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Dear Carol Robinson,

Thank you and the reviewers 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 and feel that they have substantially improved the manuscript.

Please find more details below:

- (1) our point-by-point response to the reviews
- (2) a list of all relevant changes made in the manuscript
- (3) a marked-up manuscript version

Thank you very much for your attention and consideration.

Sincerely,

Yun-wei Dong Ph. D

On behalf of all co-authors.

(1)

Point-by-point response to the reviews

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

For clarity, we keep the review's comments in blue and italic while our response is in black font.

Reply to comments of M. Byrne (Referee) #1

Q1: This manuscript by Wang et al is an interesting study of the impact of climate change stressors on limpets. Several aspects still to be revised. For instance, the significance of doing both the inducible and constitutive forms of HSP needs to be explained in the introduction. Most readers will not appreciate the difference in the two HSPs and what to expect with regard to their expression. What have other studies found? Interestingly the ramping method to assess HSP inducible is different from other more typical 1 hr shock, 1 hr recovery 'heat shock' studies. From a comparative perspective this is important to consider in the discussion.

Response to Q1: Thank you for your constructive suggestions. Responses to your comments were listed as follows:

(1) P. 5, L. 84-92. Generally, expression patterns of inducible and constitutive Hsps are different and the expression is an energy-consuming way of defending thermal stress. Therefore, we underlined the significance of studying both inducible and constitutive *hsps*. We have provided a brief introduction about HSP in the introduction, including its forms and functions in defending thermal stress.

“At the molecular level, expression of heat shock proteins (Hsps) and *hsp* genes is induced above a certain temperature, reaches maximum and finally ceases in response to heat shock (Han et al., 2013; Miller et al., 2009). Upregulation of Hsps and *hsp* genes is an energy-consuming mechanism for defense against thermal stress (Somero et al., 2016). As a commonly used biomarker, the Hsp70 multigenic family includes two proteins with divergent expression patterns (inducible Hsp70 and constitutive Hsc70). Hsp70 significantly increases in expression when animals are exposed to stressors and plays a role in maintaining protein stability (Feder and Hofmann, 1999). Hsc70, which is constitutively expressed and may be mildly induced during stress, takes part in folding and repair of denatured proteins (Dong et al., 2015).”

(2) P. 17, L. 347-357. We have added a paragraph in the discussion to compare expression patterns of *hsp70* under abrupt exposure and gradual exposure (two possible exposure scenarios experienced by intertidal limpets, suggested by Denny et al., 2006) and stated the importance of the present study in predicting how animals will cope with prolonged aerial exposure during low tide.

“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.”

Q2: I think the authors may have under sold their work. I do not think that heart beat and both HSPs have been investigated previously as combined response variables in warming-acidification studies. The authors should clearly state what is novel about this study.

Response to Q2: Cardiac responses and heat shock responses are commonly measured physiological responses to climate change (Somero et al., 2016). Although some studies have shown coordinated heart rate and expression of genes encoding to Hsps in response to elevated temperature (Han et al., 2013; Prusina et al., 2014), little is known about the patterns of heart rate and expression of *hsp* genes for coping with warming and ocean acidification. The present study provided insight into combined effects of increased temperature and $p\text{CO}_2$ on stress response, energy consumption and physiological plasticity in intertidal invertebrates by measuring both heart rate and expression of *hsp* genes.

P. 4-5, L. 78-95. “Heart rate (HR), as a measure of cardiac activity, is a useful indicator for indicating physiological response to stress in molluscs (Dong and Williams, 2011; Xing et al., 2016). Animals exhibit a stable basal HR under conditions which are not thermally stressful, and HR increases and reaches a peak followed by a sudden decrease with temperature rising (Braby and Somero, 2006; Dong and Williams, 2011). The temperature at which a sharp discontinuity in slope occurs in an Arrhenius plot (i.e. Arrhenius breakpoint temperature, ABT) can represent the limit of metabolic functioning of animals (Nickerson et al., 1989; Somero, 2002). At the molecular level, expression of heat shock proteins (Hsps) and *hsp* genes is induced above a certain temperature, reaches maximum and finally ceases in response to heat shock (Han et al., 2013; Miller et al., 2009). Upregulation of Hsps and *hsp* genes is an energy-consuming mechanism for defense against thermal stress (Somero et al., 2016). As a commonly used biomarker, the Hsp70 multigenic family includes two proteins with divergent expression patterns (inducible Hsp70 and constitutive Hsc70). Hsp70 significantly increases in expression when animals are exposed to stressors and plays a role in maintaining protein stability (Feder and Hofmann, 1999). Hsc70, which is constitutively expressed and may be mildly induced during stress, takes part in folding and repair of denatured proteins (Dong et al., 2015). Some studies have shown coordinated HR and expression of genes encoding to Hsps in response to elevated temperature (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.”

P. 6, L. 118-121. “This study provides novel information concerning the combined effects of increased temperature and $p\text{CO}_2$ on stress response, energy consumption and physiological plasticity in intertidal invertebrates, potentially providing predications of the ecological impacts of the future environmental changes.”

Q3: I am concerned that the experimental design is pseudoreplicated.

Response to Q3: P. 7, L. 130-137. During the acclimation treatment, all collected limpets were randomly allocated into one of four treatments. In each acclimation treatment, approximately 100

limpets were randomly allocated in ~ 30 containers (3 individuals in each container). All samples were acclimated under the same relative humidity and light intensity conditions with different $p\text{CO}_2$ concentration and temperature controlled by climate chambers. Therefore, we suggest that the experimental design is not pseudoreplication. In the revised manuscript, we have modified the description about acclimation treatment as follows.

“These limpets were randomly allocated into one of four treatments and temporally acclimated in different $p\text{CO}_2$ concentrations and temperatures (LTLC, 20 °C + 400 ppm, as a control treatment; LTHC, 20 °C + 1000 ppm; HTLC, 24 °C + 400 ppm; HTHC, 24 °C + 1000 ppm) for 7 d in climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which control both the $p\text{CO}_2$ concentration and temperature under the same relative humidity and light intensity conditions. In each acclimation treatment, approximately 100 limpets were randomly allocated in ~ 30 containers (3 individuals in each container), to simulate field densities of ~ 1 limpet per 10 cm².”

Q4: Title and overall interpretations need some consideration (see below)

- increasing sensitivity - is this a good or a bad thing?

- The variability increases but I do not know what can be said about this because all the limpets per treatment were housed together in one tank and so were competing in a lab environment. This may have influence the outcome.

- The limpets may have been collected from different microclimates. This would influence the outcome.

In essence - these considerations and potential caveats need to be presented and identify potential limitations of what can be said.

Response to Q4: Thanks for your kind and helpful suggestions. As suggested, we have provided clear descriptions about how samples were collected and how they were treated during the acclimation and heat process in the revised manuscript. We have identified and presented the limitations of the present study. Responses to the different questions are listed separately below:

(1) P. 14, L. 280-290. The increased sensitivity could be negative for the survive of a population. We have modified the first paragraph in the discussion to state the negative outcome of increased sensitivity.

“Short-term acclimation at elevated temperature and $p\text{CO}_2$ can increase physiological sensitivity of limpets to thermal stress. The higher thermal sensitivity of limpets acclimated to 1000 ppm indicates that the resilience of limpets to thermal stress associated with warming will be compromised under future ocean acidification. This prediction is contrary to the general thought that intertidal ectotherms, such as limpets and other gastropods, will demonstrate high tolerance to thermal stress because they are adapted to an extreme thermal environment. For example, the operative temperatures, which *C. toreuma* suffers in the field, frequently exceed 40 °C in summer along Asian coastlines and the limpet can survive at temperatures in excess of 45 °C (Dong et al., 2015). Our data show, however, that ocean acidification will lead to increased sensitivity to changes to future thermal regimes, indicating a synergistic negative effect. The change in the metabolic partitioning in individuals could ultimately lead to a decline in fitness and population-level responses in the future.”

(2) P. 17, L. 343-346. During the acclimation treatment, three individuals were kept in a container, resembling field densities of ~ 1 limpet per 10 cm². Despite this, the increased variability could also

result from the experiment design. We now acknowledge and presented this limitation as follows: “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).”

(3) Considering high temperature variation from sun-exposed rock surfaces, we only collected samples from shaded rock surfaces. Limpets mainly inhabit the mid-intertidal rocky shores at the collection site. Despite all this, we could not ensure that all samples come from exactly the same microclimate. We identified and presented this limitation in the discussion (please see above; Q4 - (2)).

P. 7, L. 125-128. More details about the sampling are now provided: “Samples were collected from shaded rock surfaces at mid-tidal level in Xiamen on a falling high tide in July (*in situ* temperature: 30.8 ± 0.8 °C). The sampling is to ensure that all limpets have similar thermal history, given the possible impacts from microclimate (Dong et al., 2017; Lathlean and Seuront, 2014).”

Abstract

Q5: I am not convinced that the authors have demonstrated physiological plasticity. To identify this, every animal would have to be treated exactly the same, but if they were in the same tank inter-individual interactions may have influence outcome.

Response to Q5: We acclimated the limpets in ‘common garden’. After acclimation in different temperatures and $p\text{CO}_2$ concentrations, we heat-shocked all the individuals on artificial walls in air separately. There was no direct inter-individual interaction during the heat shock procedure. With this experimental design, we think we can investigate the physiological plasticity. More details on specific changes of experiment designs are provided as follows.

P. 7, L. 125-140. “Samples were collected from shaded rock surfaces at mid-tidal level in Xiamen on a falling high tide in July (*in situ* temperature: 30.8 ± 0.8 °C). The sampling is to ensure that all limpets have similar thermal history, given the possible impacts from microclimate (Dong et al., 2017; Lathlean and Seuront, 2014). They were transported to the State Key Laboratory of Marine Environmental Science, Xiamen University, China within 2 h. Limpets were firstly allowed to recover at 20 °C for 3 d with a tidal cycle of approximately 6 h immersion and 6 h emersion. These limpets were randomly allocated into one of four treatments and temporally acclimated in different $p\text{CO}_2$ concentrations and temperatures (LTLC, 20 °C + 400 ppm, as a control treatment; LTHC, 20 °C + 1000 ppm; HTLC, 24 °C + 400 ppm; HTHC, 24 °C + 1000 ppm) for 7 d in climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which control both the $p\text{CO}_2$ concentration and temperature under the same relative humidity and light intensity conditions. In each acclimation treatment, approximately 100 limpets were randomly allocated in ~ 30 containers (3 individuals in each container), to simulate field densities of ~ 1 limpet per 10 cm². Control conditions (20 °C, 400 ppm) represent the average annual temperature and ambient $p\text{CO}_2$ (~ 390 ppm) at the collection site, with high temperature (24 °C) and $p\text{CO}_2$ (1000 ppm) representing the average global increase (4 °C, 600 ppm) predicted for 2100 by the Intergovernmental Panel on Climate Change (IPCC, 2007).”

P. 8-9, L. 154-167. “After a 7-day acclimation period (crossed $p\text{CO}_2 \times$ Temperature treatments, above), the heat-shock treatments were carried out to simulate the gradual temperature exposure of

limpets in the 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 aerielly 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.”

Q6: Individuals from the same population - state from where and that they are intertidal.

Response to Q6: P. 2, L. 16-18. This sentence has been changed to “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.”

