Dear Editor,

Please find 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

# (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

*The Wang et al ms is an interesting study of the impact of warming and acidification on physiological responses. The main significant effect was seen with the molecular biology – the hsp response. Some analyses of the other parameters measured (eg. heart rate) were equivocal. I suggest reduce the emphasis on the latter and concentrate on the hsp data. Reduce the text on non-significant results. I have questions on methods that need to be addressed before a full picture of the outcomes of the work can be assessed.*

**Response:** Thanks for your kind and helpful suggestions. Some text about non-significant results were reduced. Otherwise, we expanded *hsp* discussion in the discussion section. More detailed modifications were providing as follows.

*Introduction*
*Q1: L. 42-45 – Not quite correct there are many studies that show that moderate increase in temperature – within projections – reduces/ameliorates the negative effect of acidification.*

**Response to Q1:** P. 3, L. 44-48. This sentence is changed to: "Although ocean acidification can increase the growth of organism in some cases (Gooding et al., 2009), increasing evidence showed that that rising ocean acidity exacerbates global warming, reduces an organism's resistance to environmental change (Munday et al., 2009), but and subsequently affects population dynamics (Fabry et al., 2008; Hoegh-Guldberg et al., 2007; Kroeker et al., 2013; Rodolfo-Metalpa et al., 2011)."

*Q2: At the end of the introduction more contect is need about the region, species and approaches used. Some of this is in the first section of the methods and can be moved here. Also provide some predictions/hypotheses at the end of the introduction. How would you expect the limpets to response with respect to hsp, heart rate, ABT etc.*

**Response to Q2:** P. 4-5, L. 75-98. Thanks for your constructive suggestions. The introduction section is reformulated by adding region, species, approaches, and hypotheses, and details are provided as follows.

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

the community structure of the associated biofilm. Therefore, this species is a key organism for studying the relationship between physiological response to thermal stress and ocean acidification in highly variable environment on the shore.

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

Here, we investigated the importance of physiological plasticity and variability for *C. toreuma* to cope with ocean acidification and elevated temperatures by quantifying heart rates (as a proxy of metabolic performance) and expression of genes encoding heat-shock proteins after short-term acclimation in different $pCO_2$ concentrations (400 ppm and 1000 ppm) and temperatures (20 °C and 24 °C). We hypothesize that (1) limpets will increase their thermal sensitivity of metabolism and stress responses under elevated $pCO_2$ and temperatures; (2) short-term acclimation at high temperature and $pCO_2$ will cause higher inter-individual physiological variation. This study provides novel information concerning the combined effects of increased temperature and $pCO_2$ on physiological plasticity in intertidal invertebrates, and is important in allowing predications of the ecological impacts of the future environmental changes."

*Methods*

*Q3: Is 7 days a sufficient "acclimation" time – why was this selected. It seems that the limpets were placed directly in treatment – is this a shock? I do not think that with a 7-day experiment much can be said about post-acclimation, (eg. discussion) some justification is needed for this – perhaps there are other studies that have determined this for other limpets.*

**Response to Q3:** Responses were listed separately as follows:

(1) It might be proper to describe the 7-day acclimation as a short-term acclimation in the present study. Recent reviews of the literature on the ocean acidification (Doney et al., 2009; Parker et al., 2013) found that the biological responses to acidification between short-term and long-term experiments could be different for benthic invertebrates. We suggest that our study (i.e. short-term acclimation) has its significance for understanding physiological response of organisms to warming and ocean acidification, especially when considering highly variable temperature and $pCO_2$ concentration in the intertidal zone (Cai et al., 2016; Kwiatkowski et al., 2016). Meanwhile, future studies with long-term acclimation (several months) and a larger sample size are recommended in order to validate our findings.

(2) Considering that intertidal species under natural conditions can tolerate high variation of temperature and $CO_2$ (Kwiatkowski et al., 2016), we suggest that directly placing the limpets in treatment might not be a strict shock. In addition, in order to avoid the direct shock of treatments, limpets collected in the field were allowed to recover at 20 °C for 3 d with a tidal cycle of approximately 6 h immersion and 6 h emersion in the lab before allocated in treatments.

(3) As for the term "post-acclimation", according to Seebacher et al. (2015), the post-acclimation

thermal sensitivity is calculated by estimating how much a physiological rate change when animals are allowed to acclimated to different condition (i.e. across chronic acclimation conditions). Since the acclimation is a short-term process in the present study, we suggest that adding the following statement can avoid unnecessary ambiguity. P. 14, L. 275-276: "Short-term acclimation at elevated temperature and pCO2 can increase physiological sensitivity of limpets against thermal stress."

*Q4: The sample n=100 per acclimation treatment that is a big sample size, so how many in total ~ 400? How many containers were the limpets in? To use as independent data each limpet would have to be housed in several containers. What was the density of the limpets in each container? These animals have distinct density dependent behavior – shown in many studies and this may influence outcome. It is not clear to me what was done with the100's of limpets when only ~10 were used for the experimental measures – perhaps I am missing something?*

**Response to Q4:** Responses to your comments were listed as follows:

(1) There were about 100 limpets which were reared in each acclimation treatment. As there were four acclimation treatments, about 400 limpets in total were used for the present study. There were three individuals in a container, and the density was ~1 limpet per 10 $cm^2$ in each acclimation treatment. As the density in the acclimation treatment is similar to that when we collected the samples, we thought that the influence of density dependent behavior on the outcome is limited. We suggest that this paragraph could be modified as follows to make it clearer.

P. 7, L. 98-111: "Samples were collected from Xiamen, and were transported back State Key Laboratory of Marine Environmental Science, Xiamen University, China within 2 h. Limpets were firstly allowed to recover at 20 °C for 3 d with a tidal cycle of approximately 6 h immersion and 6 h emersion. These limpets were randomly allocated into four acclimation treatments and temporally acclimated in different $pCO_2$ concentrations and temperatures (LTLC, 20 °C + 400 ppm, as a control treatment; LTHC, 20 °C + 1000 ppm; HTLC, 24 °C + 400 ppm; HTHC, 24 °C + 1000 ppm) for 7 d in climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which can control the $pCO_2$ concentration. There were about 100 indiv. per acclimation treatment, and the density was ~ 1 limpet per 10 cm2 in all acclimation treatments. This density was similar to that when we collected the samples. Control temperature (20 °C) and high temperature (24 °C), respectively, represent the average annual temperature in the collection site and the average global increase (4 °C) predicted for 2100 by the Intergovernmental Panel on Climate Change (IPCC, 2007). Two pCO2 levels, 400 ppm and 1000 ppm, represent the present-day situation and scenarios for 2100 respectively, as projected by IPCC (2007)."

