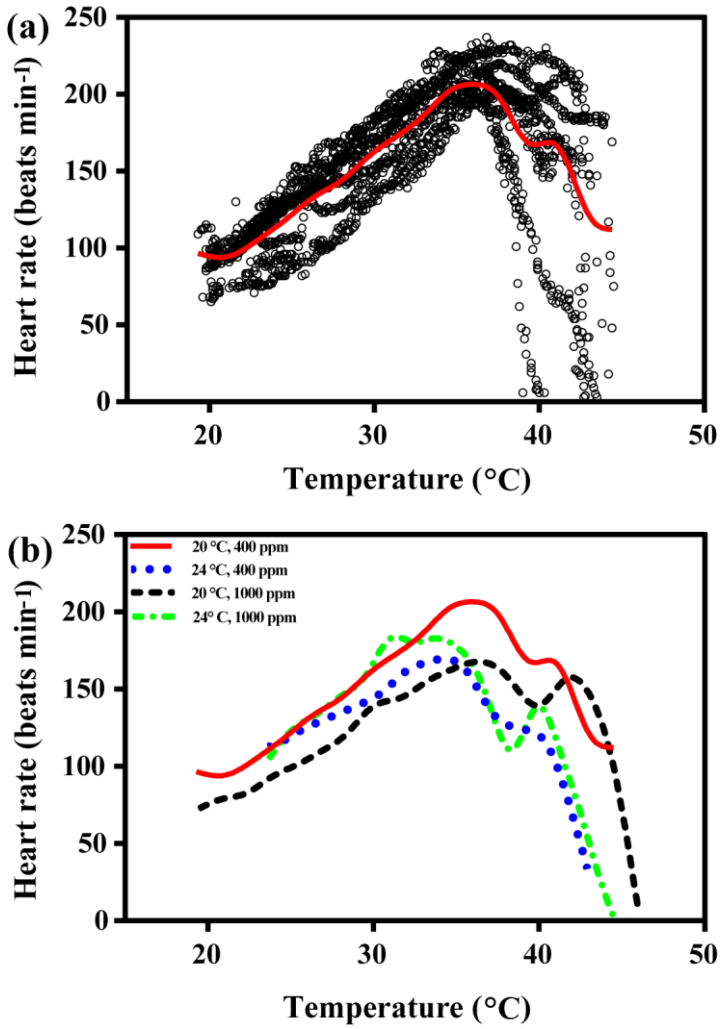
494

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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506 **Figure 1.** (a) Heart rates of all limpets acclimated to 24 °C and 400ppm, presented as an example of HR calculation

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

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

509 different experimental temperature and CO_2 conditions.