Q7: Context at lines 107 and 111 need to be added to the abstract - i.e. treatments - what are the control treatments and what based on (e.g. annual mean, temperature at time of collection etc). Where the limpets collected from exactly the same level on the shore and the same aspect wrt to insolation?

Response to Q7: P. 2, L. 18-22. More details about the treatments have been added in the abstract section: “Limpets were collected from shaded mid-intertidal rock surfaces. They were acclimated under combinations of different $p\text{CO}_2$ concentrations (400 ppm and 1000 ppm, corresponding to pH 7.8 and 8.1) 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 $p\text{CO}_2$ level at the collection site.”

Q8: Let the reader know that both the inducible and constitutive forms of HSP were investigated ...and to address for what aspect each are useful/or why used.

Response to Q8: P. 2, L. 22-27. Thanks for your suggestions. Detailed information about the HSP has been provided: “Heart rates (as a proxy for metabolic performance) and genes encoding inducible and constitutive heat-shock proteins (*hsp70* and *hsc70*) at different heat shock temperatures (26, 30, 34 and 38 °C) were measured. Hsp70 and Hsc70 play important roles in protecting cells from heat stresses, but have different expression patterns with Hsp70 significantly increased in expression during stress and Hsc70 constitutively expressed and only mildly induced during stress.”

Q9: I do not understand “better to cope physiologically”.

Response to Q9: P. 2, L. 33-34. This sentence has been changed to “... some individuals have higher physiological plasticity to cope with these conditions.”

Q10: Also provide pH levels in the abstract. Because local TA can differ regionally the levels of ppm used are not easy to compare between studies.

Response to Q10: P. 2, L. 20. The pH levels were provided: "... (400 ppm and 1000 ppm, corresponding to pH 8.1 and 7.8) ..."

Introduction

Q11: L. 43 - better to replace "acidity" with "decreased pH" - as per Gatusso's paper on how OA should be presented.

Response to Q11: P. 3, L. 49. "acidity" was replaced with "decreased pH".

Q12: L. 56 replace "climate change" with "increased temperate" so the reader understands the focus.

Response to Q12: P. 4, L. 63. "climate change" was replaced with "increased temperature".

Q13: L. 61 - correct the Gibson reference - this is the editor of the volume not the author of the paper!

Response to Q13: P. 4, L. 68. The reference has been modified and the sentence was changed to: "... organisms to warming (Byrne and Przeslawski, 2013; Byrne, 2011; Kroeker et al., 2013), ..."

Q14: The main response variables are heart rate, hspc and hspi. All of these and their use as indicators should be introduced. It will suffice to say that heart beat is often used with molluscs in stress studies (REF). Does an increase in heart rate indicate the animal is coping or is in stress - i.e. what does heart rate measures indicate (with refs). Similarly, the two HSP markers need to be introduced. How do these differ? Why do both? Cite a few previous studies. One of the authors, Dr Dong has done great research on HSPs and so will be able to address this. Is this the first study to combine these physiological markers with heart rate? Here the authors can clearly state what is novel about this study.

Response to Q14: Thank you for your constructive suggestions. We have added a paragraph to introduce heart rate and HSP markers (P. 4-5, L. 78-95), and stated the novelty of the present study (P. 6, L. 118-121.). Please see above (Q2) for more details of these changes in the revised manuscript.

Q15: L. 85 how did the authors measure plasticity - was this based on CV?? If so state this here and justify with refs>

Response to Q15: P. 6, L. 110-112. In the present study, the plasticity was measured based on post-acclimation temperature sensitivity. This sentence was changed to: "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* ..."

Methods

Q16: The animals are from fluctuating habitats (e.g. L. 51,77) but the treatments are static. I understand that it is difficult to mimic intertidal flux conditions in the lab, but the fact that the experimental conditions used do not reflect the natural conditions needs to be acknowledged and

some justification of the approach stated - perhaps add 'potential' insights?

Response to Q16: P. 17-18, L. 357-359. Thanks for your useful suggestions. We acknowledged that it is necessary to state the experimental conditions used do not reflect fluctuant conditions in natural environment. We have added a sentence to mention this fact in the final paragraph in the discussion.

“Further, since our findings are based on static experimental conditions, the results should be treated with caution when we predict organism’s response to future climate change in the highly variable natural environment.”

Q17: We are told what the habitat temperature is. What is the range of the habitat pCO₂?

Response to Q17: P. 7, L. 137-138. The habitat pCO₂ was provided in the revised manuscript: “... represent the average annual temperature and ambient pCO₂ (~ 390 ppm) at the collection site, ...”

Q18: L. 64 - microclimate is mentioned - indeed this is very important. For instance, the Lathlean papers (infrared studies) and others show that depending on aspect of the habitat rocks to the sun and even rock colours, that limpets and other intertidal invertebrates have very different thermal environments. The reader needs to be assured that the experimental animals had a similar thermal history and cite some of these studies.

Response to Q18: P. 7, L. 125-128. Thank you for your useful suggestions. All samples were collected from shaded rock surfaces at mid-intertidal level at the collection site. Therefore, we suggested that all collected limpets had similar microclimate and thermal history. In the revised manuscript, detailed information about the sampling was provided: “Samples were collected from shaded rock surfaces at mid-tidal level in Xiamen on a falling high tide in July (*in situ* temperature: 30.8 ± 0.8 °C). The sampling is to ensure that all limpets have similar thermal history, given the possible impacts from microclimate (Dong et al., 2017; Lathlean and Seuront, 2014).”

Q19: As I read the design - 100 limpets were maintained in a single container per acclimation treatment. This is pseudoreplication. The authors must acknowledge this. L. 125 - were randomly selected - that helps ... potentially to get around pseudo rep??

Response to Q19: Approximately 100 limpets were reared in each acclimation treatment and they were randomly allocated in ~ 30 containers. There were three individuals in a container, and the density was ~1 limpet per 10 cm² in each acclimation treatment, similar to that under field conditions (our field investigation) to limit the potential for density-dependent behaviour.

P. 7, L. 135-137. The sentence was changed to: “In each acclimation treatment, approximately 100 limpets were randomly allocated in ~ 30 containers (3 individuals in each container), to simulate field densities of ~ 1 limpet per 10 cm².”

Q20: However, the ramping was done on an individual basis.

Response to Q20: P. 8-9, L. 154-167. The procedure of ramping was modified for clarity. Please see above (Q5) for more details of these changes in the revised manuscript.

Q21: L 177 This is confusing and may be due to English. What does “stands for” mean. It is important that the definition is clear.

Response to Q21: P. 11, L. 213. This sentence was changed to: “Thermal sensitivity is the change in a physiological rate function ...”.

Q22: L. 179 replace “is seen” ... perhaps with “was determined”?

Response to Q22: P. 11, L. 215. “is seen” was replaced with “was determined”.

Results

Q23: L. 209 ABT - I suggest spell out each use eg.

Response to Q23: P. 12, L. 246-248. The ABTs of all four treatments were provided in the result section: “The ABTs of limpets showed a trend to be reduced for HT treatments (mean \pm 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).”

Q24: L 208 and 219 “indicating ...” This is discussion text - delete from results.

Response to Q24: They were deleted from results section.

Discussion

Q25: L. 242,254 - what is the bottom line - is sensitivity a good or bad outcome? What is the take home? Move up sentence L. 245 and 271-272. We need to understand what does change in sensitivity mean for the prospects of the limpets in the future.

Response to Q25: P. 14, L. 280-290. Thank you for your constructive suggestions. The bottom line is that the increased sensitivity and stress response of limpets under future conditions could be a negative response, especially for the survival of a population. We have modified the first paragraph in the discussion section:

“Short-term acclimation at elevated temperature and $p\text{CO}_2$ can increase physiological sensitivity of limpets to thermal stress. The higher thermal sensitivity of limpets acclimated to 1000 ppm indicates that the resilience of limpets to thermal stress associated with warming will be compromised under future ocean acidification. This prediction is contrary to the general thought that intertidal ectotherms, such as limpets and other gastropods, will demonstrate high tolerance to thermal stress because they are adapted to an extreme thermal environment. For example, the operative temperatures, which *C. toreuma* suffers in the field, frequently exceed 40 °C in summer along Asian coastlines and the limpet can survive at temperatures in excess of 45 °C (Dong et al., 2015). Our data show, however, that ocean acidification will lead to increased sensitivity to changes to future thermal regimes, indicating a synergistic negative effect. The change in the metabolic partitioning in individuals could ultimately lead to a decline in fitness and population-level responses in the future.”

Q26: L. 251 - Does this indicate a synergistic negative effect?

Response to Q26: P. 14, L.288-289. The increased sensitivity to thermal stress under the acclimation at elevated temperature and $p\text{CO}_2$ indicates that the resilience of limpets to thermal stress is reduced under future ocean acidification, which indicates a synergistic negative effect. This sentence was changed to: “... will lead to increased sensitivity to changes to future thermal regimes, indicating a synergistic negative effect.”

Q27: The HSP text is confusing - let the reader know that one paragraph is on the inducible and the other is on the constitutive forms AND the significance of these - with citations.

Response to Q27: In the discussion section, we focused on discussing the inducible *hsp70* in the second paragraph (P. 14-15, L. 291-309) and constitutive *hsc70* in the third paragraph (P. 15-16, L. 310-331).

P. 14-15, L. 291-309. “Increased temperature and CO₂ 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 CO₂ (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 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.”

P. 15-16, L. 310-331. “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 CO₂ condition changed in comparison with the LTLC treatment), there was significant upregulation of *hsc70* 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 CO₂ 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 CO₂) 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.”

Q28: Para starting L. 295. Could the “variation” (based on CV) interpreted here as “plasticity” be due to variable environmental history not still present despite a 7 day acclimation. This needs to be considered here. A longer acclimation time would be needed. Also plasticity is reversible. Essentially speak to the data and potential caveats.

Response to Q28: As suggested, the variability (based on CV) could be also caused by the variable environmental history in despite of a 7-day acclimation. In the revised manuscript, the caveat for the interpretation of the variability and long-term acclimation to validate our findings was provided as follows:

P. 17, L. 343-346. “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).”

P. 17-18, L. 357-361. “Further, since our findings are based on static experimental conditions, the results should be treated with caution when we predict organism’s response to future climate change in the highly variable natural environment. Therefore, future studies with long-term acclimation, larger sample size, and variable treatment conditions are recommended in order to validate our findings.”

Q29: The caveat of flux (natural) -vs- stable (exp) needs to be stated.

Response to Q29: The caveat of natural and experiment conditions has now been clearly stated in combination with the explanation on acclimation time. See the comment above.

Reply to comments of anonymous referee #3

Q1: This manuscript is an interesting study looking at the impact of warming and ocean acidification on the variability of physiological responses on an intertidal limpet species. The interesting idea in the manuscript is the impact of multiple stressors on the variability of the response. However, since the focus is on inter-individual variability, the sample size should be bigger than 10.

Response to Q1: We thank the reviewer for this comment. In combination with the comments from original Referee #2, it has helped to further refine the discussion of our results. While we appreciate that a larger sample size and geographical distribution of samples would be necessary to characterize the variability in responses in a species, in this study we were testing for the response of individuals within a population to altered environmental conditions. The main results that we report are the physiological and molecular responses within the population. In our results, we clearly show that there is a biological response (statistically significant) to the different conditions, meaning that the replication was, by definition, large enough to detect responses. However, we also provide information on the variation in responses as this could be an important part of how populations and species adapt to changing conditions.