(2) In the heat shock experiments, for each acclimation condition, 10 limpets were heated in each designated temperature (26, 30, 34 and 38 °C) and there was a non-heat-stressed group of 10 limpets, so there were 50 individuals in each acclimation treatment. In addition, about 10 individuals were used to test heart rates for each acclimation treatment. Considering that some individuals would die during the acclimation and heat process, ~ 100 individuals were acclimated in each treatment before experiments. The method section about the heat shock experiments was changed to: "After 7-day short-term acclimation, individuals from all four acclimation conditions (n = 10 indiv. per acclimation treatment) were randomly sampled and frozen at -80 °C as non-heated control samples. In each acclimation treatment, 40 limpets were randomly selected and were transferred to an

artificial rock (see Fig. A1). The rock was heated at a rate of 6 °C per hour (a natural heating rate, Han et al., 2013) to the designated temperatures (26, 30, 34 and 38 °C). The heat-shock treatments were carried out as described in Denny et al. (2006) (Fig. A2). After achieving the target temperature, the temperature was maintained for the allotted time, and then decreased to acclimated temperatures (20 or 24 °C) at a rate of 6 °C per hour, for a total exposure time of 7 h. After recovery at 20 or 24 °C seawater for 1 h, limpets (n = 8-10 indiv. per heat shock temperature at each acclimation condition) were immediately collected and stored at -80 °C for gene expression quantification." (P. 8, L. 147-159)

*Q5: Show a photo of the artificial rock.*

**Response to Q5:** The photo of the artificial rock (60 cm length × 30 cm width) was shown here added as shown in Figure A1. Limpets were placed on artificial rock and heated to the designated temperate.

*Q6: How where the n= 10, n=9-11 limpets selected for hsp and heart rate respectively. Were the latter in separate containers during this measurement? Use of CV is not mentioned in the stats section – also state why used.*

**Response to Q6:**

(1) Limpets were randomly selected from different containers of each acclimation treatment for both gene expression and heart rate experiments.

(2) Each limpet was placed in a separate container during the heart rate measurement.

(3) The reason why CV is chosen for the present study would be added in the statistical analysis section as follows. P. 12, L. 225-229: "The coefficient of variation (CV) of ABT, $Q_{10}$ and *hsc70* mRNA expression at 38 °C were calculated for each acclimation condition. The CV is the variance in a sample divided by the mean of that sample, providing a method to compare the variation within a sample relative to the mean. It is generally accepted that higher CV demonstrates that there is greater variation among individuals within one treatment than another (Reed et al., 2002)."

*Results*

*Q7: Just provide stats for significant results, so give the ANOVA results for the heart rate and post hoc for the heart rate but not the ABTs. For the latter just give mean and SE, say non significant and cite stats table. Same for the next paragraph.*

**Response to Q7:** More details about the analysis results would be provided in the results section (P. 12-13, L. 233-246).

"The maximal heart rate was ~ 30 % higher in limpets acclimated to control conditions (20 °C, 400 ppm) than the other treatments (Fig. 1 and Table A3) indicating reduced metabolic performance under high temperatures and *p*CO2 conditions. The ABTs of limpets ranged from 34.5 °C to 44.2 °C and showed a trend to be reduced for HT treatments (Fig. A4). Temperature (Two-way ANOVA, $F_{1, 35}$ = 3.375, P = 0.075) and $pCO_2$ (Two-way ANOVA, $F_{1, 35}$ = 0.118, P = 0.733) both had non-significant effects on ABTs, and there was a non-significant interaction between temperature and $pCO_2$ (Two-way ANOVA, $F_{1, 35}$ = 0.908, P = 0.347) (Table A4; Fig. A4).

Temperature coefficients ($Q_{10}$ rates) were higher for limpets acclimated at 20 °C than at 24 °C (Two-way ANOVA, $F_{1, 35}$ = 5.878, P = 0.02), but there was no significant difference for acclimation to different $pCO_2$ concentrations (Two-way ANOVA, $F_{1, 35}$ = 1.332, P > 0.05) and for the interaction between temperature and $pCO_2$ (Two-way ANOVA, $F_{1, 35}$ = 0.1135, P > 0.05) (Table A4; Fig. 2). The post-acclimation thermal sensitivity of limpets acclimated at low $CO_2$ (2.12) was lower than limpets at high $CO_2$ (2.95) (Fig. 2), indicating that the latter are more metabolically sensitive to temperature."

*Q8: Fig 2 – why are there no error bars on the post data – best to state why in the legend. Interesting that the hsp data was significant with just n=10 per treatment. Usually n=20 is the minimum.*

**Response to Q8:**

(1) According to the formula provided by Seebacher et al. (2015), calculation of post-acclimation $Q_{10}$ is done for the mean response of all individuals as the same individual are not used at each acclimation temperature. Therefore, no calculation of variation or error is possible. The reason why there are no error bars on the post data would be added in the legend (P. 28, L. 586-588).

"The calculation of post-acclimation $Q_{10}$ is done for the mean response of all individuals as the same individual are not used at each acclimation temperature. Therefore, there was no calculation of variation or error for post-acclimation."

(2) Some preliminary researches (e.g. Currie et al., 1999; Dong et al., 2008; Williams et al., 2011; Dong and Williams, 2011; Barshis et al., 2012) were carried out with less than 10 individuals in the heat shock experiments, and showed that such a sample size was reasonable for the *hsp* gene expression experiment. So we thought that the significance with n=10 was credible.

*Discussion*

*Q9: Paragraph 1 can be reduced – some of this is introduction type text. Only speak to the significant results and make this clear. State that higher thermal sensitivity to .... was indicated by increased heart rate.*

**Response to Q9**: P. 12-13, L. 243-253. Thanks for your useful suggestion. The first paragraph of the discussion section is reduced to: "Short-term acclimation at elevated temperature and $pCO_2$ can increase physiological sensitivity of limpets against thermal stress. Post-acclimation thermal sensitivity represents the extent to which ectothermic animals can acclimate to longer-term increases in temperature (several days to weeks) (Seebacher et al., 2015). Thus, the higher thermal sensitivity of limpets acclimated to 1000 ppm indicates that the resilience of limpets to thermal stress associated with warming will be compromised under future ocean acidification. This prediction is contrary to the general thought that intertidal ectotherms, such as limpets and other gastropods, will demonstrate high tolerance to thermal stress because they are adapted to an extreme thermal environment. For example, the operative temperatures, from which *C. toreuma* suffers in the field, frequently exceed 40 °C in summer along Asian coastlines and the limpet can survive at temperatures in excess of 45 °C (Dong et al., 2015). Our data show, however, that ocean acidification will lead to increased sensitivity to changes to future thermal regimes."

*Q10: It will be good to state what the CVs actually indicate. Overall perhaps for some measures the sample size was too low.*

**Response to Q10:** The definition of the coefficients of variation (CV) is stated as follows. "The CV is the variance in a sample divided by the mean of that sample, providing a method to compare the variation within a sample relative to the mean. It is generally accepted that higher CV demonstrates that there is greater variation among individuals within one treatment than another."

