College of Ocean and Earth Sciences Tel: 86-18659211278

State Key Laboratory of Marine Environmental Science,

Xiamen University, Xiamen, P. R. China Email: dongyw@xmu.edu.cn

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Dear Dr. Carol Robinson,

Thank you and Prof. Stephen Hawkins so much for your useful comments and suggestions for improving our manuscript, "*Ocean acidification increases the sensitivity and variability of physiological responses of an intertidal limpet to thermal stress*". We have addressed all of the reviewer's comments.

Please find more details below:

(1) our point-by-point response to the reviews
(2) a marked-up manuscript version

We feel lucky and honored that our revised paper will be judged acceptable for publication in *Biogeosciences* after minor revision.

Thanks for your assistance, I remain.

Sincerely,
Yun-wei Dong Ph. D
On behalf of all co-authors.

# (1) Point-by-point response to the reviews

We thank the referee for his positive review of this work. The comments really helped us to improve the manuscript.

## Reply to comments of Stephen J Hawkins (Referee) #4

*Q1: This is a very general readership journal. At first mention in the introduction clearly explain what "constitutive" means (perhaps in a bracket).*

**Response to Q1:** P. 5, L. 93. In the revised manuscript, a concise description has been added to explain "constitutive" at first mention in the introduction section. "… the constitutive Hsc70, which is transcribed continuously …)."

*Q2: L.31 "showed increased sensitivity" is better*

**Response to Q2:** P. 2, L. 32-33. This sentence has been changed to "These results suggested that limpets showed increased sensitivity and stress response …".

*Q3: L. 34 replace "acidic" with "reduced pH" - the pHs are above 7 - therefore alkaline... Introduction*

**Response to Q3:** P. 2, L. 35-36. This sentence has been modified to "While short-term acclimation to reduced pH seawater decreases the ability …".

*Q4: L. 50 "resistance of an organism" (try to avoid apostrophes with the possessive)*

**Response to Q4:** P. 3, L. 52. In the revised manuscript, we avoided using apostrophe with the possessive throughout the text. This sentence has been changed to "… reduce resistance of an organism to environmental change …".

*Q5: L. 53 I would contend these are "responses" not "options". Options implies conscious choice. Sessile and sedentary organisms do not "shift their ranges" - their ranges shift as individuals occur in different places. Highly mobile species may actually shift their ranges but not limpets. This sloppy wording (and I have done it too) permeates the climate change literature - species do not consciously shift their ranges - their ranges shift.*
*Suggested rephrase: Organisms can respond in three ways: exhibit shifts in distributional ranges (...), evolve adaptive changes (), or perish ().*

**Response to Q5:** P. 3, L. 55-57. We have rephrased this sentence according to your suggestion. "In the face of a changing environment, organisms can respond in three ways: exhibit shifts in distributional ranges (Parmesan and Yohe, 2003), develop adaptive changes (Hoffmann and Sgro, 2011), or perish (Fabricius et al., 2011)."

*Q6: L. 80 not a huge amount of space is saved by abbreviating Heart Rate - in full throughout? (I would not like confusion with Human Resources...)*

**Response to Q6:** We used heart rate to replace its abbreviation HR throughout the revised manuscript according to the comment.

*Q7: L. 83 ..... Breakpoint Temperature - start with capitals as a term.*

**Response to Q7:** P. 5, L. 84-85. This sentence has been changed to "… (i.e. Arrhenius Breakpoint Temperature, ABT) …".

*Q8: L. 100 ecosystems*

**Response to Q8:** P. 5, L. 103. It has been changed to "ecosystems".

*Q9: L. 104 rephrase - do you mean "subtropical high pressure systems" ???*

**Response to Q9:** P. 6, L. 107. Subtropical High refers to subtropical high pressure systems. This sentence has been modified to "Under the impact of subtropical high pressure systems, …".

*Q10: L. 107 from what pH to what pH*

**Response to Q10:** P. 6, L. 111-112. We have added the pH values and it was changed to "… have declined by 0.2 pH units from 8.05 in 1986 to 7.85 in 2012".

*Q11: L. 116 "show increased..."*

**Response to Q11:** P. 6, L. 118-119. This sentence has been changed according to suggestions.

*Q12: L. 118 "Our study, by measuring both heart rate and heats hock proteins,..."*

**Response to Q12:** P. 6, L. 121. This sentence has been rephrased according to the comment.

*Q13: L. 126 "ensured" not ensure and delete "is to"*

**Response to Q13:** P. 7, L. 129. We have rephrased this sentence according to the comment.

*Q14: L. 136,155 field not filed and check throughout.*

**Response to Q14:** Modified throughout the text.

*Q15: L. 187 "the foot muscle..."*

**Response to Q15:** P. 10, L. 190. We have modified this sentence.

*Q16: L. 247 - far too much code - in full at first mention for the different treatments to help the reader.*

**Response to Q16:** P. 7, L. 134-137. In the revised manuscript, we have spelt out the codes in full at first mention. "…in different $pCO_2$ concentrations and temperatures (LTLC, low temperature and low $CO_2$, 20 °C + 400 ppm, as a control treatment; LTHC, low temperature and high $CO_2$, 20 °C + 1000 ppm; HTLC, high temperature and low $CO_2$, 24 °C + 400 ppm; HTHC, high temperature and high $CO_2$, 24 °C + 1000 ppm) …".

*Q17: L. 276 ...condition: .... (replace comma with colon)*

**Response to Q17:** P. 14, L. 279. This sentence has been modified according to the comment.

*Q18: L. 301 Spend not cost*

**Response to Q18:** P. 15, L. 304. We have modified this sentence according to the comment.

*Q19: L. 302 Somero not Semero*

**Response to Q19:** P. 15, L. 305. Modified the spelling error.

*Q20: L. 355 combining not combing*

**Response to Q20:** P. 17, L. 358. This sentence has been revised according to the comment.

*Q21: L. 358 "the response of an organism..."*

**Response to Q21:** P. 18, L. 361. This sentence has been modified to "…we predict the response of an organism to …".

*Q22: L. Table A1 could go in the main text near the methods - this is important information.*

**Response to Q22:** P. 26. In the revised manuscript, Table A1 has been moved in the main text as Table 1.

*Q23: L. Figure 1A - it would be good to fit individual light lines for each individual limpet as well as the population average - this shows the individual variability and hence plasticity at the heart of the paper.*