P. 16-17, L. 332-346. We have added some clarification on our interpretation of variation as follows: “Variation and plasticity in both physiological and molecular responses to thermal stress are not only important for coping with future environmental change but also underpin evolutionary and adaptive changes through selective pressures (Franks and Hoffmann, 2012). In the present study, the coefficients of variation in physiological responses of limpets acclimated in simulated future conditions, including ABT, Q_{10} and *hsc70* mRNA, were higher than those in the other three acclimation conditions. Crucially, this means that a subset of individuals in our experimental population might be more physiologically pre-adapted to cope with heat shock. Once acclimated to future climate change scenario (warming and ocean acidification), this variation in physiological performance increased, indicating that in a harsher environment the physiological plasticity of some individuals allows them to modify their physiological tolerance limits and increase chances for survival and reproduction (Williams et al., 2008). Under high selective pressure, these individuals would form the basis for future generations while less plastic individuals would be removed from populations. However, differences among the coefficients of variation need to be interpreted with caution, as multiple factors can cause this type of variation, including the variable environmental history of individuals despite a 7-day acclimation, competition among individuals during the acclimation period, or the sample size (around 10 limpets per treatment).”

Q2: The material and methods is not clear. 100 individuals are placed in each acclimated treatment. Then 10 are used as a control for genes expression at the end of the acclimation before putting them on the wall. This is not really a control. The 10 individuals for control should have been put on the wall and kept at the control parameters for the same amount of time than the other individuals on the wall. The procedure for the cardiac performance measurement is unclear. How the temperature was heated? There is no control for this procedure. This is also unclear when the limpets are in the water or in the air. Since the individuals are exposed to tidal cycle of 6 hours immersion and 6 hours emersion, is the air temperature of the air the same than the water? Is the wall experiment realized under water or in the air?

Response to Q2: Responses to your comments were listed as follows:

(1) P. 8-9, L. 154-167. In the heat shock experiments, for each acclimation condition, 10 limpets were heated in each designated temperature (26, 30, 34 and 38 °C) in addition to a non-heat-stressed (control) group of 10 limpets. All samples experienced a total exposure time of 7 h in air, including the control samples under 20 or 24 °C. The bottom of artificial rock was heated in water bath, and limpets were on the surface of rock and exposed to air. In the revised manuscript, we have modified this paragraph as follows for clarity:

“After a 7-day acclimation period (crossed $p\text{CO}_2 \times$ Temperature treatments, above), the heat-shock treatments were carried out to simulate the gradual temperature exposure of limpets in the 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 aerielly 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) P. 9, L. 170-180. In the cardiac performance experiment, one individual was placed into a container, and the container was immersed in water bath and heated at a rate of 6 °C per hour which mimicked emersion in the natural environment. During the whole process of ramping, limpets were in the air. Since limpets exhibited a stable basal heat rate under no-stress condition, we suggested that no control for this procedure is acceptable. Details about the heating procedure were provided as follows:

“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. 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).”

Specific comments:

Q3: - Line 37: Widdicombe and Spicer, 2008 missing in the reference list

Response to Q3: The reference has been added in the reference list.

P. 25, L. 555-556. “Widdicombe, S. and Spicer, J. I.: Predicting the impact of ocean acidification on benthic biodiversity: what can animal physiology tell us?, *J. Exp. Mar. Biol. Ecol.*, 366, 187-197, 2008.”

Q4: - Line 43: unclear

Response to Q4: P. 3, L. 47-52. This sentence has been changed to: “Although ocean acidification can increase the growth of organisms in some cases (e.g. Gooding et al., 2009), there is increasing evidence that decreased pH exacerbates global warming, and interactions of ocean acidification and warming reduce an organism’s resistance to environmental change (Munday et al., 2009) and subsequently affect population dynamics (Fabry et al., 2008; Hoegh-Guldberg et al., 2007; Kroeker et al., 2013; Rodolfo-Metalpa et al., 2011).”

Q5: - Line 54: Pörtner et al., 2012 missing in the reference list

Response to Q5: The reference has been added in the reference list.

P. 23, L. 512-513. “Pörtner, H. O.: Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes, *Mar. Ecol. Progr. Ser.*, 470, 273-290, 2012.”

Q6: - Line 64: re-ordered the references

Response to Q6: P. 3, L. 47. The order of the references has been modified to: “... (Dong et al., 2014; Firth and Williams, 2009).”

Q7: - Line 76: only on the associated biofilm?

Response to Q7: P. 5, L. 99-101. Limpets play an important role in food chains of the intertidal zone. This sentence has been changed to: “As a common calcifier inhabiting coastal ecosystem, *C. toreuma* plays an important ecological role in food chains, grazing on biofilm and being an important food source for other species (e.g. crabs, sea birds and sea stars).”

Q8: - Line 98: when the samples were collected in the field? What was the in situ temperature?

Response to Q8: P. 7, L. 125-126. The samples were collected on a falling high tide in July. The *in situ* temperature on the shaded rock surface was around 30 °C. Details were provided as follows: “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).”

Q9: - Lines 116-117: unclear

Response to Q9: P. 8, L. 145-148. This sentence has been changed to: “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).”

Q10: - Line 120: HSO4-?

Response to Q10: It is KSO₄⁻, for the value of KSO₄ is the dissociation constant for HSO₄⁻.

Q11: - Line 121: Dickson et al. (1990)

Response to Q11: P. 8, L. 151. It has been modified to: "... total Boron was set from Millero et al. (2006), Dickson et al. (1990) and Lee et al. (2010) respectively."

Q12: - Line 178: Fig. A3

Response to Q12: P. 11, L. 214. It has been changed to Fig. A3.

Q13: - Line 185: Fig. A3

Response to Q13: P. 11, L. 221. It has been modified to Fig. A3.

Q14: - Lines 191-192: R1 and R2 were average heart rate?

Response to Q14: P. 11-12, L. 226-229. R1 and R2 were the average heart rate. The sentence has been changed to: "In each CO₂ concentration (400 ppm or 1000 ppm), the post-acclimation Q₁₀ values were calculated using the same equation as shown above, but R₂ was the average heart rate of the warm-acclimated limpets at the acclimated temperature (T₂ = 24 °C), and R₁ was the average heart rate of cold-acclimated limpets at T₁ = 20 °C (Fig. A3, modified from Seebacher et al. (2015))."

Q15: - Line 278: Fangue et al., 2006 missing in the reference list

Response to Q15: The reference has been added in the reference list.

P. 21, L. 446-448. "Fangue, N. A., Hofmeister, M., and Schulte, P. M.: Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*, J. Exp. Biol., 209, 2859-2872, 2006."

Q16: - Line 279: Chuang et al., 2007 and Teixeira et al., 2013 missing in the reference list

Response to Q16: The references have been added in the reference list.

P. 20, L. 412-413. "Chuang, K. H., Ho, S. H., and Song, Y. L.: Cloning and expression analysis of heat shock cognate 70 gene promoter in tiger shrimp (*Penaeus monodon*), Gene, 405, 10-18, 2007."
P. 24, L. 541-543. "Teixeira, T., Diniz, M., Calado, R., and Rosa, R.: Coral physiological adaptations to air exposure: heat shock and oxidative stress responses in *Veretillum cynomorium*, J. Exp. Mar. Biol. Ecol., 439, 35-41, 2013."
"

Q17: - Line 456: Year 2010 and not 2012.

Response to Q17: P. 24, L. 521. It has been modified to 2010.

Q18: - Figure 1: (a) Heart rates of all limpets acclimated at 20 °C?

Response to Q18: P. 27, L. 579. It has been changed to: "Figure 1. (a) Heart rates of all limpets acclimated to 20 °C and 400ppm, ..."

Q19: - Lines 554-555: the heart rate of limpets from the warm-acclimated...

Response to Q19: P. 33, L. 629. This sentence has been modified to: "Black line and grey line showed the heart rate of limpets from the warm-acclimated ...".

Q20: - Table A1: change all the umol by μmol and change utam by μatm

Response to Q20: P. 35. All the umol and utam have changed to μmol and μatm, respectively.

(2)

A list of all relevant changes made in the manuscript

Based on the comments of the reviewers, we have intensively discussed the revision of our manuscript. To best possibly address all reviewer's comments, some parts of the manuscript have been updated. Please find below a list of changes that have been made to the manuscript.

- *Abstract*: We provided basic information on sample collection, pH levels, treatments, and the inducible and constitutive forms of heat shock protein.

- *Introduction*: We added a paragraph to introduce heart rate and heat shock proteins and stated the novelty of the present study.

- *Material and Methods*: The descriptions about sample collection, acclimation and heat-shock treatments, cardiac performance measurement were rephrased.

- *Results*: Average values of ABTs were provided.

- *Discussion*: A paragraph was added to compare expression patterns of *hsp70* in intertidal limpets under abrupt exposure and under gradual exposure. We identified and presented limitations of the present study.

(3)

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

4

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6

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13 **Abstract.** Understanding physiological responses of organisms to warming and ocean acidification is
14 the first step towards predicting the potential population- and community-level ecological impacts of
15 these stressors. Increasingly, physiological plasticity is being recognized as important for organisms to
16 adapt to the changing microclimates. Here, we evaluate the importance of physiological plasticity for
17 coping with ocean acidification and elevated temperature, and its variability among individuals ~~from the~~
18 ~~same population~~, of the intertidal limpet *Cellana toreuma* from the same population in Xiamen. Limpets
19 were collected from shaded mid-intertidal rock surfaces. They were acclimated under combinations of
20 different $p\text{CO}_2$ concentrations (400 ppm and 1000 ppm, corresponding to pH 8.1 and 7.8) and
21 temperatures (20 °C and 24 °C) in a short-term period (7 days), with the control condition (20 °C and
22 400 ppm) representing the average annual temperature and present-day $p\text{CO}_2$ level at the collection site.
23 Heart rates (as a proxy for metabolic performance) and genes encoding inducible and constitutive heat-
24 shock proteins (*hsp70* and *hsc70*) at different heat shock temperatures (26, 30, 34 and 38 °C) were
25 measured ~~at different heat shock temperatures (26, 30, 34 and 38 °C) in individuals temporally acclimated~~
26 ~~(7 d) under combinations of different $p\text{CO}_2$ concentrations (400 ppm and 1000 ppm) and temperatures~~
27 ~~(20 °C and 24 °C).~~ Hsp70 and Hsc70 play important roles in protecting cells from heat stresses, but have
28 different expression patterns with Hsp70 significantly increased in expression during stress and Hsc70
29 constitutively expressed and only mildly induced during stress. Analysis of heart rate showed
30 significantly higher temperature coefficients (Q_{10} rates) for limpets at 20 °C than at 24 °C and post-
31 acclimation thermal sensitivity of limpets at 400 ppm was lower than at 1000 ppm. Expression of *hsp70*
32 linearly increased with the increasing heat-shock temperatures, with the largest slope occurring in limpets
33 acclimated under a future scenario (24 °C and 1000 ppm $p\text{CO}_2$). These results suggested that limpets
34 increased sensitivity and stress response under future conditions. Furthermore, the increased variation in
35 physiological response under the future scenario indicated that some individuals have higher
36 physiological plasticity ~~were better~~ to cope ~~physiologically~~ with these conditions. While short-term
37 acclimation at acidic seawater decreases the ability of partial individuals against thermal stress,
38 physiological plasticity and variability seem to be crucial in allowing some intertidal animals to survive
39 in a rapidly changing environment.
40

41 1 **Introduction**

42 Benthic organisms living in the intertidal zone will be exposed to increasingly variable and extreme
43 environmental conditions, such as temperature, oxygen and CO₂, due to climatic change (IPCC, 2013;
44 Kwiatkowski et al., 2016). These highly fluctuating environmental variables can significantly affect the
45 physiological performance of coastal species (Helmuth et al., 2006; Hofmann and Todgham, 2010;
46 Somero, 2012; Widdicombe and Spicer, 2008). Therefore, understanding the interaction of multiple
47 environmental stressors on the physiological performance is crucial for predicting the consequences of
48 environmental change on ecosystems (Deutsch et al., 2015). For example, salinity fluctuations coupled
49 with high temperatures during emersion can have both sub-lethal physiological effects and lethal effects
50 on intertidal molluscs ([Dong et al., 2014](#); Firth and Williams, 2009; ~~[Dong et al., 2014](#)~~). Although ocean
51 acidification can increase the growth of organisms in some cases (e.g. Gooding et al., 2009), [there is](#)
52 increasing evidence ~~showed that~~ [decreased pH rising ocean acidity](#) exacerbates global warming, [and](#)
53 [interactions of ocean acidification and warming](#) reduces an organism's resistance to environmental
54 change (Munday et al., 2009); and subsequently affects population dynamics (Fabry et al., 2008; Hoegh-
55 Guldberg et al., 2007; Kroeker et al., 2013; Rodolfo-Metalpa et al., 2011).