We aware that our results should be validated by a larger sample size, even though such a sample size (around 10 individuals for each treatment) is reasonable for the *hsp* gene expression experiment as it has been shown in some researches (e.g. Currie et al., 1999; Dong et al., 2008; Williams et al., 2011; Dong and Williams, 2011; Barshis et al., 2012). Therefore, we recommend that future research should be undertaken with a larger sample size.

*Q11: The hsp text could be expanded with regard to the species and methods comparisons. For instance, a lot of the work by Tomanek and colleagues involves other intertidal molluscs and on different heights on the shore etc. Are there any other studies of limpets etc.*

**Response to Q11**: P. 14-15, L. 287-306. The *hsp* text is expanded by comparing present study with previous researches on intertidal molluscs as follows.

"Increased temperature and $CO_2$ increase the sensitivity of heat shock responses to thermal stress. The expression of *hsp70* mRNA steadily increased from 20°C to 38°C for individuals across all experimental treatments. However, rates of upregulation of *hsp70* mRNA in limpets acclimated at high temperature and high $CO_2$ (HTHC) were significantly higher than those of limpets acclimated at the other three acclimation conditions. As a molecular chaperon, *Hsp70* plays crucial roles in maintaining protein stability with the expense of a large amount of energy (Feder and Hofmann, 1999; Tomanek and Sanford, 2003). By comparing the expression patterns of Hsp70 of different *Chlorostoma* species (formerly *Tegula*) that have distinct vertical distribution, Tomanek and Somero (1999, 2000) found that there existed interspecific difference in the frequency of the induction of *Hsp70* synthesis and interspecific divergence of the time-course of Hsp70 synthesis.

These studies from genus *Chlorostoma* suggested that species that live higher in the intertidal cost more energy for proteostasis and restore proteostasis to cope with a second consecutive day of high temperatures (Semero et al., 2016). Usually, the expression of *Hsp70* of less thermal-tolerant species is more sensitive to increases in temperature (limpet *Lottia*, Dong et al., 2008; snail *Chlorostoma*, Tomanek, 2002), and the rapid upregulation of *hsp70* mRNA in limpets exposed to future conditions potentially represents a high sensitivity of limpets to thermal stress in the face of ocean acidification. Due to the expensive energy consumption during the synthesis and function of *hsp70*, the more rapid upregulation of *hsp70* mRNA in these limpets also indicates more energy was allocated into cellular homeostasis, which then can affect the limpet's growth and reproduction. This change in the metabolic partitioning in individuals could ultimately lead to a decline in fitness and population-level responses."

*Q12: For the hsp – the sample size may have been too low to discern between constitutive and induced expression.*

**Response to Q12:** In the present study, the PCR primers (please see Table A2) were used to amplify induced *hsp70* gene, which could discern between constitutive and induced expression of *hsp70*.

*Q13: What studies have used gene expression–vs-protein expression. This might influence the comparisons being made. Just because the gene is expressed we really do not know if the protein is also expressed.*

**Response to Q13:** We assume that the protein is expressed when gene expression occurs for limpets which are heated to designated temperatures, considering that the expression patterns of heat shock protein gene (Zhang et al., 2014; Dong et al., 2014) are similar to the expression patterns of heat shock protein (Tomanek and Somero, 2002; Tomanek, 2002; Tomanek and Sanford, 2003; Dong et al., 2008; Dong and Williams, 2011) for some intertidal gastropods. One of the similar patterns is that both HSP gene expression and protein expression can be rapidly upregulated in respond to heat shock treatment (> 1000 folds more than the control and relatively low temperature shock). Therefore, we suggest that the high-throughput hsp gene expression in respond to heat shock can be translated to heat shock protein in the present study. This speculation needs further experimental evidence in the future study.

**General comments –**

*Q14: L. 21 state 7 days*

**Response to Q14:** P. 2, L. 20-21. It is changed to: "… individuals temporally acclimated (7 d) under combinations of different $pCO_2$ (400 ppm and 1000 ppm) and temperature (20 °C and 24 °C) regimes"

*Q15: For a short results section – 6 pages of references seems excessive –*

**Response to Q15:** In the revised manuscript, some redundant references have been deleted.

*Q16: L. 35 Scheffers et al could be deleted*

**Response to Q16:** This reference is deleted.

*Q16: L 46-49 – This is a general sentence – one ref will suffice*

**Response to Q17:** P. 3, L. 49-52. This sentence is change to: "In the face of a changing environment, organisms have three main options; shift their geographical distribution (Parmesan and Yohe, 2003), develop evolutionary adaptive changes (Hoffmann and Sgro, 2011), or perish (Fabricius et al., 2011)."

*Q17: L. 98-99 can delete much of this detail (eg falling high tide)*

**Response to Q18:** P. 7, L98-99. This sentence is reduced to: **"**Samples were collected from Xiamen, and were transported back State Key Laboratory of Marine Environmental Science, Xiamen University, China within 2 h."

*Q18: L. 367 – this is a discussion paper – not fully peer review – delete*

**Response to Q18:** This reference is deleted.

**References:**

Barshis, D. J., Ladner, J. T., Oliver, T. A., Seneca, F. O., Traylor-Knowles, N., & Palumbi, S. R. (2013). Genomic basis for coral resilience to climate change. *Proceedings of the National Academy of Sciences*, 110(4), 1387-1392.

Cai, M., Liu, Y., Chen, K., Huang, D., and Yang, S.: Quantitative analysis of anthropogenic influences on coastal water–A new perspective, Ecol. Indic., 67, 673-683, 2016.

Currie, S., Tufts, B. L., & Moyes, C. D. (1999). Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 276(4), R990-R996.

Doney, S. C., Fabry, V. J., Feely, R. A., & Kleypas, J. A. (2009). Ocean acidification: the other CO2 problem. *Annual review of marine science*, 1, 169-192.

Dong, Y. W., Han, G. D., & Huang, X. W. (2014). Stress modulation of cellular metabolic sensors: interaction of stress from temperature and rainfall on the intertidal limpet *Cellana toreuma*. *Molecular ecology*, 23(18), 4541-4554.

Dong, Y., Miller, L. P., Sanders, J. G., & Somero, G. N. (2008). Heat-shock protein 70 (Hsp70) expression in four limpets of the genus Lottia: interspecific variation in constitutive and inducible

synthesis correlates with in situ exposure to heat stress. *The Biological Bulletin*, 215(2), 173-181.

Dong, Y. W., & Williams, G. A. (2011). Variations in cardiac performance and heat shock protein expression to thermal stress in two differently zoned limpets on a tropical rocky shore. *Marine biology*, 158(6), 1223-1231.

Kwiatkowski, L., Gaylord, B., Hill, T., Hosfelt, J., Kroeker, K. J., Nebuchina, Y., ... & Caldeira, K. (2016). Nighttime dissolution in a temperate coastal ocean ecosystem increases under acidification. *Scientific reports*, 6.

Parker, L. M., Ross, P. M., O'Connor, W. A., Pörtner, H. O., Scanes, E., & Wright, J. M. (2013). Predicting the response of molluscs to the impact of ocean acidification. Biology, 2(2), 651-692.