**Response to Q23:** P. 28. We have modified Figure 1A to show the individual variability according to the comment.

**(2)**

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

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

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

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

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

**Corresponding to*: Yun-wei Dong (dongyw@xmu.edu.cn)

**Abstract.** Understanding physiological responses of organisms to warming and ocean acidification is the first step towards predicting the potential population- and community-level ecological impacts of these stressors. Increasingly, physiological plasticity is being recognized as important for organisms to adapt to the changing microclimates. Here, we evaluate the importance of physiological plasticity for coping with ocean acidification and elevated temperature, and its variability among individuals, of the intertidal limpet *Cellana toreuma* from the same population in Xiamen. Limpets were collected from shaded mid-intertidal rock surfaces. They were acclimated under combinations of different $pCO_2$ concentrations (400 ppm and 1000 ppm, corresponding to pH 8.1 and 7.8) and temperatures (20 °C and 24 °C) in a short-term period (7 days), with the control condition (20 °C and 400 ppm) representing the average annual temperature and present-day $pCO_2$ level at the collection site. Heart rates (as a proxy for metabolic performance) and expression of genes encoding inducible and constitutive heat-shock proteins (*hsp70* and *hsc70*) at different heat shock temperatures (26, 30, 34 and 38 °C) were measured. Hsp70 and Hsc70 play important roles in protecting cells from heat stresses, but have different expression patterns with Hsp70 significantly increased in expression during stress and Hsc70 constitutively expressed and only mildly induced during stress. Analysis of heart rate showed significantly higher temperature coefficients ($Q_{10}$ rates) for limpets at 20 °C than at 24 °C and post-acclimation thermal sensitivity of limpets at 400 ppm was lower than at 1000 ppm. Expression of *hsp70* linearly increased with the increasing heat-shock temperatures, with the largest slope occurring in limpets acclimated under a future scenario (24 °C and 1000 ppm $pCO_2$). These results suggested that limpets showed increased sensitivity and stress response under future conditions. Furthermore, the increased variation in physiological response under the future scenario indicated that some individuals have higher physiological plasticity to cope with these conditions. While short-term acclimation ~~at~~ to ~~acidic~~ reduced pH seawater decreases the ability of partial individuals against thermal stress, physiological plasticity and variability seem to be crucial in allowing some intertidal animals to survive in a rapidly changing environment.

## 1 Introduction

Benthic organisms living in the intertidal zone will be exposed to increasingly variable and extreme environmental conditions, such as temperature, oxygen and $CO_2$, due to climatic change (IPCC, 2013; Kwiatkowski et al., 2016). These highly fluctuating environmental variables can significantly affect the physiological performance of coastal species (Helmuth et al., 2006; Hofmann and Todgham, 2010; Somero, 2012; Widdicombe and Spicer, 2008). Therefore, understanding the interaction of multiple environmental stressors on the physiological performance is crucial for predicting the consequences of environmental change on ecosystems (Deutsch et al., 2015). For example, salinity fluctuations coupled with high temperatures during emersion can have both sub-lethal physiological effects and lethal effects on intertidal molluscs (Dong et al., 2014; Firth and Williams, 2009). Although ocean acidification can increase the growth of organisms in some cases (e.g. Gooding et al., 2009), there is increasing evidence that decreased pH exacerbates global warming, and interactions of ocean acidification and warming reduce resistance of an organism ~~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 can respond in three ways: ~~have three main options;~~ exhibit shifts in distributional ranges ~~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~~ heart rate under conditions which are not thermally stressful, and ~~HR~~ heart rate increases and

reaches a peak followed by a sudden decrease with temperature rising (Braby and Somero, 2006; Dong and Williams, 2011). The temperature at which a sharp discontinuity in slope occurs in an Arrhenius plot (i.e. Arrhenius ~~breakpoint~~ Breakpoint ~~temperature~~Temperature, ABT) can represent the limit of metabolic functioning of animals (Nickerson et al., 1989; Somero, 2002). At the molecular level, expression of heat shock proteins (Hsps) and *hsp* genes is induced above a certain temperature, reaches maximum and finally ceases in response to heat shock (Han et al., 2013; Miller et al., 2009). Upregulation of Hsps and *hsp* genes is an energy-consuming mechanism for defense against thermal stress (Somero et al., 2016). As a commonly used biomarker, the Hsp70 multigenic family includes two proteins with divergent expression patterns (inducible Hsp70 and constitutive Hsc70). The inducible Hsp70 significantly increases in expression when animals are exposed to stressors and plays a role in maintaining protein stability (Feder and Hofmann, 1999~~). .~~); on the other hand, the constitutive Hsc70, which is ~~constitutively expressed~~transcribed continuously and may be mildly induced during stress, takes part in folding and repairing of denatured proteins (Dong et al., 2015) and plays a role in the formation of mitotic structures (Sconzo et al., 1999). Some studies have shown coordinated ~~HR~~ heart rate and expression of genes encoding to Hsps in response to elevated temperate (Han et al., 2013; Prusina et al., 2014). However, little is known about the patterns of heart rate and expression of *hsp* genes for coping with combined warming and ocean acidification.

The limpet *C. toreuma* is a keystone species on rocky shores in the western Pacific (Dong et al., 2012), occupying the mid–low intertidal zones (Morton and Morton, 1983). This species is a gonochoric and broadcast spawner, whose embryos develop into planktonic trocophore larvae and later into juvenile veligers before becoming fully grown adults (Ruppert et al., 2004). As a common calcifier inhabiting coastal ecosystems, *C. toreuma* plays an important ecological role in food chains, gazing on biofilm and

being an important food source for other species (e.g. crabs, sea birds and sea stars). Therefore, this species is a key organism for studying the relationship between physiological response to thermal stress and ocean acidification in highly variable environment on the shore.

Under the impact of subtropical high pressure systems~~Subtropical High~~, Xiamen (118°14′ E, 24°42′ N) is one of the hottest areas in China (Dong et al. 2017). The coastal seawater of this area is experiencing rapid temperature rise and acidification (Bao and Ren, 2014). The sea surface temperature (SST) in Xiamen coastal water has increased a total of 1 °C since 1960, and is rising at a mean annual rate of 0.02 °C (Yan et al., 2016). The annual pH values of seawater in Xiamen Bay have declined by 0.2 pH units from 8.05 in 1986 to 7.85 in 2012, a trend which is predicted to continue based on simulations (Cai et al., 2016).

Here, we investigated the importance of physiological plasticity (based on the measurement of post-acclimation temperature sensitivity; see Seebacher et al., 2015) and variability (based on coefficient of variation) for *C. toreuma* to cope with ocean acidification and elevated temperatures by quantifying heart rates (as a proxy of metabolic performance) and expression of genes encoding inducible and constitutive heat-shock proteins (Hsp70 and Hsc70) after short-term acclimation in different $pCO_2$ concentrations (400 ppm and 1000 ppm) and temperatures (20 °C and 24 °C). We hypothesize that (1) limpets will show increased ~~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~~ Our study, by measuring both heart rate and heat shock protein gene expression, provides novel information concerning the combined effects of increased temperature and $pCO_2$ on stress response, energy consumption and physiological plasticity in intertidal invertebrates, potentially providing predications of the ecological impacts of the future environmental changes.