56 In the face of a changing environment, organisms have three main options; shift their geographical
57 distribution (Parmesan and Yohe, 2003), develop evolutionary adaptive changes (Hoffmann and Sgro,
58 2011), or perish (Fabricius et al., 2011). Prior to mortality or range-shifts, environmental changes can
59 often drive physiological adaptation or the evolution of phenotypic plasticity (Chevin et al., 2010;
60 Sanford and Kelly, 2011). Yet, warming and ocean acidification are not unidirectional, but rather
61 combined with rapid fluctuations on daily to seasonal and decadal time-scales. Thus, the changing
62 environment often does not provide clear signals to drive strong directional selection of traits, meaning

63 that, usually, physiological plasticity is the more important factor in acclimation to changing
64 environmental conditions (Hoffmann and Sgro, 2011; Pörtner et al., 2012; Somero et al., 2012). In a
65 recent meta-analysis, Seebacher et al. (2015) demonstrated that acclimation to higher temperatures
66 decreased the sensitivity to increased temperature climate change in both freshwater and marine animals.
67 While this response suggests that acclimation could reduce the impact of warming on organisms, the
68 responses were only tested for shifts in mean temperature. Yet, organisms inhabiting variable
69 environments, such as the intertidal zone, will be exposed to increasing extremes in temperature
70 concomitant with increasing $p\text{CO}_2$, or ocean acidification (OA), in the future. While OA has been
71 suggested to increase the sensitivity of organisms to warming (Byrne and Przeslawski, 2013; Byrne,
72 Gibson et al., 2011; Kroeker et al., 2013), physiological plasticity and variation in responses may provide
73 the basis for populations to survive.

74 Physiological variation, or plasticity, within population is important for adapting to local
75 microclimate and for evolution (Dong et al., 2017; Oleksiak et al., 2002; Prosser, 1955). For example,
76 different color morphs of the gastropod *Littorina saxatilis* have enhanced physiological performance
77 which leads to increased survival under extreme conditions, indicating physiological differences may
78 provide a selective advantage for those color morphs under extremely fluctuating salinity and
79 temperature regime in estuaries (Sokolova and Berger, 2000). For the limpet *Cellana toreuma*, highly
80 variable expressions of genes related to stress responses and energy metabolism are important for
81 surviving the harsh environment on subtropical rocky shores (Dong et al., 2014).

82 Heart rate (HR), as a measure of cardiac activity, is a useful indicator for indicating physiological
83 response to stress in molluscs (Dong and Williams, 2011; Xing et al., 2016). Animals exhibit a stable
84 basal HR under conditions which are not thermally stressful, and HR increases and reaches a peak

85 followed by a sudden decrease with temperature rising (Braby and Somero, 2006; Dong and Williams,
86 2011). The temperature at which a sharp discontinuity in slope occurs in an Arrhenius plot (i.e. Arrhenius
87 breakpoint temperature, ABT) can represent the limit of metabolic functioning of animals (Nickerson et
88 al., 1989; Somero, 2002). At the molecular level, expression of heat shock proteins (Hsps) and *hsp* genes
89 is induced above a certain temperature, reaches maximum and finally ceases in response to heat shock
90 (Han et al., 2013; Miller et al., 2009). Upregulation of Hsps and *hsp* genes is an energy-consuming way
91 of mechanism for defense against thermal stress (Somero et al., 2016). As a commonly used biomarker,
92 the Hsp70 multigenic family includes two proteins with divergent expression patterns (inducible Hsp70
93 and constitutive Hsc70). Hsp70 significantly increases in expression when animals are exposed to
94 stressors and plays a role in maintaining protein stability (Feder and Hofmann, 1999). Hsc70, which is
95 constitutively expressed and may be mildly induced during stress, takes part in folding and repair of
96 denatured proteins (Dong et al., 2015). Some studies have shown coordinated HR and expression of
97 genes encoding to Hsps in response to elevated temperature (Han et al., 2013; Prusina et al., 2014).
98 However, little is known about the patterns of heart rate and expression of *hsp* genes for coping with
99 combined warming and ocean acidification.

100 The limpet *C. toreuma* is a keystone species on rocky shores in the ~~Western-western~~ Pacific (Dong
101 et al., 2012) ~~and, occupies occupying the~~ mid-low intertidal zones (Morton and Morton, 1983). This
102 species is a gonochoric and broadcast spawner, whose embryos develop into planktonic trocophore larvae
103 and later into juvenile veligers before becoming fully grown adults (Ruppert et al., 2004). As a common
104 calcifier inhabiting coastal ecosystem, *C. toreuma* plays an important ecological role in food chains,
105 gazing on biofilm and being an important food source for other species (e.g. crabs, sea birds and sea stars)
106 affecting the community structure of the associated biofilm. Therefore, this species is a key organism for

107 studying the relationship between physiological response to thermal stress and ocean acidification in
108 highly variable environment on the shore.

109 Under the impact of Subtropical High, Xiamen (118°14' E, 24°42' N) is one of the hottest areas in
110 China. The coastal seawater of this area is experiencing rapid temperature rise and acidification (Bao and
111 Ren, 2014). The sea surface temperature (SST) in Xiamen coastal ~~area-water~~ has ~~risen-increased~~ a total
112 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
113 of seawater in Xiamen Bay have declined by 0.2 pH units from 1986 to 2012, a trend which is predicted
114 to continue based on simulations (Cai et al., 2016).

115 Here, we investigated the importance of physiological plasticity (based on the measurement of post-
116 acclimation temperature sensitivity; see Seebacher et al., 2015) and variability (based on coefficient of
117 variation) for *C. toreuma* to cope with ocean acidification and elevated temperatures by quantifying heart
118 rates (as a proxy of metabolic performance) and expression of genes encoding inducible and constitutive
119 heat-shock proteins (Hsp70 and Hsc70) after short-term acclimation in different $p\text{CO}_2$ concentrations
120 (400 ppm and 1000 ppm) and temperatures (20 °C and 24 °C). We hypothesize that (1) limpets will
121 increase their thermal sensitivity of metabolism and stress responses under elevated $p\text{CO}_2$ and
122 temperatures; (2) short-term acclimation at high temperature and $p\text{CO}_2$ will cause higher inter-individual
123 physiological variation. This study provides novel information concerning the combined effects of
124 increased temperature and $p\text{CO}_2$ on stress response, energy consumption and physiological plasticity in
125 intertidal invertebrates, ~~potentially providing and is important in allowing~~ predictions of the ecological
126 impacts of the future environmental changes. _

127

128 2 Material and Methods

129 2.1 Limpet collection and experiment treatments

130 Samples were collected from ~~shaded rock surfaces at mid-tidal level in Xiamen, and on a falling~~
131 ~~high tide in July (*in situ* temperature: 30.8 ± 0.8 °C). The sampling is to ensure that all limpets have~~
132 ~~similar thermal history, given the possible impacts from microclimate (Dong et al., 2017; Lathlean and~~
133 ~~Seuront, 2014). They~~ were transported ~~to the back~~ State Key Laboratory of Marine Environmental
134 Science, Xiamen University, China within 2 h. Limpets were firstly allowed to recover at 20 °C for 3 d
135 with a tidal cycle of approximately 6 h immersion and 6 h emersion. These limpets were randomly
136 allocated into ~~one of~~ four ~~acclimation~~ treatments and temporally acclimated in different $p\text{CO}_2$
137 concentrations and temperatures (LTLC, 20 °C + 400 ppm, as a control treatment; LTHC, 20 °C + 1000
138 ppm; HTLC, 24 °C + 400 ppm; HTHC, 24 °C + 1000 ppm) for 7 d in climate chambers (RXZ280A,
139 Jiangnan Instrument Company, Ningbo, China), which ~~can~~ control both the $p\text{CO}_2$ concentration and
140 temperature under the same relative humidity and light intensity conditions. In each acclimation
141 treatment, There were three individuals were placed in a container (approx. about 100 indiv. per
142 acclimation treatment), to simulate field densities of and the density was ~ one 1 limpet per 10 cm² in
143 all acclimation treatments. This density was similar to that when we collected the samples. Control
144 conditions temperature (20 °C, 400 ppm) and high temperature (24 °C), respectively, represent the
145 average annual temperature and ambient $p\text{CO}_2$ (~ 390 ppm) in at the collection site, with high
146 temperature (24 °C) and $p\text{CO}_2$ (1000 ppm) representing and the average global increase (4 °C, 600 ppm)
147 predicted for 2100 by the Intergovernmental Panel on Climate Change (IPCC, 2007). Two $p\text{CO}_2$ levels,
148 400 ppm and 1000 ppm, represent the present day situation and scenarios for 2100 respectively, as
149 projected by IPCC (2007).

150 Animals were kept in a simulated tidal cycle with 6 h aerial exposure and 6 h seawater immersion.
151 Seawater was pre-bubbled with air containing the corresponding $p\text{CO}_2$ concentrations in advance. pH
152 was measured before and after the acclimation in seawater each time with PB-10 pH meter (Sartorius
153 Instruments, Germany) calibrated with National Institute of Standards and Technology standard pH
154 solutions (NIST, USA). Total dissolved inorganic carbon (DIC) was measured before and after the
155 acclimation in seawater each time using ~~a Li-Cor[®] non-dispersive infrared (NDIR) detector (Li-6252) by~~
156 ~~a dissolved inorganic carbon analyzer (As-C3, Apollo SciTech, Colorado, USA), using a Li-Cor[®] non-~~
157 ~~dispersive infrared detector (Li-6252)~~ with a precision of 0.1% (Cai, 2003). Seawater carbonate
158 chemistry parameters were estimated based on the measured values of pH, DIC, temperature and salinity
159 with the software CO2Calc v4.0.9 (Robbins et al., 2010). For CO2Calc settings, the NBS scale was
160 applied as the pH scale, and the CO_2 constant, the KHSO_4^- constant and the total Boron was set from
161 Millero et al. (2006), Dickson et al. (~~1999~~1990) and Lee et al. (2010) respectively. The information of
162 the measured and calculated seawater chemistry parameters is summarized (Table A1).