Peck, L. S., Morley, S. A., Richard, J., & Clark, M. S.: Acclimation and thermal tolerance in Antarctic marine ectotherms. J. Exp. Biol., 217, 16-22, 2014.

Prusina, I., Sarà, G., De Pirro, M., Dong, Y. W., Han, G. D., Glamuzina, B., & Williams, G. A.: Variations in physiological responses to thermal stress in congeneric limpets in the Mediterranean Sea. J. Exp. Mar. Biol. Ecol., 456, 34-40, 2014.

Seebacher, F., White, C. R., & Franklin, C. E. (2015). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nature Climate Change*, 5(1), 61-66.

Tomanek, L. (2002). The heat-shock response: its variation, regulation and ecological importance in intertidal gastropods (genus Tegula). *Integrative and Comparative Biology*, 42(4), 797-807.

Tomanek, L., & Somero, G. N. (2002). Interspecific-and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus Tegula): implications for regulation of hsp gene expression. *Journal of Experimental Biology*, 205(5), 677-685.

Tomanek, L., & Sanford, E. (2003). Heat-shock protein 70 (Hsp70) as a biochemical stress indicator: an experimental field test in two congeneric intertidal gastropods (Genus: Tegula). *The Biological Bulletin*, 205(3), 276-284.

Williams, G. A., De Pirro, M., Cartwright, S., Khangura, K., Ng, W. C., Leung, P. T., & Morritt, D. (2011). Come rain or shine: the combined effects of physical stresses on physiological and protein-level responses of an intertidal limpet in the monsoonal tropics. *Functional Ecology*, 25(1), 101-110.

Zhang, S., Han, G. D., & Dong, Y. W. (2014). Temporal patterns of cardiac performance and genes encoding heat shock proteins and metabolic sensors of an intertidal limpet Cellana toreuma during sublethal heat stress. *Journal of thermal biology*, 41, 31-37.

## Reply to comments of anonymous referee #2

*Q1: The methods, most of all, were well explained, facilitating the understanding of the experiments. However, the limpets were acclimated for a short period of time (7 days) and submitted to different heat shock treatments for a maximum period of 7 h, only once during the whole experiment. No evidence of actual acclimation of these animals was presented (methods for assessing acclimation are discussed by Peck et al. in J. Exp. Biology (2014) 217, 16-22, doi: 10.1242 / jeb.089946). Therefore, contrary to the authors' conclusion the results of these experiments allow predictions of future scenario in a very limited way. The authors argue about a large variability of the physiological response in the population based on the coefficient of variation of the analyzed parameters. However, this coefficient is derived from a standard deviation that will be reliable if obtained from large population samples, which was not the case (around 10 individuals per treatment). Therefore, this could weaken the argument about the physiological plasticity.*

**Response to Q1**: According to the review by Peck and colleagues (Peck et al., 2014), changes in acute thermal tolerance (upper and lower critical and lethal temperatures, $CT_{min}$, $CT_{max}$, UTL and LTL) were used to assess the complete acclimation. Though the authors of this review suggested that Antarctic marine invertebrates required 2-5 months to complete whole-animal acclimation, they also pointed out that this conclusion should be noted as the successful acclimation was only observed in a very limited number of species. On the other hand, they suggested that the time needed to acclimate for temperate species is several times lower than that of Antarctic species. In the present study, we did not test the $CT_{max}$ and thus could not assess the complete acclimation at the whole-animal level in this respect. However, it is also difficult to deny that the short-term acclimation in the present study is not enough for the successful acclimation. As you suggested, we should be careful when making the conclusion that the present results allowed for the prediction of future scenario. We suggest that underlining the short-term acclimation in the conclusion section is important for correctly comprehending the results and conclusions of the present study.

There is no doubt that larger sample size can increase the reliability of the CVs. We aware that using the CVs with the sample size (10 individuals per treatment) might weaken the inference about the physiological plasticity. Therefore, in the discussion section we state that: "However, the results about the coefficients of variation need to be interpreted with caution, as the sample size (around 10 limpets per treatment) in the present study may affect the prediction accuracy."

**Specific comments**

*Q2: Title - The authors obtained evidence that only hsp70 expression was affected in acclimated limpets under HTHC conditions. CO2 level did not affect Q10, and the highest temperature decreased Q10. Therefore, the ocean acidification affected only hsp70. Then, the title does not specifically reflect the content.*

**Response to Q2**: Three main findings show the physiological plasticity of limpets acclimated at different conditions. (1) The post-acclimation $Q_{10}$ of limpets which were acclimated at high $pCO_2$ is much higher than those acclimated at low $pCO_2$, indicating the higher physiological plasticity of limpets to combined environmental stresses. (2) The Coefficients of variation (%) of Arrhenius

break temperature (ABT), temperature coefficients ($Q_{10}$) and *hsc70* mRNA expression at 38°C of limpets acclimated at high $CO_2$ are higher than those of the limpets acclimated at low $CO_2$. (3) The rates of upregulation of *hsp70* mRNA in limpets acclimated at high temperature and high $CO_2$ (HTHC) were significantly higher than those of limpets acclimated at the other three acclimation conditions. Therefore, we suggest that this title can reflect these three main findings. If the title only presents the significant upregulation of *hsp70* mRNA, some other important findings would be lost.

*Q3: The paragraph between lines 86 and 93 should be in the introduction. The determination of seawater characteristics (lines 112 - 122) should be in a separate item.*

**Response to Q3**: It is a useful advice and this adjustment would make the manuscript readable. We have added the paragraph in the introduction.

*Q4: The authors should make it clear if the limpets were kept in a chamber with constant CO2 concentration in the air during thermal shock.*

**Response to Q4**: During the thermal shock, the limpets were exposed to air, instead of a chamber with constant $CO_2$ concentration.

*Q5: On the line 267, the phrase "If only one environmental factor changed (i.e., temperature or CO2) ..." is not sufficiently clear to me.*

**Response to Q5**: This sentence is rephrased to make it clear. “In the present study, for limpets acclimated under HTLC and LTHC (i.e., only temperature or $CO_2$ condition changed in comparison with the LTLC treatment), there was significant upregulation of *hsc70* mRNA when the heat shock temperatures were beyond 30 °C.”

*Q6: The discussion about why the expression of hsc70 was not affected by the treatments is insufficient. Why was this protein chosen to analysis? Is it sensitive to temperature rise in other species? Do other factors affect its expression? The discussion needs to be expanded. The conclusion and abstract must be rewritten because an incomplete acclimatization may have occurred and the experiment did not reproduce with reasonable fidelity a future scenario in which the limpets would be exposed to thermal shock.*

**Response to Q6**:

(1) *Hsc70* is the constitutively expressed protein and is important for the chaperoning function under unstressed conditions, while the *Hsp70* is inducible protein and crucial when species suffering acute stress. Basically, *Hsc70* and *Hsp70* have different expression patterns. However, some studies showed that *Hsc70* and *Hsp70* have similar response patterns to stress (please see a review by Morris et al. 2013). Also, the response patterns may reflect adaptive strategy to the environment. Therefore, choosing both *hsp70* and *hsc70* is helpful for us to understand how limpets respond to the heat stress at both constitutive and inducible expression levels.