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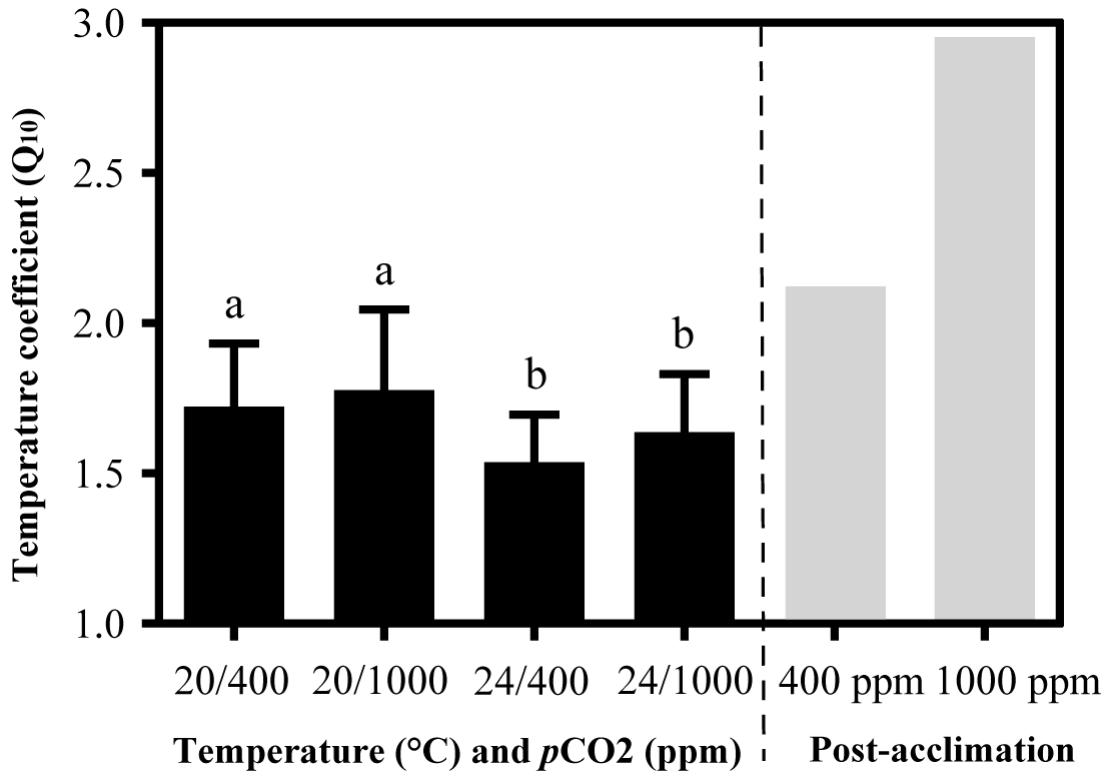
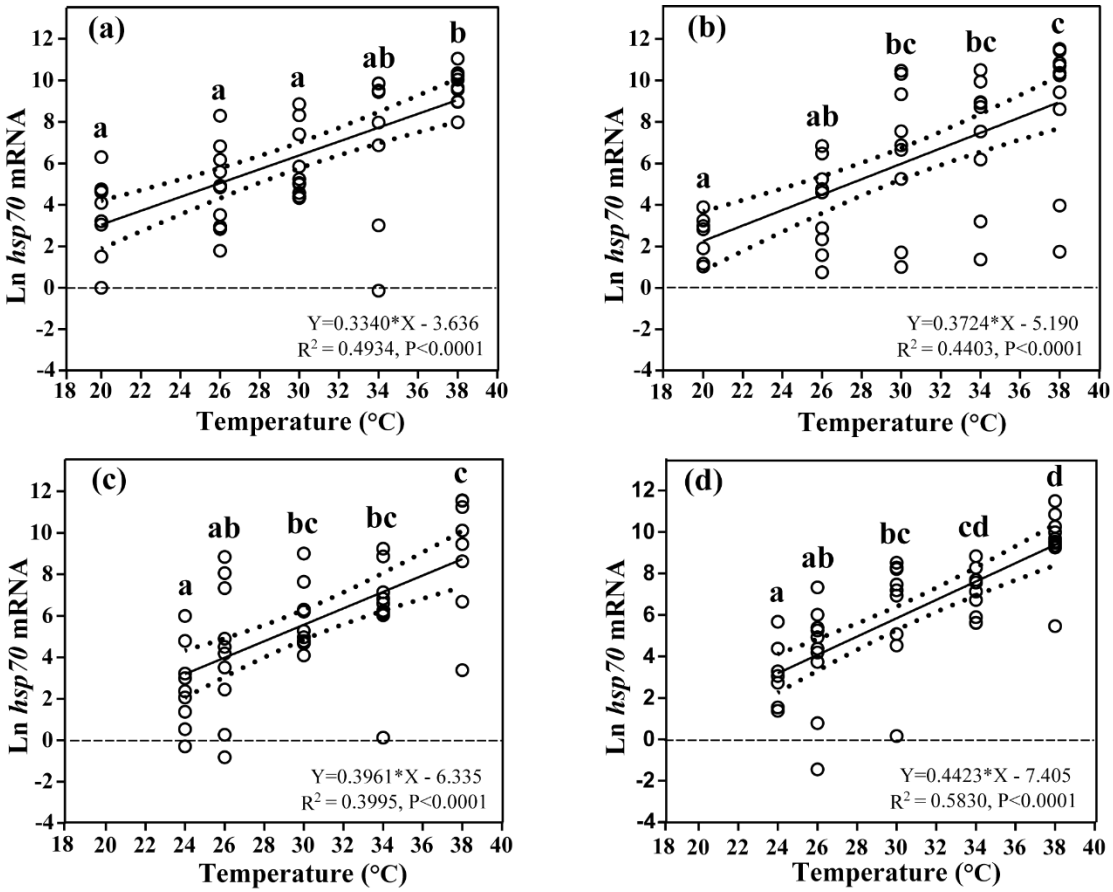


Figure 2. Temperature coefficients (Q10) of limpets acclimated at different temperatures (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). The temperature coefficient (Q10) values were calculated for all limpets using heart rate data from 24 to 33°C. Post-acclimation temperature sensitivity was 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. The calculation of post-acclimation Q_{10} is done for the mean response of all individuals as the same individual are not used at each acclimation temperature. Therefore, there was no calculation of variation or error for post-acclimation. Different letters represent significant differences in the Q_{10} among different acclimation treatments.