## 2 Material and Methods

### 2.1 Limpet collection and experiment treatments

Samples were collected from shaded rock surfaces at mid-tidal level in Xiamen on a falling high tide in July (*in situ* temperature: 30.8 ± 0.8 °C). The sampling ~~is to~~ ensured that all limpets have similar thermal history, given the possible impacts from microclimate (Dong et al., 2017; Lathlean and Seuront, 2014). They were transported to the State Key Laboratory of Marine Environmental Science, Xiamen University, China within 2 h. Limpets were firstly allowed to recover at 20 °C for 3 d with a tidal cycle of approximately 6 h immersion and 6 h emersion. These limpets were randomly allocated into one of four treatments and temporally acclimated in different $pCO_2$ concentrations and temperatures (LTLC, low temperature and low $CO_2$, 20 °C + 400 ppm, as a control treatment; LTHC, low temperature and high $CO_2$, 20 °C + 1000 ppm; HTLC, high temperature and low $CO_2$, 24 °C + 400 ppm; HTHC, high temperature and high $CO_2$, 24 °C + 1000 ppm) for 7 d in climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which control both the *p*CO2 concentration and temperature under the same relative humidity and light intensity conditions. In each acclimation treatment, approximately 100 limpets were randomly allocated in ~ 30 containers (3 individuals in each container), to simulate ~~filed~~ field densities of ~ 1 limpet per 10 $cm^2$. Control conditions (20 °C, 400 ppm) represent the average annual temperature and ambient $pCO_2$ (~ 390 ppm) at the collection site, with high temperature (24 °C) and $pCO_2$ (1000 ppm) representing the average global increase (4 °C, 600 ppm) predicted for 2100 by the Intergovernmental Panel on Climate Change (IPCC, 2007).

Animals were kept in a simulated tidal cycle with 6 h aerial exposure and 6 h seawater immersion.

Seawater was pre-bubbled with air containing the corresponding $pCO_2$ concentrations in advance. pH was measured before and after the acclimation in seawater each time with PB-10 pH meter (Sartorius 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 dissolved inorganic carbon analyzer (As-C3, Apollo SciTech, Colorado, USA), using a Li-Cor® non-dispersive infrared detector (Li-6252) with a precision of 0.1% (Cai, 2003). Seawater carbonate chemistry parameters were estimated based on the measured values of pH, 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. (1990) and Lee et al. (2010) respectively. The information of the measured and calculated seawater chemistry parameters is summarized (Table ~~A~~1).

After a 7-day acclimation period (crossed $pCO_2$ × Temperature treatments, above), the heat-shock treatments were carried out to simulate the gradual temperature exposure of limpets in the ~~filed~~ field as described in Denny et al. (2006) (Fig. A1). For each heat-shock treatment, 10 limpets were randomly selected from each of four acclimation conditions (40 indiv. total) and transferred to artificial rocks (Fig. A2), with individuals from LTLC and LTHC on one rock and individuals from HTLC and HTHC on another rock. The artificial rocks were separately placed in 20 °C water baths and 24 °C water baths, and heated at a rate of 6 °C per hour that simulated emersion in the natural condition at the collection site (Han et al., 2013) to the designated temperatures (26, 30, 34 and 38 °C). After achieving the target temperature, the temperature was maintained for the allotted time, and then decreased to the acclimation temperature (20 or 24 °C) at a rate of 6 °C per hour, for a total exposure time of 7 h. Individuals from all

four acclimation conditions (n = 10 indiv. per treatment) were randomly selected, transferred to artificial rocks and aerially exposed at 20 or 24 °C for 7 h, as non-heated control samples. After recovery at 20 or 24 °C seawater for 1 h, limpets were immediately collected and stored at -80 °C for gene expression analysis.

### 2.2 Cardiac performance measurement

The cardiac performance of limpets was recorded during whole heating processes from the acclimated temperature (20 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. The containers were immersed in water baths, allowing the temperature in the container to be increased at a rate of 6 °C per hour that simulated emersion in the natural environment. 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. Variations in the light-dependent current produced by the heartbeat were amplified, filtered and recorded using an infrared signal amplifier (AMP03, Newshift, Leiria, Portugal) and Powerlab AD converter (4/30, ADInstruments, March-Hugstetten, Germany). Data were viewed and analyzed using Lab Chart (version 7.0).

For determining the Arrhenius ~~breakpoint~~ Breakpoint ~~temperatures~~ Temperature (ABT) 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; the foot muscle was cut off immediately using RNA-free scissors (180 °C before using); the muscle (~ 50 mg) was cut into pieces in a 1.5 ml EP tube containing RNA lysis buffer provided by Eastep reagent kit (Promega, USA); total RNA was isolated using Eastep reagent kit (Promega, USA). The first strand of cDNA was synthesized using total RNA as a template. Reverse transcriptase (RT) reactions were performed using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Shiga, Japan).

The levels of mRNA of genes encoding two heats hock proteins, inducible heat-shock protein 70 (*hsp70*) and constitutive heat shock protein 70 (*hsc70*), were measured using real-time quantitative PCRs in CFX96™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules CA, USA) followed the methods described by Han et al. (2013) with specific primers (Table ~~A2~~A1). For normalizing expression of genes, we examined expression of *18S ribosomal RNA*, *β-actin*, *β-tubulin* genes, which typically have relatively stable expression levels. The expression stability of these housekeeping genes was evaluated using the GeNorm Algorithm (Primer Design, Ltd., Southampton University, Highfield Campus, Southampton Hants, UK) as described by Etschmann et al. (2006). Based on the expression stability measures (M values), all the three genes were selected as the reference genes for normalizing the level of expression of stress-induced genes. All samples were measured in triplicates. Ct (dR) values were analyzed using the CFX Manager™ Software Version 3.0 (Bio-Rad). The expression of *hsp70* and *hsc70* was determined relative to the value of *18S*, *β-actin* and *β-tublin* from a reference individual.