(2) The expression of *hsc70* is the constitutively expressed form and only mildly induced during

heat stress. Some studies, however, showed that thermal stress could significantly induce the up-regulation of both *hsc70* gene and *Hsc70* protein, such as 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).

The discussion section about *hsc70* was expanded as follows. "The expression patterns of *hsc70* mRNA were different among limpets at the four acclimation conditions. *Hsc70* is constitutively expressed and is a molecular chaperone involved in the *in vivo* folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain similar promoter regions, there are differential expressions to a given stimulus between them (Hansen et al., 1991). Some studies showed that thermal stress could significantly induce the up-regulation of both *hsc70* gene and *Hsc70* protein in the killifish *Fundulus heteroclitus* (Fangue et al., 2006), the shrimp *Penaeus monodon* (Chuang et al., 2007), and the coral *Veretillum cynomorium* (Teixeira et al., 2013). In the present study, for limpets acclimated under HTLC and LTHC (i.e., only temperature or $CO_2$ condition changed in comparison with the LTLC treatment), there was significant upregulation of *hsc*70 mRNA when the heat shock temperatures were beyond 30 °C. However, the expression of *hsc70* mRNA showed no significant difference among different heat-shock temperatures under predicated future environmental conditions (HTHC: 24 °C and 1000 ppm). These results indicate that the upregulation of *hsc70* mRNA in response to heat shock represents an increasing capability for coping with the enhanced protein denaturation and more energy allocated into the somatic maintenance after being exposed to either warming or high $CO_2$ environment. The insignificant upregulation of *hsc70* in response to thermal stress indicates that limpets acclimated under HTHC may employ a "preparative defense" strategy (Dong et al., 2008) to maintain high constitutive levels of *hsc70* as a mechanism to copy with unpredictable heat stress. However, the absence of significant upregulation of *hsc70* mRNA in limpets acclimated to future conditions (warming and elevated $CO_2$) might also be attributed to the very high variation of gene expression at 38°C (CV, 90.36 %). In the context of future conditions, multiple environmental stressors can induce diverse physiological responses among different individuals, which might be an evolutionary adaptation to the harsh environment on the shore."

(3) In addition to heat, other factors like cold, heavy metals, ethanol, toxin, hypoxia and acidosis can also increase the expression of *hsc70* (see reviews by Roberts et al., 2010; Liu et al., 2012).

(4) The present study has only investigated the physiological responses of limpets to heat stress after short-term acclimation. Consequently, the abstract and conclusion sections should be rephrased.

The conclusion section was changed to: "In conclusion, the resilience of intertidal limpets to thermal stress is weakened after exposure to predicted future conditions for a short-term acclimation period (7 d). Yet, the combination of elevated temperature and $CO_2$ concentration prompted divergence of physiological and molecular responses. These results suggest that while organisms may be able to protect themselves from the damaging effects of thermal stress in the short-term, changes to multiple environmental conditions may drive population-level responses through physiological responses (e.g. Giomi et al., 2016). Further, the increased variation in responses, and the observation that some individuals were more capable to physiologically cope with the conditions, may be associated with intergenerational adaptation, but this speculation needs further evidence. As the "weaker" individuals are lost, the offspring in the next generation will be better physiologically

adapted to warming under high-$CO_2$ conditions. Therefore, while elevated $CO_2$ and the associated ocean acidification decrease the ability of many individuals to respond to thermal stress, it appears that physiological plasticity and variability could be adaptive mechanisms in at least some populations of intertidal organisms. Our research underlined the importance of physiological plasticity and variability for coastal species coping with warming and ocean acidification. However, the present study has only examined the physiological responses of limpets to heat stress after short-term acclimation. Future studies with long-term acclimation and a larger sample size are therefore recommended in order to validate our findings."

**References:**


Chuang, K., Ho, S., & Song, Y. (2007). Cloning and expression analysis of heat shock cognate 70 gene promoter in tiger shrimp (Penaeus monodon). Gene, 405(1), 10-18.

Dong, Y., Han, G., Ganmanee, M., & Wang, J. (2015). Latitudinal variability of physiological responses to heat stress of the intertidal limpet Cellana toreuma along the Asian coast. Marine Ecology Progress Series, 107-119.

Fangue, N. A., Hofmeister, M., & Schulte, P. M. (2006). Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, Fundulus heteroclitus. The Journal of Experimental Biology, 209(15), 2859-2872.

Giomi, F., Mandaglio, C., Ganmanee, M., Han, G., Dong, Y., Williams, G. A., & Sara, G. (2016). The importance of thermal history: costs and benefits of heat exposure in a tropical, rocky shore oyster. The Journal of Experimental Biology, 219(5), 686-694.

Liu, T., Daniels, C. K., & Cao, S. (2012). Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. Pharmacology & therapeutics, 136(3), 354-374.

Morris, J. P., Thatje, S., & Hauton, C. (2013). The use of stress-70 proteins in physiology: a re-appraisal. Molecular Ecology, 22(6), 1494-1502.

Peck, L. S., Morley, S. A., Richard, J., & Clark, M. S. (2014). Acclimation and thermal tolerance in Antarctic marine ectotherms. The Journal of Experimental Biology, 217(1), 16-22.

Roberts, R. J., Agius, C., Saliba, C., Bossier, P., & Sung, Y. Y. (2010). Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. Journal of fish diseases, 33(10), 789-801.

Teixeira, T., Diniz, M. S., Calado, R., & Rosa, R. (2013). Coral physiological adaptations to air exposure: Heat shock and oxidative stress responses in Veretillum cynomorium. Journal of Experimental Marine Biology and Ecology, 35-41.

## (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 underlined the short-term acclimation of the present study and corresponding conclusion was rephrased.

- *Introduction*: The paragraphs about region and species were moved here from methods section. Approaches and hypotheses were added. Some redundant literatures were removed.

- *Material and Methods*: The description about the heating treatment was rephrased. The use of coefficient of variation and the reason why used were stated.

- *Results*: We added some detailed results of the Two-way ANOVA for the analysis of cardiac performance and a table (Table A4) was added in the Appendix.

- *Discussion*: As recommended by the reviewers, the discussion of *hsp70* and *hsc70* should be expanded. The discussion about the responses of heat shock protein was expanded by comparing present study and with previous researches on intertidal molluscs. We mentioned that the conclusion of the present study was made based on the short-term acclimation.

- *Appendix*: A photo of the artificial rock was added in the appendix section. A table of the Two-way ANOVA analysis for the heart rate was provided.