163 After a 7-day ~~short-term~~ acclimation period (crossed $p\text{CO}_2 \times$ Temperature treatments, above), the
164 heat-shock treatments were carried out to simulate the gradual temperature exposure of limpets in the
165 filed as described in Denny et al. (2006) (Fig. A1). ~~individuals from all four acclimation conditions (n =~~
166 ~~10 indiv. per acclimation treatment) were randomly sampled and frozen at -80 °C as non-heated control~~
167 ~~samples. In~~ For each acclimation-heat-shock treatment, 40-10 limpets were randomly selected from each
168 of four acclimation conditions (40 indiv. total) and ~~and were~~ transferred to ~~an~~ artificial rocks (Fig. A2),
169 with individuals from LTLC and LTHC on one rock and individuals from HTLC and HTHC on another
170 rock. (see Fig. A1). ~~The artificial rocks were separately placed in 20 °C water baths and 24 °C water~~
171 baths, and ~~The rock was~~ heated at a rate of 6 °C per hour ~~(that simulated emersion in the~~ natural heating

172 ~~rate condition at the collection site;~~ (Han et al., 2013) to the designated temperatures (26, 30, 34 and
173 38 °C). ~~The heat shock treatments were carried out as described in Denny et al. (2006) (Fig. A2).~~ After
174 achieving the target temperature, the temperature was maintained for the allotted time, and then
175 decreased to ~~acclimated~~ the acclimation temperatures (20 or 24 °C) at a rate of 6 °C per hour, for a total
176 exposure time of 7 h. ~~Individuals from all four acclimation conditions (n = 10 indiv. per treatment)~~
177 ~~were randomly selected, transferred to artificial rocks and aerially exposed at 20 or 24 °C for 7h, as non-~~
178 ~~heated control samples.~~ After recovery at 20 or 24 °C seawater for 1 h, limpets (~~n = 8-10 indiv. per heat~~
179 ~~shock temperature at each acclimation condition~~) were immediately collected and stored at -80 °C for
180 gene expression ~~quantification~~ analysis.

181

182 2.2 Cardiac performance measurement

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

193 viewed and analyzed using Lab Chart (version 7.0).

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

198

199 **2.3 Quantifying genes expression**

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

206 The levels of mRNA of genes encoding two heats hock proteins, inducible heat-shock protein 70
207 (*hsp70*) and constitutive heat shock protein 70 (*hsc70*), were measured using real-time quantitative PCRs
208 in CFX96™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules CA, USA) followed the methods
209 described by Han et al. (2013) with specific primers (Table A2). For normalizing expression of genes,
210 we examined expression of *18S ribosomal RNA*, *β-actin*, *β-tubulin* genes, which typically have relatively
211 stable expression levels. The expression stability of these housekeeping genes was evaluated using the
212 GeNorm Algorithm (Primer Design, Ltd., Southampton University, Highfield Campus, Southampton
213 Hants, UK) as described by Etschmann et al. (2006). Based on the expression stability measures (M

214 values), all the three genes were selected as the reference genes for normalizing the level of expression
215 of stress-induced genes. All samples were measured in triplicates. Ct (dR) values were analyzed using
216 the CFX Manager™ Software Version 3.0 (Bio-Rad). The expression of *hsp70* and *hsc70* was determined
217 relative to the value of *18S*, *β-actin* and *β-tublin* from a reference individual.

218

219 2.4 Statistical analysis

220 The general additive mixed model (GAMM) was used to compare thermal sensitivities of heart rate
221 among limpets acclimated at different temperatures (20 or 24 °C) and CO₂ concentrations (400 or 1000
222 ppm). Analyses were conducted with the *mgcv* (Wood, 2004) and *nlme* (Pinheiro et al., 2013) libraries in
223 R Version 3.0 (R Core Team, 2014). The generalized additive model (GAM), describing heart rate as a
224 function of temperature, was used to test for how heart rates of limpets from each treatment deviated
225 from those of limpets from control conditions (20 °C, 400 ppm) (Angilletta et al., 2013).

226 Thermal sensitivity ~~is stands for~~ the change in a physiological rate function reacting to a rapid
227 change in environmental temperature within the same acclimation set temperature (Fig. [A2A3](#), modified
228 from Seebacher et al. (2015)). In the present study, thermal sensitivity ~~was determined is seen~~ in the
229 temperature coefficient (Q₁₀) values of heart rate. Q₁₀ was calculated using heart-rate data from the
230 temperature at which the experiment started (T₁ = 24 °C) to the temperature to which temperature
231 increased 10 °C (T₂ = 33 °C) with Eq. (1):

$$232 \quad Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \quad (1)$$

233 where R is the heart rate (R₁ and R₂ are the heart rate at T₁ and T₂ respectively), and T is the temperature
234 (Kelvin) (Fig. [A2A3](#), modified from Seebacher et al. (2015)). The differences in Q₁₀ among the four

235 acclimation conditions with different CO₂ concentrations (400 ppm vs. 1000 ppm) and temperatures
236 (20 °C vs. 24 °C) were analyzed using two-way ANOVA with Duncan's *post hoc* analysis using the SPSS
237 20.0 for Windows statistical package (IBM SPSS Statistics, Chicago, USA). Post-acclimation thermal
238 sensitivity of limpets in different CO₂ concentrations were calculated as described by Seebacher et al.
239 (2015). In each CO₂ concentration (400 ppm or 1000 ppm), the post-acclimation Q₁₀ values were
240 calculated using the same equation as shown above, but R₂ was the average heart rate of the warm-
241 acclimated limpets at the acclimated temperature (T₂ = 24 °C), and R₁ was the average heart rate of cold-
242 acclimated limpets at T₁ = 20 °C (Fig. A3, modified from Seebacher et al. (2015)). It is worth noting that
243 post-acclimation thermal sensitivity should be considered with caution, as in the present study the
244 acclimation period (7 days) may not have been sufficient for full acclimation to altered conditions.

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

250 The coefficient of variation (CV) of ABT, Q₁₀ and *hsc70* mRNA expression at 38 °C were
251 calculated for each acclimation condition. The CV is the variance in a sample divided by the mean of
252 that sample, providing a method to compare the variation within a sample relative to the mean. It is
253 generally accepted that higher CV demonstrates that there is greater variation among individuals within
254 one treatment than another (Reed et al., 2002).

255

256 **3 Results**

257 **3.1 Cardiac performance**

258 The maximal heart rate was ~ 30 % higher in limpets acclimated to control conditions (20 °C, 400
259 ppm) than the other treatments (Fig. 1 and Table A3) ~~indicating reduced metabolic performance under~~
260 ~~high temperatures and pCO₂ conditions~~. The ABTs of limpets ~~ranged from 34.5 °C to 44.2 °C and~~ showed
261 a trend to be reduced for HT treatments (mean ± SD: LTLC, 38.9 ± 2.9 °C; HTLC, 38.2 ± 1.8 °C; LTHC,
262 40.0 ± 3.3 °C; HTHC, 37.7 ± 2.3 °C) (Fig. A4). Temperature (Two-way ANOVA, $F_{1,35} = 3.375$, $P = 0.075$)
263 and $p\text{CO}_2$ (Two-way ANOVA, $F_{1,35} = 0.118$, $P = 0.733$) both had non-significant effects on ABTs, and
264 there was a non-significant interaction between temperature and $p\text{CO}_2$ (Two-way ANOVA, $F_{1,35} = 0.908$,
265 $P = 0.347$) (Table A4; Fig. A4).

266 Temperature coefficients (Q_{10} rates) were higher for limpets acclimated at 20 °C than at 24 °C (Two-
267 way ANOVA, $F_{1,35} = 5.878$, $P = 0.02$), but there was no significant difference for acclimation to different
268 $p\text{CO}_2$ concentrations (Two-way ANOVA, $F_{1,35} = 1.332$, $P > 0.05$) and for the interaction between
269 temperature and $p\text{CO}_2$ (Two-way ANOVA, $F_{1,35} = 0.1135$, $P > 0.05$) (Table A4; Fig. 2). The post-
270 acclimation thermal sensitivity of limpets acclimated at low CO_2 (2.12) was lower than limpets at high
271 CO_2 (2.95) (Fig. 2), ~~indicating that the latter are more metabolically sensitive to temperature~~.

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

276

277 **3.2 Gene expression**

278 Levels of *hsp70* mRNA (log-transformed) linearly increased with the increasing heat-shock
279 temperatures (Fig. 3). ANCOVA analysis showed that the slopes of the linear regressions were
280 significantly different among different acclimation conditions ($F_{4, 189} = 42.62$, $P < 0.001$), and the slope
281 of HTHC limpets was higher than those of the other three acclimation conditions. Thus, the rate of
282 increase in production of *hsp70* mRNA in response to warming was greater at the elevated CO₂
283 concentration.

284 The responses of *hsc70* mRNA to heat shock were divergent among the four acclimation conditions
285 (Fig. 4). For HTHC limpets, there were no significant differences among different heat shock
286 temperatures ($F_{4, 42} = 2.11$, $P = 0.096$). For LTLC, LTHC and HTLC limpets, levels of *hsc70* mRNA after
287 being heat-shocked at 38°C were higher than corresponding levels of *hsc70* mRNA at 20 °C or 24 °C
288 (Duncan's *post hoc* analysis, $F_{4, 42} = 4.389$, $P = 0.005$; $F_{4, 44} = 8.521$, $P < 0.0001$; $F_{4, 42} = 5.713$, $P = 0.001$).
289 The coefficients of variation of *hsc* mRNA after heat shock of 38°C were different among different
290 acclimation conditions, HTHC (90.36%) > LTHC (80.44%) ≈ HCLT (80.12%) > LCLT (56.20%) (Table
291 1).

292
293 **4 Discussion**

294 Short-term acclimation at elevated temperature and $p\text{CO}_2$ can increase physiological sensitivity of
295 limpets ~~against to~~ thermal stress. The higher thermal sensitivity of limpets acclimated to 1000 ppm
296 indicates that the resilience of limpets to thermal stress associated with warming will be compromised
297 under future ocean acidification. Post-acclimation thermal sensitivity represents the extent to which
298 ectothermic animals can acclimate to longer term increases in temperature (several days to weeks)

299 ~~(Seebacher et al., 2015). Thus, the higher thermal sensitivity of limpets acclimated to 1000 ppm indicates~~
300 ~~that the resilience of limpets to thermal stress associated with warming will be compromised under future~~
301 ~~ocean acidification.~~ This prediction is contrary to the general thought that intertidal ectotherms, such as
302 limpets and other gastropods, will demonstrate high tolerance to thermal stress because they are adapted
303 to an extreme thermal environment. For example, the operative temperatures, ~~from~~ which *C. toreuma*
304 suffers in the field, frequently exceed 40 °C in summer along Asian coastlines and the limpet can survive
305 at temperatures in excess of 45 °C (Dong et al., 2015). Our data show, however, that ocean acidification
306 will lead to increased sensitivity to changes to future thermal regimes, indicating a synergistic negative
307 effect. The change in the metabolic partitioning in individuals could ultimately lead to a decline in fitness
308 and population-level responses in the future.