213 **2.4 Statistical analysis**

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

220 Thermal sensitivity is the change in a physiological rate function reacting to a rapid change in
221 environmental temperature within the same acclimation set temperature (Fig. A3, modified from
222 Seebacher et al. (2015)). In the present study, thermal sensitivity was determined in the temperature
223 coefficient ($Q_{10}$) values of heart rate. $Q_{10}$ was calculated using heart-rate data from the temperature at
224 which the experiment started ($T_1$ = 24 °C) to the temperature to which temperature increased 10 °C ($T_2$
225 = 33 °C) with Eq. (1):

226 $$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \quad (1)$$

227 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
228 (Kelvin) (Fig. A3, modified from Seebacher et al. (2015)). The differences in $Q_{10}$ among the four
229 acclimation conditions with different $CO_2$ concentrations (400 ppm vs. 1000 ppm) and temperatures
230 (20 °C vs. 24 °C) were analyzed using two-way ANOVA with Duncan's *post hoc* analysis using the SPSS
231 20.0 for Windows statistical package (IBM SPSS Statistics, Chicago, USA). Post-acclimation thermal
232 sensitivity of limpets in different $CO_2$ concentrations were calculated as described by Seebacher et al.
233 (2015). In each $CO_2$ concentration (400 ppm or 1000 ppm), the post-acclimation $Q_{10}$ values were
234 calculated using the same equation as shown above, but $R_2$ was the average heart rate of the warm-

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

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

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

## 3 Results

### 3.1 Cardiac performance

The maximal heart rate was ~ 30 % higher in limpets acclimated to control conditions (20 °C, 400 ppm) than the other treatments (Fig. 1 and Table ~~A3~~A2). The ABTs of limpets showed a trend to be reduced for ~~HT~~ high temperature treatments (mean ± SD: LTLC, 38.9 ± 2.9 °C; HTLC, 38.2 ± 1.8 °C; LTHC, 40.0 ± 3.3 °C; HTHC, 37.7 ± 2.3 °C) (Fig. A4). Temperature (Two-way ANOVA, $F_{1, 35}$ = 3.375,

P = 0.075) and $pCO_2$ (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~~A3; Fig. A4).

Temperature coefficients ($Q_{10}$ rates) were higher for limpets acclimated at 20 °C than at 24 °C (Two-way ANOVA, $F_{1, 35} = 5.878$, P = 0.02), but there was no significant difference for acclimation to different $pCO_2$ 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~~A3; Fig. 2). The post-acclimation thermal sensitivity of limpets acclimated at low $CO_2$ (2.12) was lower than that of limpets at high $CO_2$ (2.95) (Fig. 2).

The coefficients of variations (CV) of ABT in the four different acclimation conditions were different (Table ~~1~~2). 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 $Q_{10}$ under LTHC condition was the highest in all the four acclimation conditions (Table ~~1~~2).

### 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~~ condition 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 $CO_2$

277 concentration.

278 The responses of *hsc70* mRNA to heat shock were divergent among the four acclimation conditions
279 (Fig. 4). For HTHC limpets, there were no significant differences among different heat shock
280 temperatures ($F_{4, 42} = 2.11$, $P = 0.096$). For LTLC, LTHC and HTLC limpets, levels of *hsc70* mRNA after
281 being heat-shocked at 38°C were higher than corresponding levels of *hsc70* mRNA at 20 °C or 24 °C
282 (Duncan's *post hoc* analysis, $F_{4, 42} = 4.389$, $P = 0.005$; $F_{4, 44} = 8.521$, $P < 0.0001$; $F_{4, 42} = 5.713$, $P = 0.001$).
283 The coefficients of variation of *hsc* mRNA after heat shock of 38°C were different among different
284 acclimation conditions~~,~~: HTHC (90.36%) > LTHC (80.44%) ≈ HCLT (80.12%) > LCLT (56.20%) (Table
285 ~~1~~2).
286

287 **4 Discussion**

288 Short-term acclimation at elevated temperature and $pCO_2$ can increase physiological sensitivity of
289 limpets to thermal stress. The higher thermal sensitivity of limpets acclimated to 1000 ppm indicates that
290 the resilience of limpets to thermal stress associated with warming will be compromised under future
291 ocean acidification. This prediction is contrary to the general thought that intertidal ectotherms, such as
292 limpets and other gastropods, will demonstrate high tolerance to thermal stress because they are adapted
293 to an extreme thermal environment. For example, the operative temperatures, which *C. toreuma* suffers
294 in the field, frequently exceed 40 °C in summer along Asian coastlines and the limpet can survive at
295 temperatures in excess of 45 °C (Dong et al., 2015). Our data show, however, that ocean acidification
296 will lead to increased sensitivity to changes to future thermal regimes, indicating a synergistic negative
297 effect. The change in the metabolic partitioning in individuals could ultimately lead to a decline in fitness
298 and population-level responses in the future.

Increased temperature and $CO_2$ elevated the sensitivity of heat shock responses to thermal stress. The expression of inducible *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 protein plays crucial roles in maintaining protein stability with the expense of a large amount of energy (Feder and Hofmann, 1999; Tomanek and Sanford, 2003). By comparing the expression patterns of Hsp70 of different *Chlorostoma* species (formerly *Tegula*) that have distinct vertical 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 zone ~~cost~~ spend more energy for proteostasis and restore proteostasis to cope with a second consecutive day of high temperatures (Somero et al., 2016). Usually, the expression of Hsp70 of less thermal-tolerant species is more sensitive to increases in temperature (limpet *Lottia*, Dong et al., 2008; snail *Chlorostoma*, Tomanek, 2002), and the rapid upregulation of *hsp70* mRNA in limpets exposed to future conditions potentially represents a high sensitivity of limpets to thermal stress in the face of ocean acidification. Due to the expensive energy consumption during the synthesis and function of *hsp70*, the more rapid upregulation of *hsp70* mRNA in these limpets also indicates more energy was allocated into cellular homeostasis, which then can affect the ~~limpet's~~ growth and reproduction of limpets.

The expression patterns of constitutive *hsc70* mRNA were different among limpets acclimated at the four acclimation conditions. Hsc70 is constitutively expressed and is a molecular chaperone involved in the *in vivo* folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain

similar promoter regions, there are differential expressions to a given stimulus between them (Hansen et al., 1991). Some studies showed that thermal stress could significantly induce the 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 changes but also underpin evolutionary and adaptive changes through selective pressures (Franks and Hoffmann, 2012). In the present study, the coefficients

of variation in physiological responses of limpets acclimated to~~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 combining both abrupt and gradual exposure may offer insight into how intertidal species respond to climate change

and extreme weather events in the future. Further, since our findings are based on static experimental conditions, the results should be treated with caution when we predict ~~organism's~~ the response of an organism to future climate change in the highly variable natural environment. Therefore, future studies with long-term acclimation, larger sample size, and variable treatment conditions are recommended in order to validate our findings.