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523

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

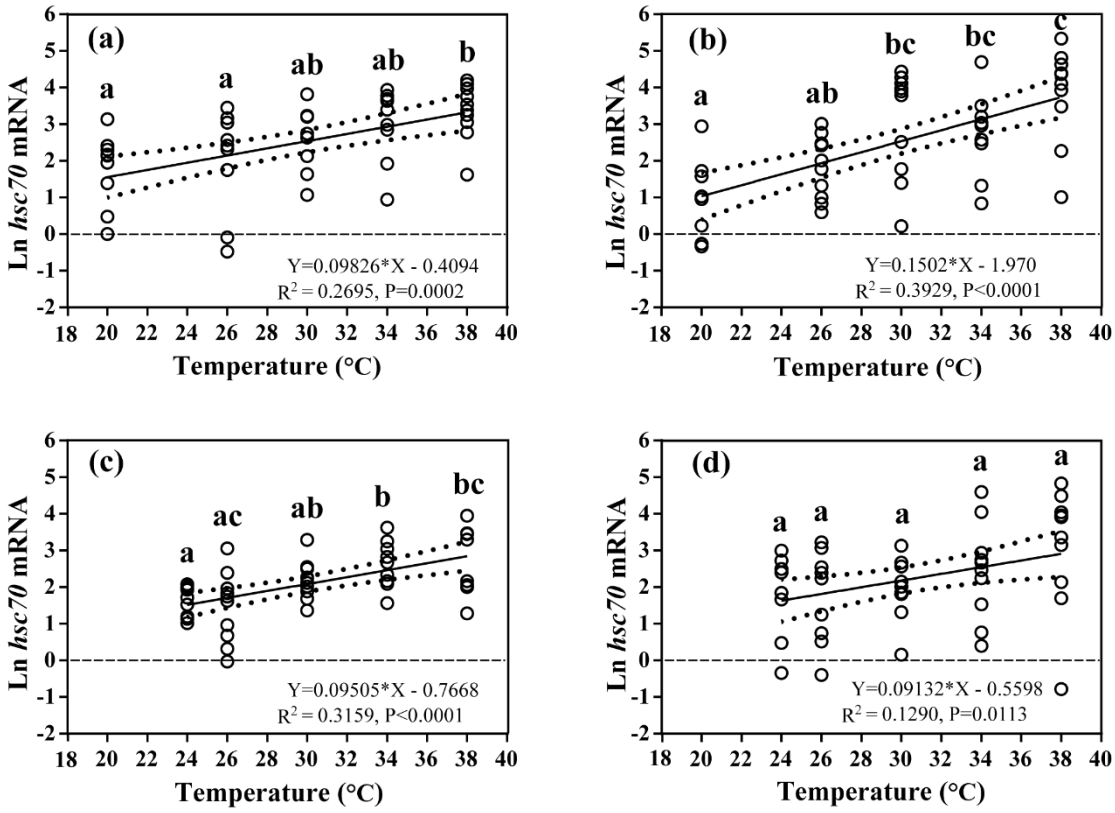
525 and 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

528 among different heat-shock temperatures.

529



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

533 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

536 among different heat-shock temperatures.

537

Appendix:

Figure A1. The photo of artificial rock (60 cm length × 30 cm width). Limpets were placed on artificial rock and heated to the designated temperate.