309 Increased temperature and CO₂ ~~elevated increase~~ the sensitivity of heat shock responses to thermal
310 stress. The expression of inducible *hsp70* mRNA steadily increased from 20°C to 38°C for individuals
311 across all experimental treatments. However, rates of upregulation of *hsp70* mRNA in limpets acclimated
312 at high temperature and high CO₂ (HTHC) were significantly higher than those of limpets acclimated at
313 the other three acclimation conditions. As a molecular chaperon, Hsp70 protein plays crucial roles in
314 maintaining protein stability with the expense of a large amount of energy (Feder and Hofmann, 1999;
315 Tomanek and Sanford, 2003). By comparing the expression patterns of Hsp70 of different *Chlorostoma*
316 species (formerly *Tegula*) that have distinct vertical distribution, Tomanek and Somero (1999, 2000)
317 found that there existed interspecific difference in the frequency of the induction of Hsp70 synthesis and
318 interspecific divergence of the time-course of Hsp70 synthesis. These studies from genus *Chlorostoma*
319 suggested that species that live higher in the intertidal cost more energy for proteostasis and restore
320 proteostasis to cope with a second consecutive day of high temperatures (Semero et al., 2016). Usually,

321 the expression of Hsp70 of less thermal-tolerant species is more sensitive to increases in temperature
322 (limpet *Lottia*, Dong et al., 2008; snail *Chlorostoma*, Tomanek, 2002), and the rapid upregulation of
323 *hsp70* mRNA in limpets exposed to future conditions potentially represents a high sensitivity of limpets
324 to thermal stress in the face of ocean acidification. Due to the expensive energy consumption during the
325 synthesis and function of *hsp70*, the more rapid upregulation of *hsp70* mRNA in these limpets also
326 indicates more energy was allocated into cellular homeostasis, which then can affect the limpet's growth
327 and reproduction. ~~This change in the metabolic partitioning in individuals could ultimately lead to a~~
328 ~~decline in fitness and population level responses.~~

329 The expression patterns of constitutive *hsc70* mRNA were different among limpets acclimated at the
330 four acclimation conditions. Hsc70 is constitutively expressed and is a molecular chaperone involved in
331 the *in vivo* folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain
332 similar promoter regions, there are differential expressions to a given stimulus between them (Hansen et
333 al., 1991). Some studies showed that thermal stress could significantly induce the up-regulation of both
334 *hsc70* gene and Hsc70 protein in the killifish *Fundulus heteroclitus* (Fangue et al., 2006), the shrimp
335 *Penaeus monodon* (Chuang et al., 2007), and the coral *Veretillum cynomorium* (Teixeira et al., 2013). In
336 the present study, for limpets acclimated under HTLC and LTHC (i.e. only temperature or CO₂ condition
337 changed in comparison with the LTLC treatment), there was significant upregulation of *hsc70* mRNA
338 when the heat shock temperatures were beyond 30 °C. However, the expression of *hsc70* mRNA showed
339 no significant difference among different heat-shock temperatures under predicated future environmental
340 conditions (HTHC: 24 °C and 1000 ppm). These results indicate that the upregulation of *hsc70* mRNA
341 in response to heat shock represents an increasing capability for coping with the enhanced protein
342 denaturation and more energy allocated into the somatic maintenance after being exposed to either

343 warming or high CO₂ environment. The insignificant upregulation of *hsc70* in response to thermal stress
344 indicates that limpets acclimated under HTHC may employ a “preparative defense” strategy (Dong et al.,
345 2008) to maintain high constitutive levels of *hsc70* as a mechanism to cope with unpredictable heat stress.
346 However, the absence of significant upregulation of *hsc70* mRNA in limpets acclimated to future
347 conditions (warming and elevated CO₂) might also be attributed to the very high variation of gene
348 expression at 38°C (CV, 90.36 %). In the context of future conditions, multiple environmental stressors
349 can induce diverse physiological responses among different individuals, which might be an evolutionary
350 adaptation to the harsh environment on the shore.

351 Variation and plasticity in both physiological and molecular responses to thermal stress are not only
352 important for coping with future environmental change but also underpin evolutionary and adaptive
353 changes through selective pressures (Franks and Hoffmann, 2012). In the present study, the coefficients
354 of variation in physiological responses of limpets acclimated in simulated future conditions, including
355 ABT, Q₁₀ and *hsc70* mRNA, were higher than those in the other three acclimation conditions. Crucially,
356 this means that a subset of individuals in our experimental population might be more physiologically
357 pre-adapted to cope with heat shock. Once acclimated to future climate change scenario (warming and
358 ocean acidification), this variation in physiological performance increased, indicating that in a harsher
359 environment the physiological plasticity of some individuals allows them to modify their physiological
360 tolerance limits and increase chances for survival and reproduction (Williams et al., 2008). Under high
361 selective pressure, these individuals would form the basis for future generations while less plastic
362 individuals would be removed from populations. However, ~~the results about~~differences among the
363 coefficients of variation need to be interpreted with caution, as multiple factors can cause this type of the
364 variation, including the variable environmental history of individuals despite a 7-day acclimation.

365 competition among individuals during the acclimation period, or the sample size (around 10 limpets per
366 treatment) in the present study may affect the prediction accuracy.

367 Intertidal limpets may experience two sorts of stressful temperature exposures in the field,
368 abrupt or gradual exposure (Denny et al., 2006). The present study showed the upregulation of *hsp70*
369 and *hsc70* expression in *C. toreuma* under gradual exposure. Similar expression patterns have been also
370 observed in *Hsp70* under gradual thermal exposure in other intertidal limpets (Dong et al., 2008; Miller
371 et al., 2009). Importantly, the gradual experimental change in thermal environment used here mimics
372 conditions that most intertidal species experience in the field and is important for predicting how animals
373 will resolve prolonged aerial exposure during low tide. Conversely, experimentally simulating abrupt
374 thermal change helps us understand physiological responses to some extreme conditions, such as heat
375 wave (upregulation of *hsp70* in intertidal limpets, Prusina et al., 2014). Therefore, future works combing
376 both abrupt and gradual exposure may offer insight into how intertidal species respond to climate change
377 and extreme weather events in the future. Further, since our findings are based on static experimental
378 conditions, the results should be treated with caution when we predict organism's response to future
379 climate change in the highly variable natural environment. Therefore, future studies with long-term
380 acclimation, larger sample size, and variable treatment conditions are recommended in order to validate
381 our findings.

382 In conclusion, the resilience of intertidal limpets to thermal stress is weakened after exposure to
383 predicted future conditions for a short-term acclimation period (7 d). Yet, the combination of elevated
384 temperature and CO₂ concentration prompted divergence of physiological and molecular responses.
385 These results suggest that while organisms may be able to protect themselves from the damaging effects
386 of thermal stress in the short-term, changes to multiple environmental conditions may drive population-

387 level responses through physiological responses (e.g. Giomi et al., 2016). Further, the increased variation
388 in responses, and the observation that some individuals were more capable to physiologically cope with
389 the conditions, may be associated with intergenerational adaptation, but this speculation needs further
390 evidence. As the “weaker” individuals are lost, the offspring in the next generation will be better
391 physiologically adapted to warming under high-CO₂ conditions. Therefore, while elevated CO₂ and the
392 associated ocean acidification decrease the ability of many individuals to respond to thermal stress, it
393 appears that physiological plasticity and variability could be adaptive mechanisms in at least some
394 populations of intertidal organisms. Our research underlines~~s~~ the importance of physiological plasticity
395 and variability for coastal species coping with warming and ocean acidification. ~~However, the present~~
396 ~~study has only examined the physiological responses of limpets to heat stress after short-term acclimation.~~
397 ~~Future studies with long-term acclimation and a larger sample size are therefore recommended in order~~
398 ~~to validate our findings.~~

399

400 **Authors' contributions**

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

404

405 **Competing interests**

406 The authors declare no conflict of interests.

407

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413

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595

596 **Table 1.** Coefficients of variation (%) of Arrhenius break temperature (ABT), temperature coefficients (Q₁₀) and

597 *hsc70* mRNA expression at 38 °C^{1,2}

598

Temperature	CO ₂	ABT	Q ₁₀	<i>hsc70</i> 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

599 ¹Temperature coefficients (Q₁₀) were calculated using heart rate from 24 to 33 °C

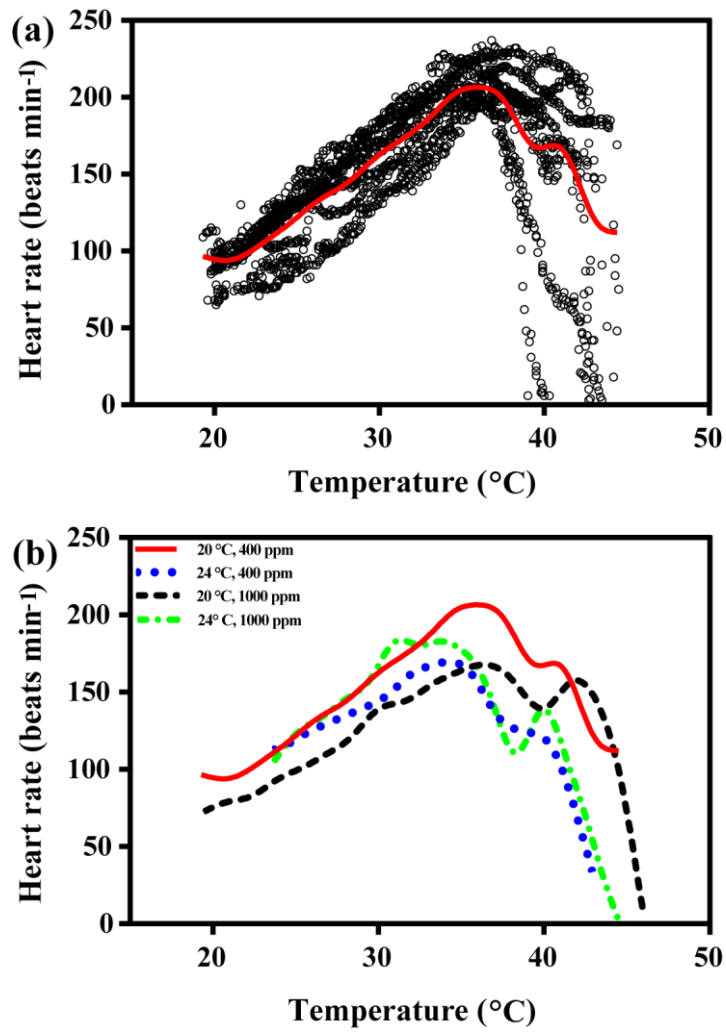
600 ²After acclimated at different CO₂ and temperature for one week, limpets (n = 8-10) from each acclimation treatment

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

602 acclimation treatments were used for calculating coefficients of variation.