In conclusion, the resilience of intertidal limpets to thermal stress is weakened after exposure to predicted future conditions for a short-term acclimation period (7 d). Yet, the combination of elevated temperature and $CO_2$ concentration prompted divergence of physiological and molecular responses. These results suggest that while organisms may be able to protect themselves from the damaging effects of thermal stress in the short-term, changes to multiple environmental conditions may drive population-level responses through physiological responses (e.g. Giomi et al., 2016). Further, the increased variation in responses, and the observation that some individuals were more capable to physiologically cope with the conditions, may be associated with intergenerational adaptation, but this speculation needs further evidence. As the "weaker" individuals are lost, the offspring in the next generation will be better physiologically adapted to warming under high-$CO_2$ conditions. Therefore, while elevated $CO_2$ and the associated ocean acidification decrease the ability of many individuals to respond to thermal stress, it appears that physiological plasticity and variability could be adaptive mechanisms in at least some populations of intertidal organisms. Our research underlines the importance of physiological plasticity and variability for coastal species coping with warming and ocean acidification.

**Authors' contributions**

B.D.R and Y.-W.D. designed experiments. W.J. and M.-W.D. conducted experiments. Y.-W.D., B.D.R,

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

**Competing interests**

The authors declare no conflict of interests.

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

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

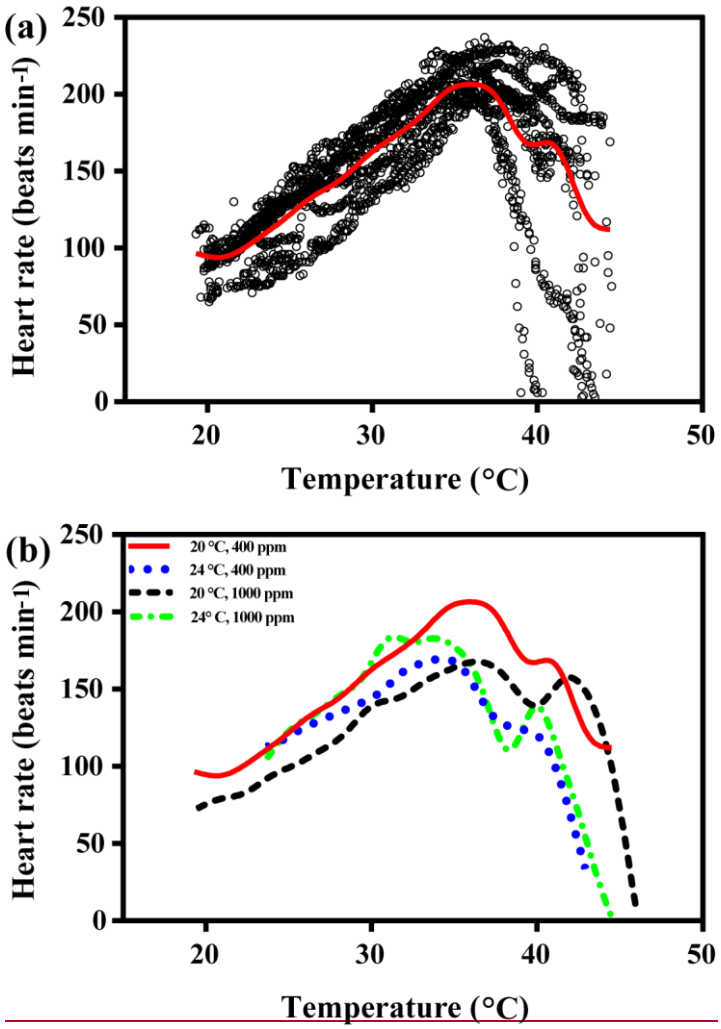
[1]Seawater temperature, salinity, pH and total dissolved inorganic carbon ($C_T$) were monitored every 6 h. Total alkalinity ($A_T$), $pCO_2$, $CO_3^{2-}$ and $\Omega_{cal}$ were calculated using CO2SYS software. Results were pooled and averaged over sampling times. Values are given as mean ± SD.

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

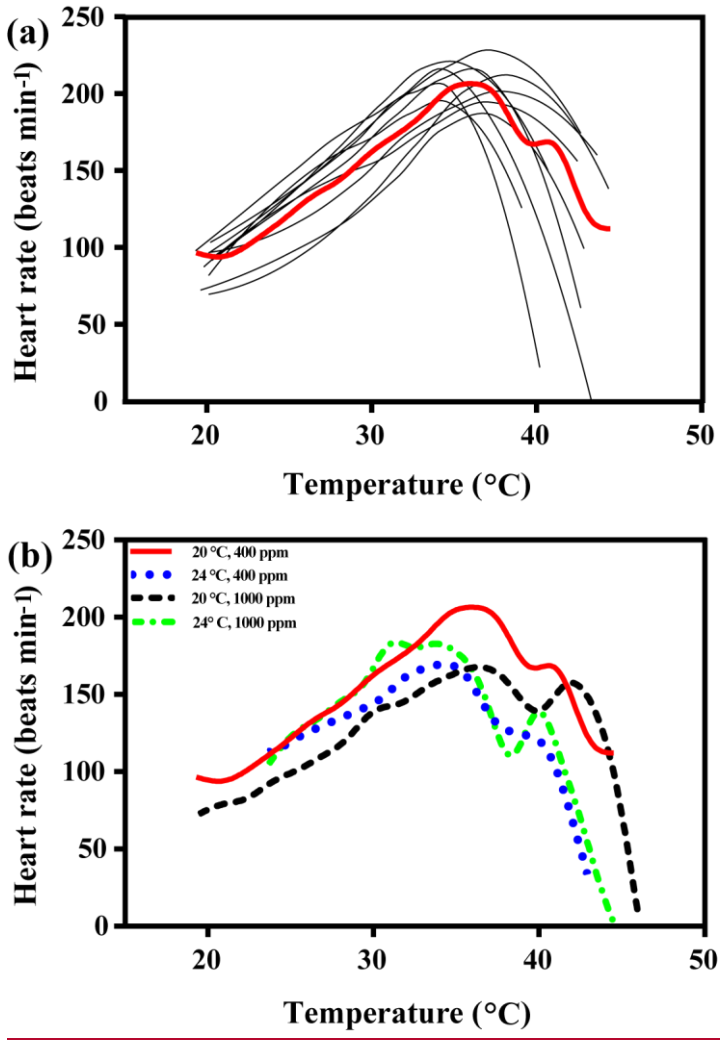
| Temperature | $CO_2$ | ABT | $Q_{10}$ | *hsc70* mRNA |
|---|---|---|---|---|
| 20 | 400 | 7.34 | 10.23 | 56.20 |
| | 1000 | 8.22 | 15.08 | 80.44 |
| 24 | 400 | 4.48 | 10.08 | 80.12 |
| | 1000 | 6.08 | 11.82 | 90.36 |

[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.