**(3)**

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

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

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

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

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

**Abstract.** Understanding physiological responses of organisms to warming and ocean acidification is the first step towards predicting the potential population~~,~~ and community-level, ~~and~~ ecological impacts of these stressors. Increasingly, physiological plasticity is being recognized as important for organisms to adapt to the changing microclimates. Here, we evaluate the importance of physiological plasticity for coping with ocean acidification and elevated temperature, and its variability among individuals from the same population, of the limpet *Cellana toreuma*. Heart rates (as a proxy for metabolic performance) and genes encoding heat-shock proteins were measured at different heat shock temperatures (26, 30, 34~~,~~ and 38 °C) in individuals temporally acclimated (7 d) under combinations of different $pCO_2$ (400 ppm~~,~~ and 1000 ppm) and temperature (20 °C~~,~~ and 24 °C) regimes. Analysis of heart rate showed significantly higher temperature coefficients ($Q_{10}$ rates) for limpets at 20 °C than at 24 °C and ~~lower~~ post-acclimation thermal sensitivity of limpets at 400 ppm was lower than at 1000 ppm. ~~*hsp70* expression~~Expression of *hsp70* linearly increased with the increasing heat-shock temperatures, with the largest slope occurring in limpets acclimated under a future scenario (24 °C and 1000 ppm *p*CO2). These results suggested that limpets~~ will have~~ increased sensitivity and ~~energy consumption~~stress response under future conditions. Furthermore, the increased variation in physiological response under the future scenario indicated that some individuals were better to cope physiologically with these conditions. ~~Therefore, w~~While short-term acclimation at acidic seawater ~~ocean acidification~~ decreases the ability of ~~many~~ partial individuals ~~to respond to~~against thermal stress, physiological plasticity and variability seem to be crucial in allowing some intertidal animals to survive in a rapidly changing environment.

## 1 Introduction

Benthic organisms living in the intertidal zone will be exposed to increasingly variable and extreme environmental conditions, such as temperature, oxygen and $CO_2$, due to climatic change (IPCC, 2013; Kwiatkowski et al., 2016~~; Scheffers et al., 2016~~). These highly fluctuating environmental variables can significantly affect the physiological performance of coastal species (Helmuth et al., 2006; Hofmann and Todgham, 2010; Somero, 2012; Widdicombe and Spicer, 2008). Therefore, understanding the interaction of multiple environmental stressors on the physiological performance is crucial for predicting the consequences of environmental change on ecosystems (Deutsch et al., 2015). For example, salinity fluctuations coupled with high temperatures during emersion can have both sub-lethal physiological effects and lethal effects on intertidal molluscs (Firth and Williams, 2009; Dong et al., 2014). ~~Indeed, it is increasingly being recognized that the interaction between global warming and ocean acidification may not only~~ Although ocean acidification can increase the growth of organisms in some cases (Gooding et al., 2009), increasing evidence showed that rising ocean acidity exacerbates global warming, reduces an organism's resistance to environmental change (Munday et al., 2009), ~~but~~ and subsequently affects population dynamics (Fabry et al., 2008; Hoegh-Guldberg et al., 2007; Kroeker et al., 2013; Rodolfo-Metalpa et al., 2011).

In the face of a changing environment, organisms have three main options; shift their geographical distribution (~~Barry et al., 2011; Bellard et al., 2012;~~ Parmesan and Yohe, 2003~~; Perry et al., 2005; Sunday et al., 2012~~), develop evolutionary adaptive changes (Hoffmann and Sgro, 2011~~; Sunday et al., 2014~~), or perish (Fabricius et al., 2011). Prior to mortality or range-shifts, environmental changes can often drive physiological adaptation or the evolution of phenotypic plasticity (Chevin et al., 2010; Sanford and Kelly, 2011). Yet, warming and ocean acidification are not unidirectional, but rather combined with rapid

55 fluctuations on daily to seasonal and decadal time-scales. Thus, the changing environment often does not
56 provide clear signals to drive strong directional selection of traits, meaning that, usually, physiological
57 plasticity is the more important factor in acclimation to changing environmental conditions (Hoffmann
58 and Sgro, 2011; Pörtner et al., 2012; Somero et al., 2012). In a recent meta-analysis, Seebacher et al.
59 (2015) demonstrated that acclimation to higher temperatures decreased the sensitivity to climate change
60 in both freshwater and marine animals. While this response suggests that acclimation could reduce the
61 impact of warming on organisms, the responses were only tested for shifts in mean temperature. Yet,
62 organisms inhabiting variable environments, such as the intertidal zone, will be exposed to increasing
63 extremes in temperature concomitant with increasing $pCO_2$, or ocean acidification (OA), in the future.
64 While OA has been suggested to increase the sensitivity of organisms to warming (Byrne and Przeslawski,
65 2013; Gibson et al., 2011; Kroeker et al., 2013), physiological plasticity and variation in responses may
66 provide the basis for populations to survive.

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

75 The limpet *C. toreuma* is a keystone species on rocky shores in the Western Pacific (Dong et al.,
76 2012) and occupies mid–low intertidal zones (Morton and Morton 1983). This species is a gonochoric

and broadcast spawner, whose embryos develop into planktonic trocophore larvae and later into juvenile veligers before becoming fully grown adults (Ruppert et al., 2004). As a common calcifier inhabiting coastal ecosystem, *C. toreuma* plays an important ecological role in affecting the community structure of the associated biofilm. Therefore, this species is a key organism for studying the relationship between physiological response to thermal stress and ocean acidification in highly variable environment on the shore.

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

~~Therefore,~~Here, we investigated the importance of physiological plasticity and variability for *C. toreuma* to cope with ocean acidification and elevated temperatures by quantifying heart rates (as a proxy of metabolic performance) and expression of genes encoding heat-shock proteins after short-term acclimation in different $pCO_2$ concentrations (400 ppm and 1000 ppm) and temperatures (20 °C and 24 °C). We hypothesize that (1) limpets will increase their thermal sensitivity of metabolism and stress responses under elevated $pCO_2$ and temperatures; (2) short-term acclimation at high temperature and $pCO_2$ will cause higher inter-individual physiological variation. This study provides novel information concerning the combined effects of increased temperature and $pCO_2$ on physiological plasticity in intertidal invertebrates, and is important in allowing predications of the ecological impacts of the future environmental changes.

## 2 Material and Methods

### ~~2.1~~1.1 ~~Sample locality and study organism~~

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

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

### ~~2.2~~2.1 Limpet collection and experiment treatments

~~The following experiments were conducted for the first time in July 2014 and the same experiment was repeated in July 2016, which was to improve the quantity and quality of the data.~~ Samples were

collected from Xiamen ~~on a falling high tide~~, and were transported back State Key Laboratory of Marine Environmental Science, Xiamen University, China within 2 h. Limpets were firstly allowed to recover at 20 °C for 3 d with a tidal cycle of approximately 6 h immersion and 6 h emersion. These limpets were randomly allocated into four acclimation treatments ~~(about 100 indiv. per acclimation treatment)~~and temporally acclimated ~~for 7 d~~ in different $pCO_2$ concentrations and temperatures (LTLC, 20 °C + 400 ppm, as a control treatment; LTHC, 20 °C + 1000 ppm; HTLC, 24 °C + 400 ppm; HTHC, 24 °C + 1000 ppm) for 7 d in climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which can control the *p*CO2 concentration. There were about 100 indiv. per acclimation treatment, and the density was ~ 1 limpet per 10 $cm^2$ in all acclimation treatments. This density was similar to that when we collected the samples. Control temperature (20 °C) and high temperature (24 °C), respectively, represent the average annual temperature in the collection site and the average global increase (4 °C) predicted for 2100 by the Intergovernmental Panel on Climate Change (IPCC, 2007). Two $pCO_2$ levels, 400 ppm and 1000 ppm, represent the present-day situation and scenarios for 2100 respectively, as projected by IPCC (2007).