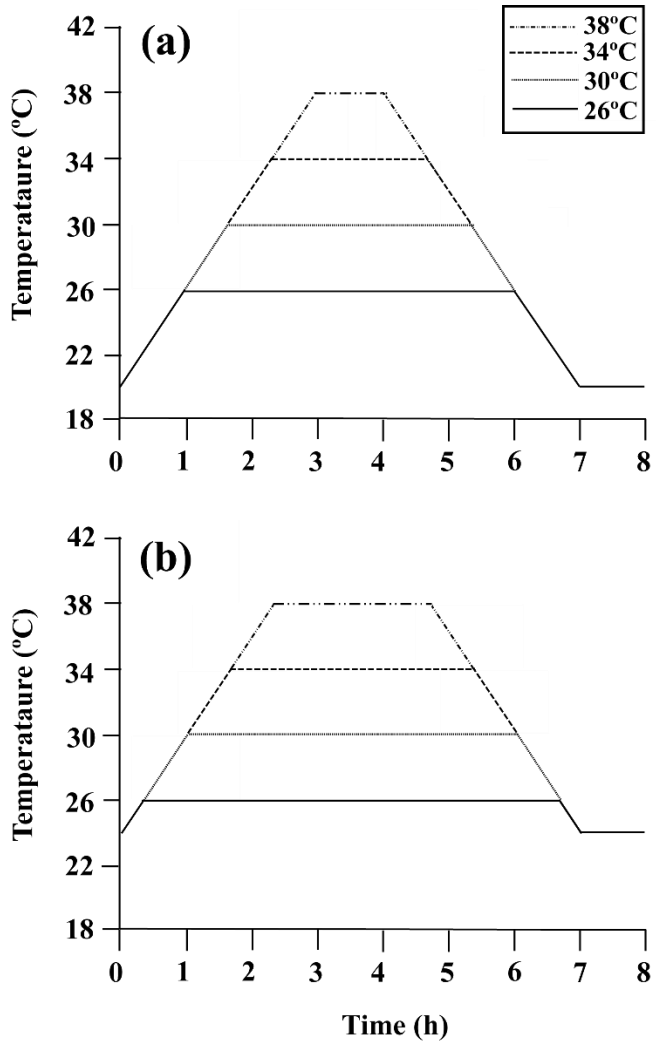
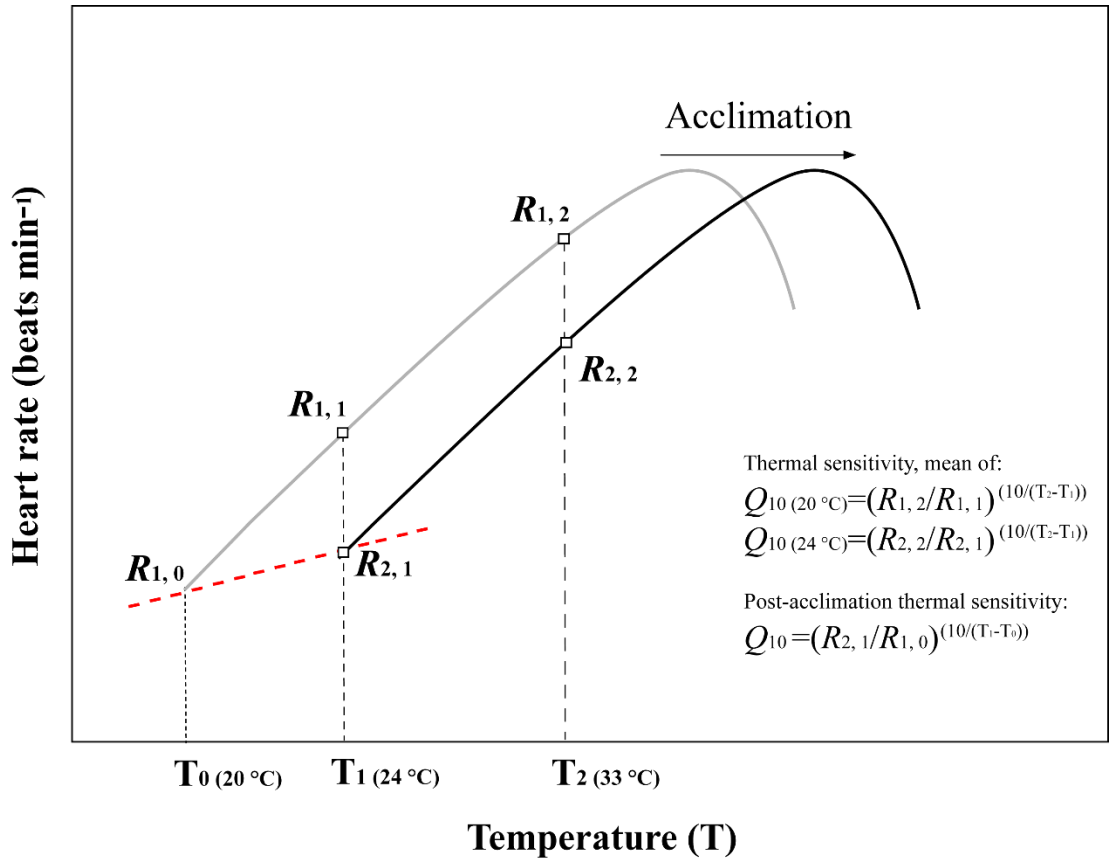


Figure A2. Diagram of the heating protocol for (a) limpets acclimated at 20 °C and (b) limpets acclimated at 24 °C. Limpets were heated at a rate of 6°C per hour from acclimation temperatures (20 or 24 °C) to designated temperatures (26, 30, 34 and 38 °C) for simulating a natural heating rate in summer. 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.