(a)
Heart rate (beats min-1)
250
200
150
100
50
0
20
30
40
50
Temperature (°C)
(b)
Heart rate (beats min-1)
20 °C, 400 ppm
24 °C, 400 ppm
20 °C, 1000 ppm
24° C, 1000 ppm
250
200
150
100
50
0
20
30
40
50
Temperature (°C)

595

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597

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

599 calculation for limpets in all treatments. The black lines correspond to smoothed fits (using the loess algorithm) of

600 heart rates for each of the individual limpets. The red line represents the most likely general additive mixed model

601 (GAMM) to depict the trajectory of heart~~s~~ rates for limpets with increasing temperature; (b) GAMM lines of limpets

602 acclimated at the different experimental temperature and $CO_2$ conditions.

603

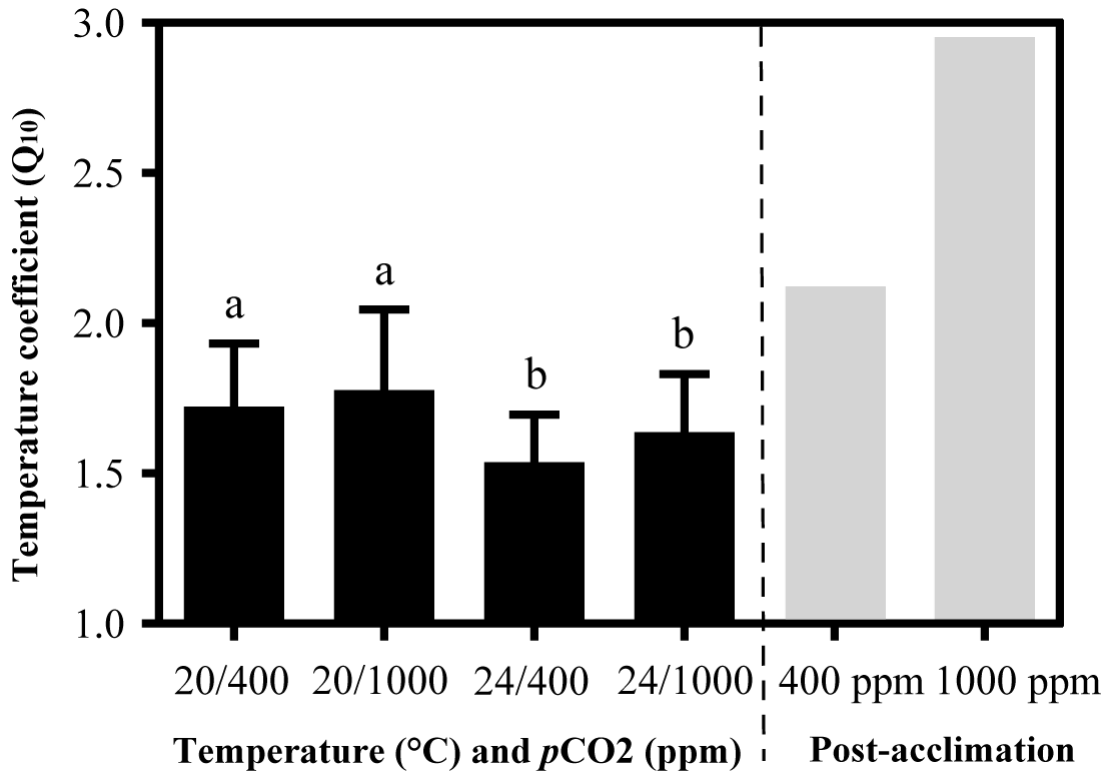
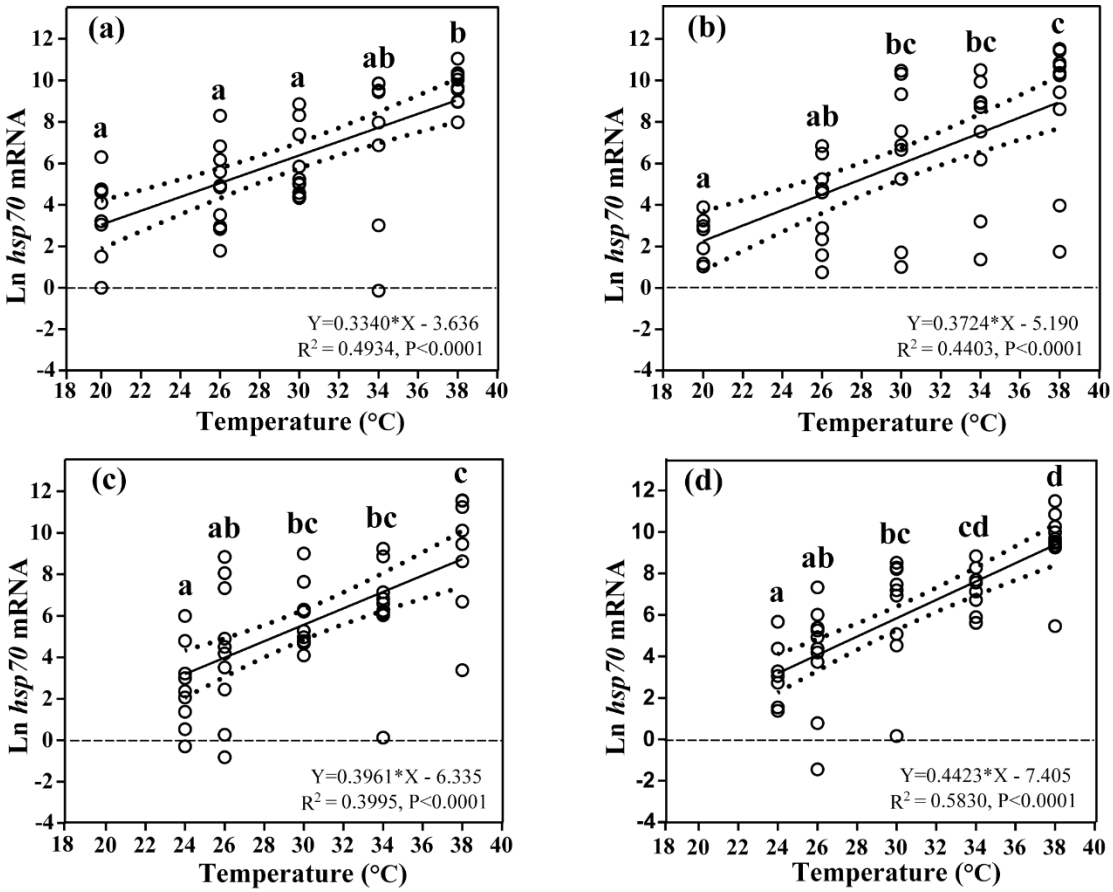



**Figure 2.** Temperature coefficients ($Q_{10}$) of limpets acclimated at different temperatures (20 or 24 °C) and $CO_2$ concentrations (400 or 1000 ppm). The temperature coefficient ($Q_{10}$) values were calculated for all limpets using heart rate data from 24 to 33°C. Post-acclimation temperature sensitivity was calculated 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, it is not possible to calculate an estimate of variation or error for post-acclimation $Q_{10}$. Different letters represent significant differences in the $Q_{10}$ among different acclimation treatments.