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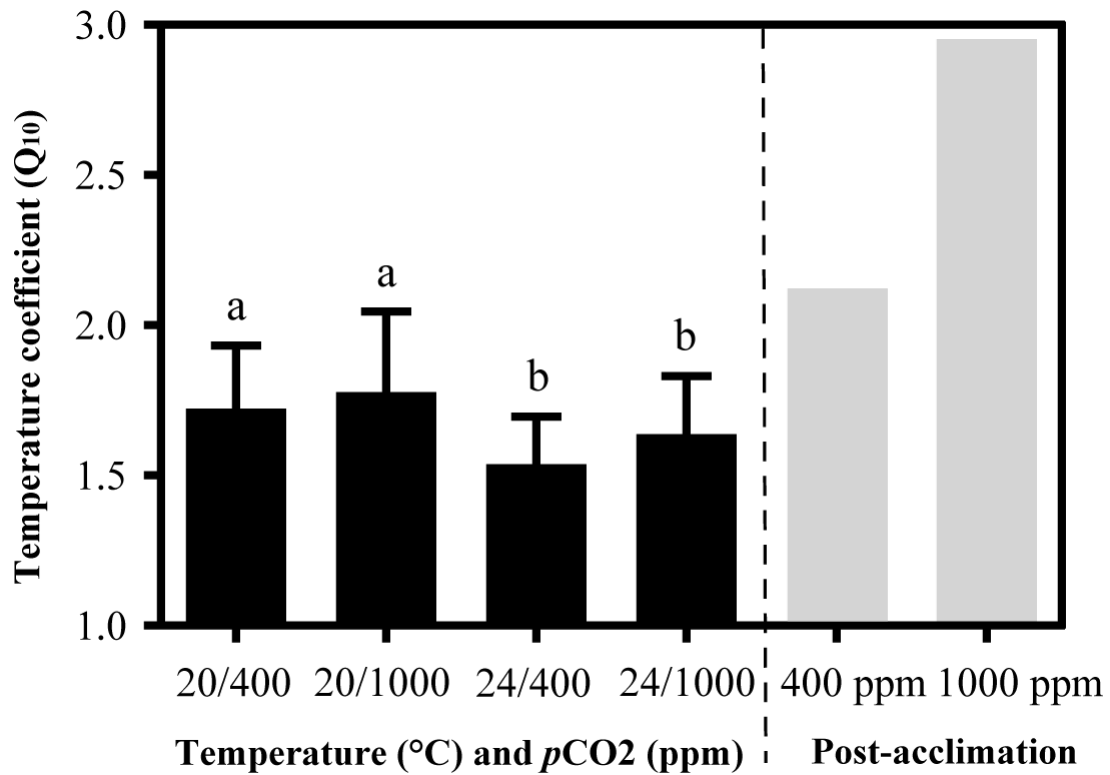
607 **Figure 1.** (a) Heart rates of all limpets acclimated to 24-20 °C and 400ppm, presented as an example of HR

608 calculation for limpets in all treatments. The red line represents the most likely general additive mixed model

609 (GAMM) to depict the trajectory of hearts rate for limpets with increasing temperature; (b) GAMM lines of limpets

610 acclimated at the different experimental temperature and CO₂ conditions.

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614 **Figure 2.** Temperature coefficients (Q₁₀) of limpets acclimated at different temperatures (20 or 24 °C) and CO₂

615 concentrations (400 or 1000 ppm). The temperature coefficient (Q₁₀) values were calculated for all limpets using

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

617 acclimated at 20 and 24°C (grey bars; *sensu* Seebacher et al., 2015) for each CO₂ concentration, where higher thermal

618 sensitivity indicates less acclimation to thermal stress. The calculation of post-acclimation Q₁₀ is done for the mean

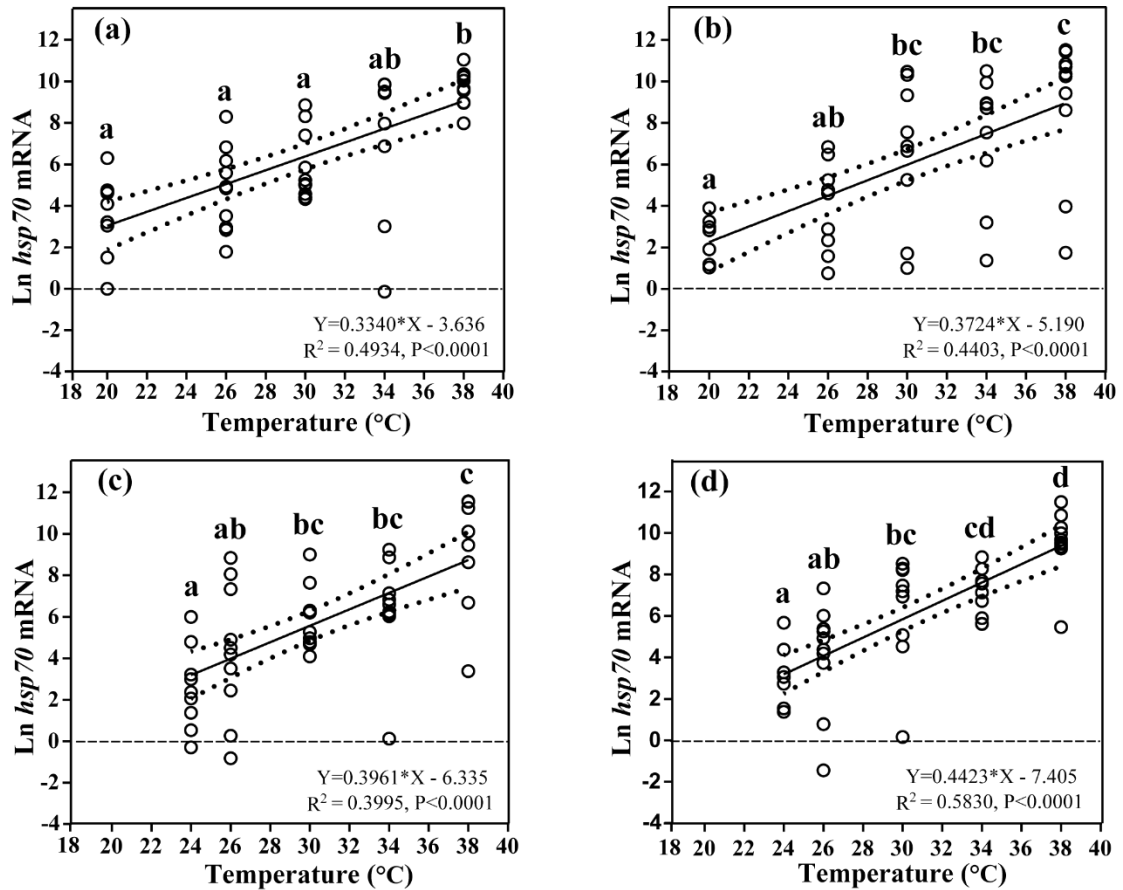
619 response of all individuals as the same individual are not used at each acclimation temperature. Therefore, it is not

620 possible to calculate an estimate of variation or error for post-acclimation Q₁₀. Therefore, there was no calculation of

621 variation or error for post acclimation. Different letters represent significant differences in the Q₁₀ among different

622 acclimation treatments.

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625

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

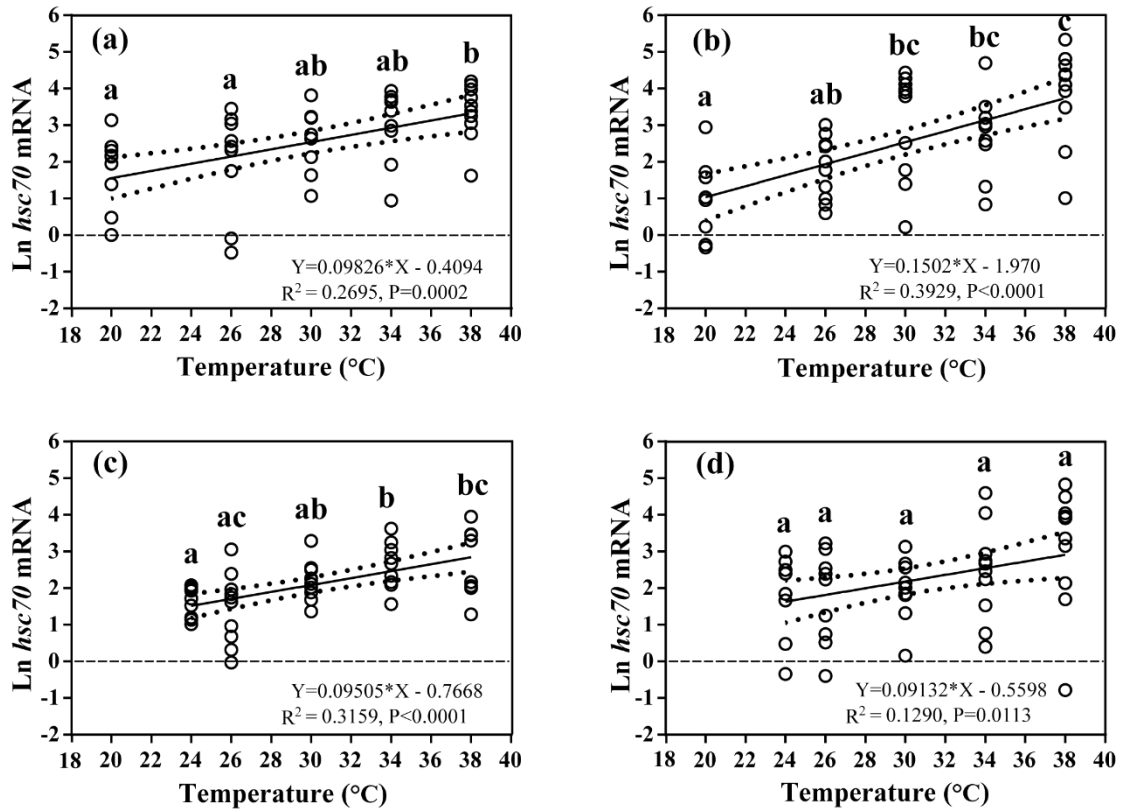
627 and 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

630 among different heat-shock temperatures.

631



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633

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

635 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

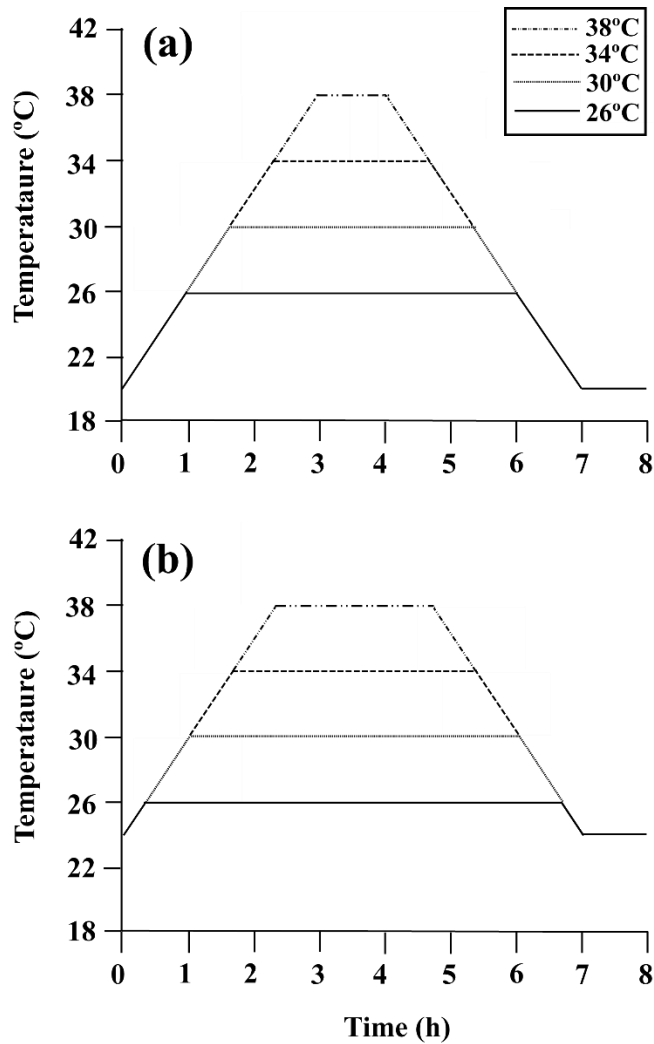
636 heat-shock temperature and log-transformed gene expression of *hsc70* was fitted using linear regressions with 95%

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

638 among different heat-shock temperatures.