Animals were kept in a simulated tidal cycle with 6 h aerial exposure and 6 h seawater immersion. Seawater was pre-bubbled with air containing the corresponding $pCO_2$ concentrations in advance. pH was measured before and after the acclimation in seawater each time with PB-10 pH meter (Sartorius Instruments, Germany) calibrated with National Institute of Standards and Technology standard pH solutions (NIST, USA). Total dissolved inorganic carbon (DIC) was measured before and after the acclimation in seawater each time using a Li-Cor® non-dispersive infrared (NDIR) detector (Li-6252) by a dissolved inorganic carbon analyzer (As-C3, Apollo SciTech, Colorado, USA) with a precision of 0.1% (Cai, 2003). Seawater carbonate chemistry parameters were estimated based on the measured values of

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

After 7-day short-term acclimation, individuals from all four acclimation conditions (n = 10 indiv. per acclimation treatment) were randomly sampled and frozen at -80 °C as non-heated control samples. ~~The remaining limpets were transferred to an artificial rock and heated at a rate of 6 °C per hour, to simulate a natural heating rate in summer during low tide in Xiamen Bay as described by Han et al. (2013), to designated temperatures (26, 30, 34 and 38 °C).~~ In each acclimation treatment, 40 limpets were randomly selected and were transferred to an artificial rock (see Fig. A1). The rock was heated at a rate of 6 °C per hour (a natural heating rate, Han et al., 2013) to the designated temperatures (26, 30, 34 and 38 °C). The heat-shock treatments were carried out as described in Denny et al. (2006) (Fig. ~~A1~~A2). After achieving the target temperature, the temperature was maintained for the allotted time, and then decreased to acclimated temperatures (20 or 24 °C) at a rate of 6 °C per hour, for a total exposure time of 7 h. After recovery at 20 or 24 °C seawater for 1 h, limpets (n = 8-10 indiv. per heat shock temperature at each acclimation condition) were immediately collected and stored at -80 °C for gene expression quantification.

### ~~2.3~~2.2 Cardiac performance measurement

The cardiac performance of limpets was recorded during whole heating processes from the

acclimated temperature (20 or 24 °C) to the temperature where the heart stopped beating (n = 9-11 indiv. per acclimation treatment). Each limpet was placed in a separate container during the measurement. Heart rates were measured using a non-invasive method (Chelazzi et al., 2001; Dong and Williams, 2011). The heartbeat was detected by means of an infrared sensor fixed with Blue-Tac (Bostik, Staffordshire, UK) on the limpet shell at a position above the heart. Variation in the light-dependent current produced by the heartbeat were amplified, filtered and recorded using an infrared signal amplifier (AMP03, Newshift, Leiria, Portugal) and Powerlab AD converter (4/30, ADInstruments, March-Hugstetten, Germany). Data were viewed and analyzed using Lab Chart (version 7.0).

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

### ~~2.4~~2.3 Quantifying genes expression

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

The levels of mRNA of genes encoding two heats hock proteins, inducible heat-shock protein 70

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

### ~~2.5~~2.4 Statistical analysis

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

Thermal sensitivity stands for the change in a physiological rate function reacting to a rapid change in environmental temperature within the same acclimation set temperature (Fig. A2, modified from

Seebacher et al. (2015)). In the present study, thermal sensitivity is seen in the temperature coefficient ($Q_{10}$) values of heart rate. $Q_{10}$ was calculated using heart-rate data from the temperature at which the experiment started ($T_1$ = 24 °C) to the temperature to which temperature increased 10 °C ($T_2$ = 33 °C) with Eq. (1):

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

where R is the heart rate ($R_1$ and $R_2$ are the heart rate at $T_1$ and $T_2$ respectively), and T is the temperature (Kelvin) (Fig. A2, modified from Seebacher et al. (2015)). The differences in $Q_{10}$ among the four acclimation conditions with different $CO_2$ concentrations (400 ppm vs. 1000 ppm) and temperatures (20 °C vs. 24 °C) were analyzed using two-way ANOVA with Duncan's *post hoc* analysis using the SPSS 20.0 for Windows statistical package (IBM SPSS Statistics, Chicago, USA). Post-acclimation thermal sensitivity of limpets in different $CO_2$ concentrations were calculated as described by Seebacher et al. (2015). In each $CO_2$ concentration (400 ppm or 1000 ppm), the post-acclimation $Q_{10}$ values were calculated using the same equation as shown above, but $R_2$ was the heart rate of the warm-acclimated limpets at the acclimated temperature ($T_2$ = 24 °C), and $R_1$ was the heart rate of cold-acclimated limpets at $T_1$ = 20 °C (Fig. ~~A2~~A3, modified from Seebacher et al. (2015)).

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

The coefficient of variation (CV) of ABT, $Q_{10}$ and *hsc70* mRNA expression at 38 °C were calculated for each acclimation condition. The CV is the variance in a sample divided by the mean of

that sample, providing a method to compare the variation within a sample relative to the mean. It is generally accepted that higher CV demonstrates that there is greater variation among individuals within one treatment than another (Reed et al., 2002).

## 3 Results

### 3.1 Cardiac performance

The maximal heart rate was ~ 30 % higher in limpets acclimated to control conditions (20 °C, 400 ppm) than the other treatments (Fig. 1 and Table A3) indicating reduced metabolic performance under high temperatures and $pCO_2$ conditions. The ABTs of limpets ranged from 34.5 °C to 44.2 °C and showed a trend to be reduced for HT treatments (Fig. A4), ~~but did not differ statistically (Fig. A3; Two-way ANOVA, P > 0.05)~~. Temperature (Two-way ANOVA, $F_{1, 35} = 3.375$, $P = 0.075$) and $pCO_2$ (Two-way ANOVA, $F_{1, 35} = 0.118$, $P = 0.733$) both had non-significant effects on ABTs, and there was a non-significant interaction between temperature and $pCO_2$ (Two-way ANOVA, $F_{1, 35} = 0.908$, $P = 0.347$) (Table A4; Fig. A4).