615

616

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

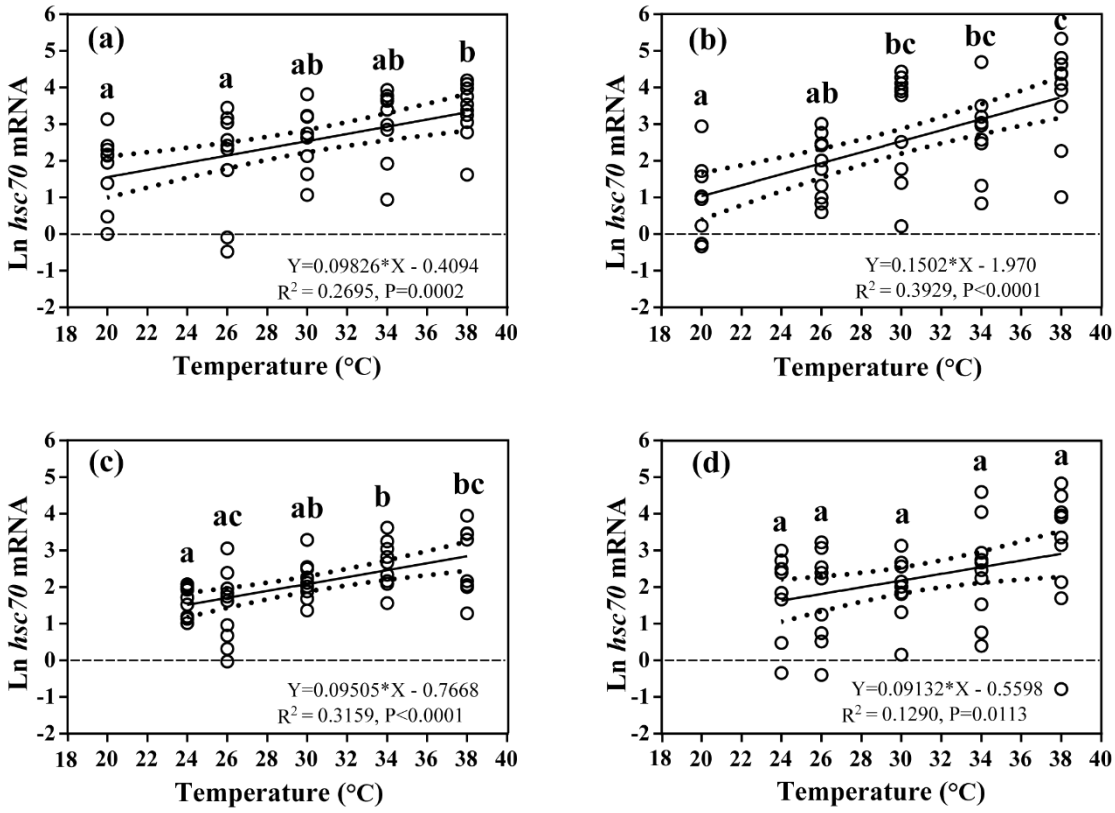
618 and 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

621 among different heat-shock temperatures.

622




623

624

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

626 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

629 among different heat-shock temperatures.

630

**Appendix:**

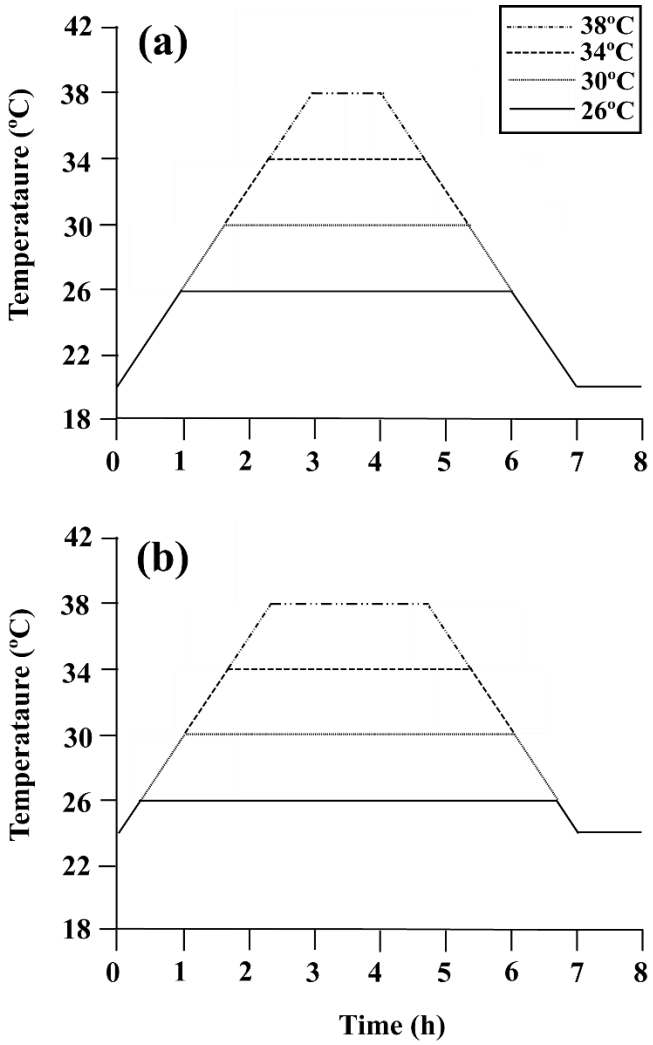



**Figure A1.** Diagram of the heating protocol for (a) limpets acclimated at 20 °C and (b) limpets acclimated at 24 °C. Limpets were heated at a rate of 6°C per hour from acclimation temperatures (20 or 24 °C) to designated temperatures (26, 30, 34 and 38 °C) for simulating a natural heating rate in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