639

640 Appendix:



641

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

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

644 (26, 30, 34 and 38 °C) for simulating a natural heating rate in summer. After achieving the target temperature, the

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

646 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,

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

648 measurement.

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Figure A1A2. The photo of artificial rock (60 cm length × 30 cm width). Limpets were placed on artificial rock

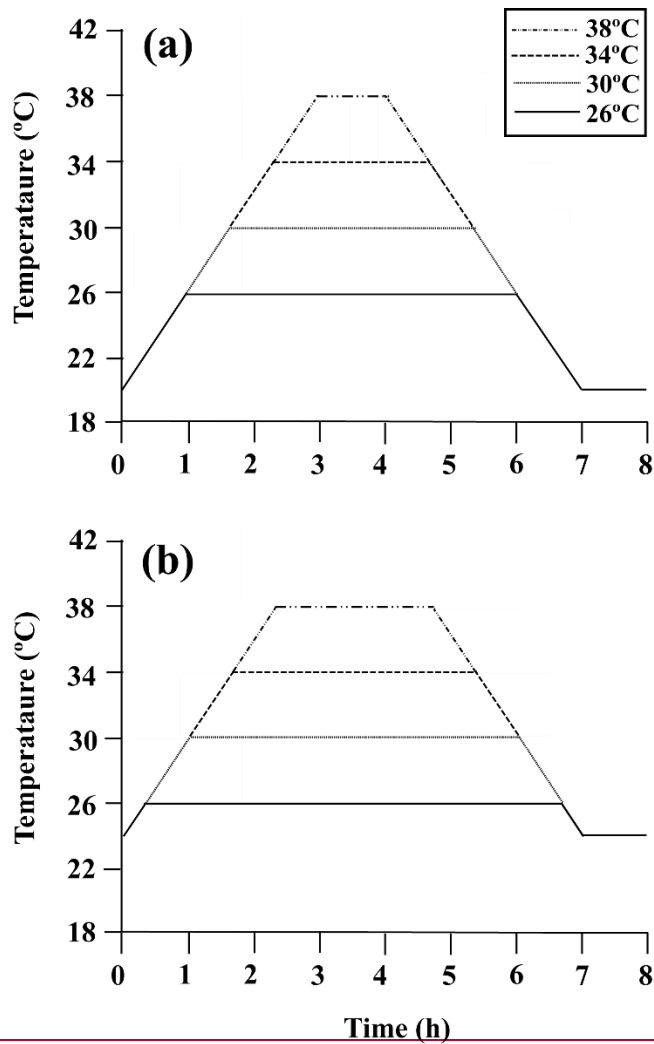
652

and heated to the designated temperate.

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657 **Figure A2.** Diagram of the heating protocol for (a) limpets acclimated at 20 °C and (b) limpets acclimated at 24 °C.

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

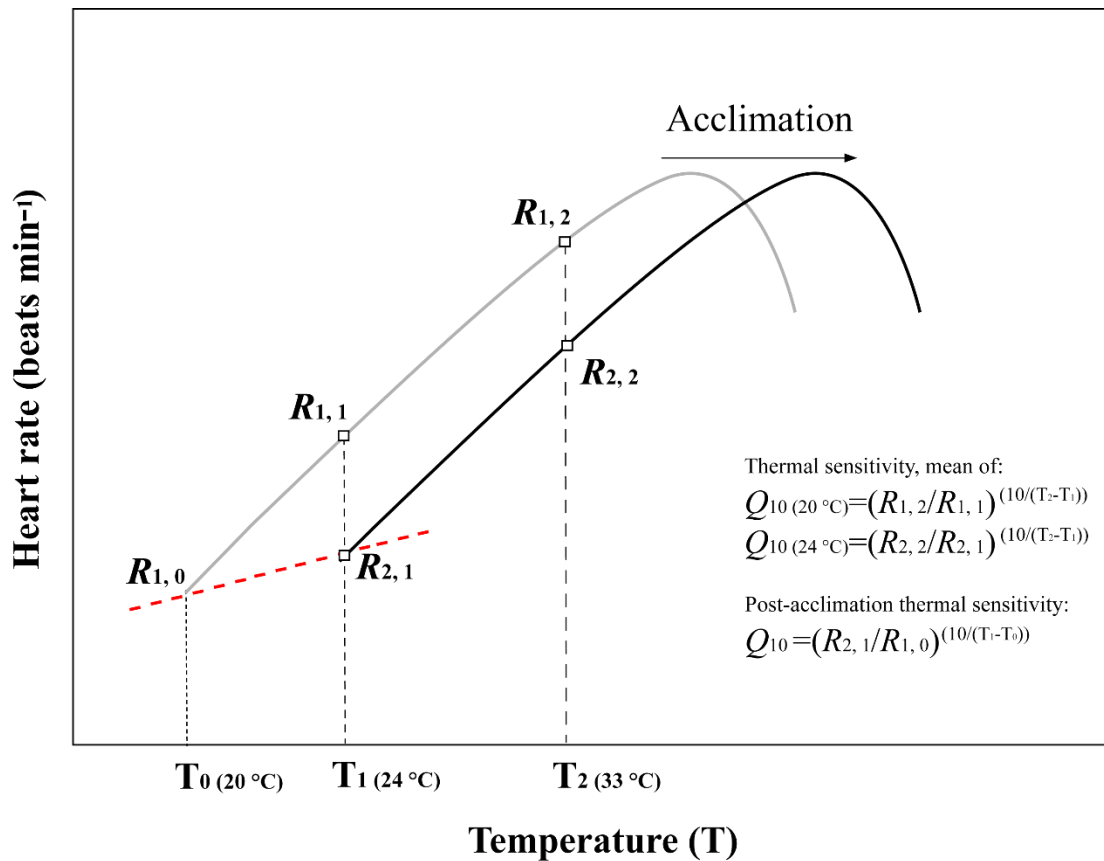
659 (26, 30, 34 and 38 °C) for simulating a natural heating rate in summer. After achieving the target temperature, the

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

661 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,

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

663 measurement.



664

665

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

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

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

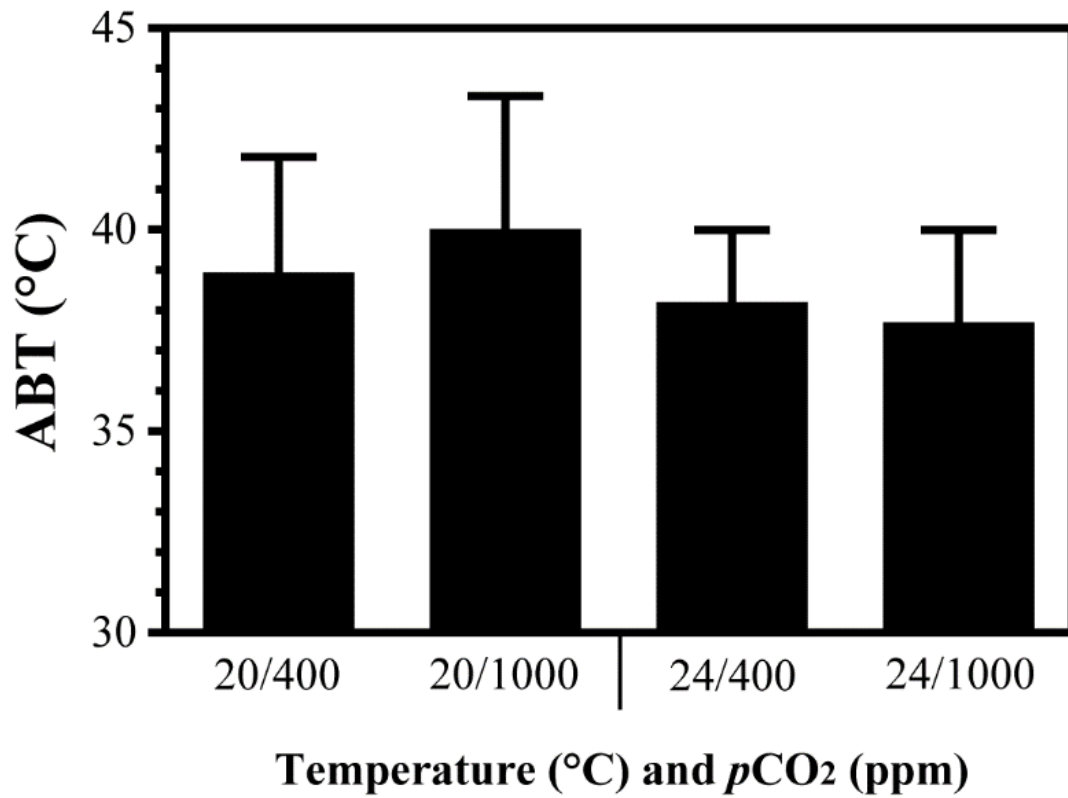
669 thermal sensitivities were calculated from data for limpets kept at an acclimation treatment in which heart rate were

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

671 two temperature acclimation conditions under the same $p\text{CO}_2$ condition.

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676 **Figure A4.** Arrhenius break-point temperature of heart rate (ABT) of limpets acclimated at different temperatures
 677 (20 or 24 °C) and CO₂ concentrations (400 or 1000 ppm). After acclimation in different conditions, limpets were
 678 heated continuously from acclimation temperatures to the heart stopped beating. During the heating process, heart
 679 rates were recorded and ABTs were calculated.

680

681

682 **Table A1.** Measured and calculated seawater carbonate chemistry variables of each acclimation treatment during the
 683 experimental period¹
 684

	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 ($\mu\text{mol/kg}$)	2082.70±191.28	2083.016±190.58	2081.19±165.93	2083.29±163.58
C_T ($\mu\text{mol/kg}$)	1910.57±174.42	1910.57±174.42	1992.76±157.22	1992.15±149.76
$p\text{CO}_2$ (μatm)	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-} ($\mu\text{mol/kg}$)	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

685 ¹Seawater temperature, salinity, pH and total dissolved inorganic carbon (C_T) were monitored every 6 h. Total
 686 alkalinity (A_T), $p\text{CO}_2$, CO_3^{2-} and Ω_{cal} were calculated using CO2SYS software. Results were pooled and averaged
 687 over sampling times. Values are given as mean ± SD.
 688

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

690

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

691

692

693 **Table A3.** Inferential statistics for the most likely general additive mixed models (GAMM) of heart rate during
 694 continuous warming of limpet *Cellana toreuma* acclimated at different temperatures (20 and 24 °C) and $p\text{CO}_2$ (400
 695 and 1000 ppm)¹

696

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

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

700

701 **Table A4.** Two-way ANOVA to investigate the effects of temperature (20 °C and 24 °C) and $p\text{CO}_2$ (400 ppm and
 702 1000 ppm) on Arrhenius break-point temperature of heart rate (ABT) and temperature coefficients (Q_{10}) on
 703 *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
$p\text{CO}_2$	1	0.790	0.790	0.118	0.733
Temperature \times $p\text{CO}_2$	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
$p\text{CO}_2$	1	0.058	0.058	1.332	0.256
Temperature \times $p\text{CO}_2$	1	0.005	0.005	0.1135	0.738
Residual	35	1.527	0.0436		

704