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553 **Figure A3.** Schematic diagram of temperature coefficients (Q_{10}) and post-acclimation Q_{10} calculations. This figure

554 was modified from Seebacher et al. (2015). Black line and grey line showed the heart rate of limpets at the warm-

555 acclimated temperature (24 °C) and the cold-acclimated temperature (20 °C), respectively. Q_{10} values for thermal

556 sensitivities were calculated from data for limpets kept at an acclimation treatment in which heart rate were measured

557 at two different temperatures. Q_{10} value for post-acclimation thermal sensitivities was calculated across two

558 temperature acclimation conditions under the same pCO_2 condition.

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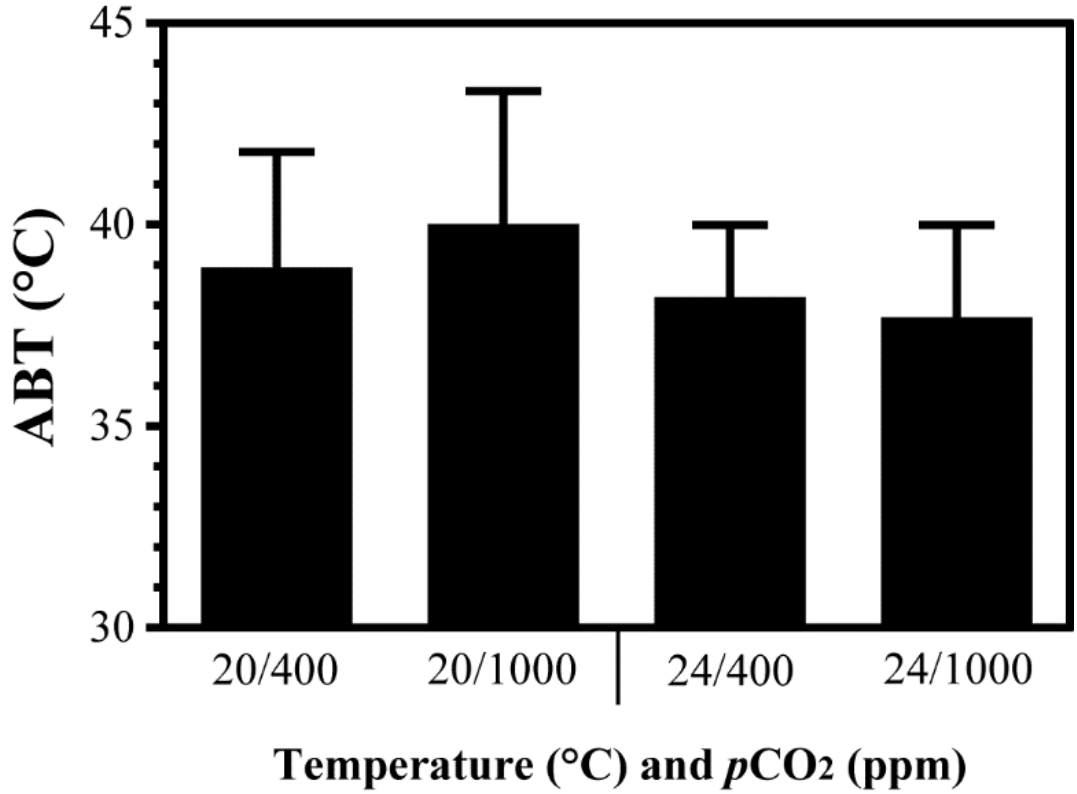

Figure A4. Arrhenius break point temperature of heart rate (ABT) of limpets acclimated at different temperatures (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). After acclimation in different conditions, limpets were heated continuously from acclimation temperatures to the heart stopped beating. During the heating process, heart rates were recorded and ABTs were calculated.

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

570 experimental period[1]

571

	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

572 [1]Seawater temperature, salinity, pH and total dissolved inorganic carbon (C_T) were monitored every 6 h. Total

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

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

575

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

577

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

578

579

580 **Table A3.** Inferential statistics for the most likely general additive mixed models (GAMM) of heart rate during

581 continuous warming of limpet *Cellana toreuma* acclimated at different temperatures (20 and 24 °C) and pCO_2 (400

582 and 1000 ppm)[1]

583

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**

584 [1]The generalized additive model describes heart rate as a function of temperature, or *f*(T), instead of using a fixed

585 parameter to describe the effect of temperature. Additional functions were included to describe how heart rates of *C.*

586 *toreuma* from each treatment deviated from those of *C. toreuma* from 20 °C and 400 ppm.

587

588 **Table A4.** Two-way ANOVA to investigate the effects of temperature (20 °C and 24 °C) and pCO_2 (400 ppm and

589 1000 ppm) on Arrhenius break point temperature of heart rate (ABT) and temperature coefficients (Q_{10}) on

590 *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		

591