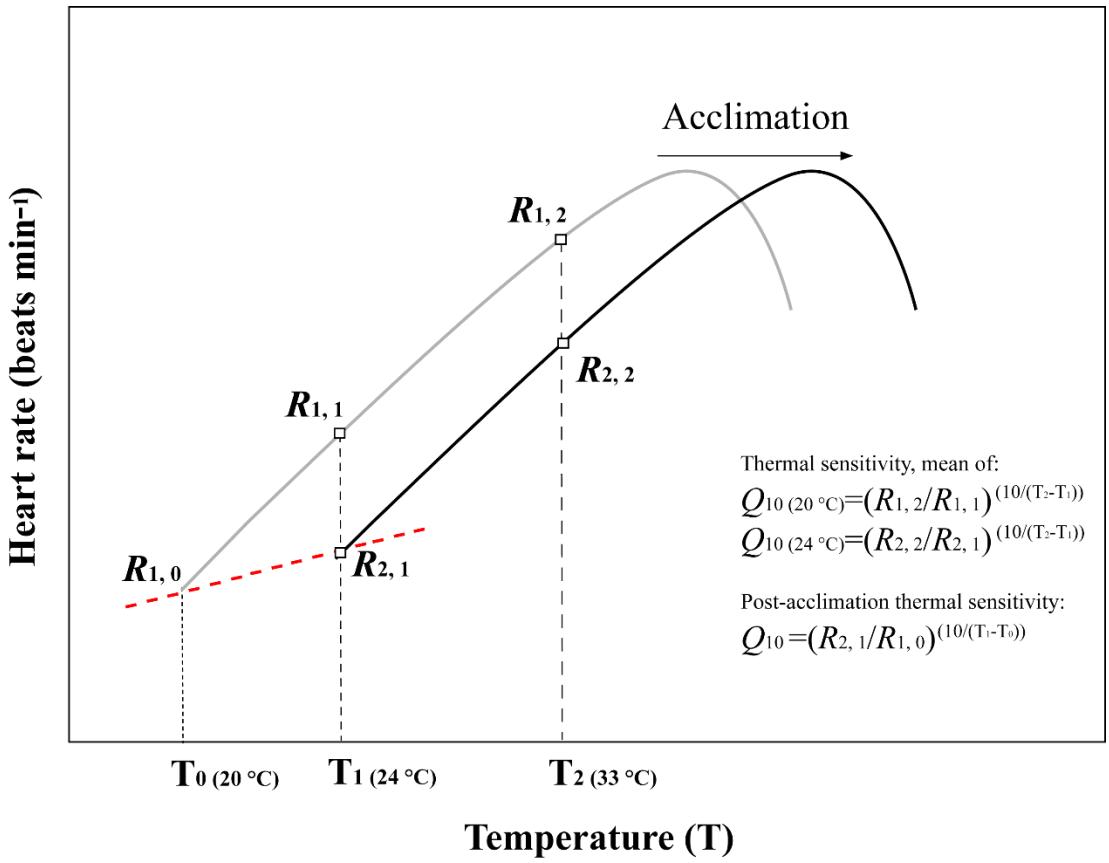
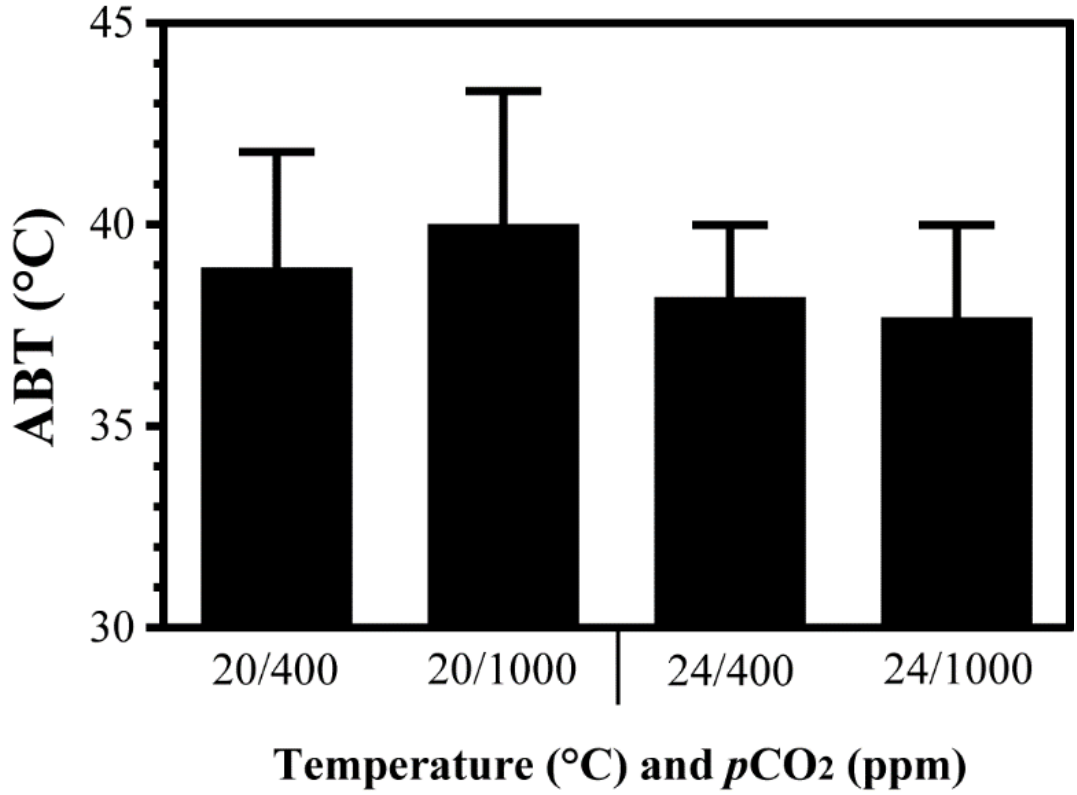



**Figure A3.** Schematic diagram of temperature coefficients ($Q_{10}$) and post-acclimation $Q_{10}$ calculations. This figure was modified from Seebacher et al. (2015). Black line and grey line showed the heart rate of limpets from the warm-acclimated temperature (24 °C) and the cold-acclimated temperature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




656

657

658 **Figure A4.** Arrhenius ~~breakpoint~~ Breakpoint ~~temperature~~ Temperature (ABT) of heart rate ~~(ABT)~~ of limpets

659 acclimated at different temperatures (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). After acclimation in

660 different conditions, limpets were heated continuously from acclimation temperatures to the heart stopped beating.

661 During the heating process, heart rates were recorded and ABTs were calculated.

662

663

**Table ~~A2~~A1.** Functions and primers of selected genes of *Cellana* limpet

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

668 **Table ~~A3~~A2.** Inferential statistics for the most likely general additive mixed models (GAMM) of heart rate during

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

670 and 1000 ppm)[1]

671

| 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** |

672 [1]The generalized additive model describes heart rate as a function of temperature, or *f*(T), instead of using a fixed

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

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

675

**Table ~~A4~~A3.** Two-way ANOVA to investigate the effects of temperature (20 °C and 24 °C) and $pCO_2$ (400 ppm and 1000 ppm) on Arrhenius ~~breakpoint~~ Breakpoint ~~temperature~~ Temperature (ABT) 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 | | |