Temperature coefficients ($Q_{10}$ rates) were higher for limpets acclimated at 20 °C than at 24 °C (~~Fig. 2,~~ Two-way ANOVA, $F_{1, 35} = 5.878$, $P = 0.02$), but there was no significant difference for acclimation to different $pCO_2$ concentrations (Two-way ANOVA, $F_{1, 35} = 1.332$, $P > 0.05$) and for the interaction between temperature and $pCO_2$ (Two-way ANOVA, $F_{1, 35} = 0.1135$, $P > 0.05$) (Table A4; Fig. 2). The post-acclimation thermal sensitivity of limpets acclimated at low $CO_2$ (2.12) was lower than limpets at high $CO_2$ (2.95) (Fig. 2), indicating that the latter are more metabolically sensitive to temperature.

The coefficients of variations (CV) of ABT in the four different acclimation conditions were

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

### 3.2 Gene expression

Levels of *hsp70* mRNA (log-transformed) linearly increased with the increasing heat-shock temperatures (Fig. 3). ANCOVA analysis showed that the slopes of the linear regressions were significantly different among different acclimation conditions ($F = 42.62$, $P < 0.001$), and the slope of HTHC limpets was higher than those of the other three acclimation conditions. Thus, the rate of increase in production of *hsp70* mRNA in response to warming was greater at the elevated $CO_2$ concentration.

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

## 4 Discussion

~~Ocean acidification and thermal stress are inherently linked to rising atmospheric $pCO_2$ and will be~~

~~manifested in combination in the future (Bijma et al., 2013; Connelll and Russell, 2009; Hale et al., 2011; Walther et al., 2009). Despite this certainty and the likelihood that ocean acidification will affect the physiological plasticity to thermal stress (Pörtner et al., 2010), there is currently limited information on how this may manifest in populations of organisms which inhabit stressful environments (Dupont and Thorndyke, 2009; Dupont and Pörtner, 2013). Here, we show that the thermal sensitivity of limpets acclimated to current atmospheric $CO_2$ (~ 400 ppm) is lower than that of limpets acclimated to 1000 ppm (2.12 vs. 2.95, respectively).~~ Short-term acclimation at elevated temperature and $pCO_2$ can increase physiological sensitivity of limpets against thermal stress. Post-acclimation thermal sensitivity represents the extent to which ectothermic animals can acclimate to longer-term increases in temperature (several days to weeks) (Seebacher et al., 2015). Thus, the higher thermal sensitivity of limpets acclimated to 1000 ppm indicates that the resilience of limpets to thermal stress associated with warming will be compromised under future ocean acidification. This prediction is contrary to the general thought that intertidal ectotherms, such as limpets and other gastropods, will demonstrate high tolerance to thermal stress because they are adapted to an extreme thermal environment. For example, the operative temperatures, from which *C. toreuma* suffers in the field, frequently exceed 40 °C in summer along Asian coastlines and the limpet can survive at temperatures in excess of 45 °C (Dong et al., 2015). Our data show, however, that ocean acidification will lead to increased sensitivity to changes to future thermal regimes.

Increased temperature and $CO_2$ increase the sensitivity of heat shock responses to thermal stress. The expression of *hsp70* mRNA steadily increased from 20°C to 38°C for individuals across all experimental treatments. However, rates of upregulation of *hsp70* mRNA in limpets acclimated at high temperature and high $CO_2$ (HTHC) were significantly higher than those of limpets acclimated at the other three

acclimation conditions. As a molecular chaperon, *Hsp70* plays crucial roles in maintaining protein stability with the expense of a large amount of energy (Feder and Hofmann, 1999; Tomanek and Sanford, 2003). By comparing the expression patterns of *Hsp70* of different *Chlorostoma* species (formerly *Tegula*) that have distinct vertical distribution, Tomanek and Somero (1999, 2000) found that there existed interspecific difference in the frequency of the induction of *Hsp70* synthesis and interspecific divergence of the time-course of *Hsp70* synthesis. These studies from genus *Chlorostoma* suggested that species that live higher in the intertidal cost more energy for proteostasis and restore proteostasis to cope with a second consecutive day of high temperatures (Semero et al., 2016). Usually, the expression of ~~*hsp70*~~ *Hsp70* of less thermal-tolerant species is more sensitive to increases in temperature (limpet *Lottia*, Dong et al., 2008; snail *Chlorostoma*, Tomanek, 2002), and the rapid upregulation of *hsp70* mRNA in limpets exposed to future conditions potentially represents a high sensitivity of limpets to thermal stress in the face of ocean acidification. Due to the expensive energy consumption during the synthesis and function of *hsp70*, the more rapid upregulation of *hsp70* mRNA in these limpets also indicates more energy was allocated into cellular homeostasis, which then can affect the limpet's growth and reproduction. This change in the metabolic partitioning in individuals could ultimately lead to a decline in fitness and population-level responses.

The expression patterns of *hsc70* mRNA were different among limpets at the four acclimation conditions. *Hsc70* is constitutively expressed and is a molecular chaperone involved in the *in vivo* folding and repair of denatured proteins (Dong et al., 2015). Although *hsp70* and *hsc70* contain similar promoter regions, there are differential expressions to a given stimulus between them (Hansen et al., 1991). Some studies showed that thermal stress could significantly induce the up-regulation of both *hsc70* gene and *Hsc70* protein in the killifish *Fundulus heteroclitus* (Fangue et al., 2006), the shrimp *Penaeus monodon*

(Chuang et al., 2007), and the coral *Veretillum cynomorium* (Teixeira et al., 2013). In the present study, for limpets acclimated under HTLC and LTHC (i.e. only temperature or $CO_2$ condition changed in comparison with the LTLC treatment), there was significant upregulation of *hsc*70 mRNA when the heat shock temperatures were beyond 30 °C. However, the expression of *hsc70* mRNA showed no significant difference among different heat-shock temperatures under predicated future environmental conditions (HTHC: 24 °C and 1000 ppm). ~~If only one environmental factor changed (i.e., temperature or $CO_2$), however, there was significant upregulation of *hsc70* mRNA when the heat shock temperatures were beyond 30 °C. These results indicate that expression of *hsc70* mRNA is relatively constitutive. That is,~~ These results indicate that the upregulation of *hsc70* mRNA in response to heat shock represents an increasing capability for coping with the enhanced protein denaturation and more energy allocated into the somatic maintenance after being exposed to either warming or high $CO_2$ environment ~~for weeks~~. The insignificant upregulation of *hsc70* in response to thermal stress indicates that limpets acclimated under HTHC may employ a "preparative defense" strategy (Dong et al., 2008) to maintain high constitutive levels of *hsc70* as a mechanism to copy with unpredictable heat stress. However, the absence of significant upregulation of *hsc70* mRNA in limpets acclimated to future conditions (warming and elevated $CO_2$) might also be attributed to the very high variation of gene expression at 38°C (CV, 90.36 %). In the context of future conditions, multiple environmental stressors can induce diverse physiological responses among different individuals, which might be an evolutionary adaptation to the harsh environment on the shore.

Variation and plasticity in both physiological and molecular responses to thermal stress are not only important for coping with future environmental change but also underpin evolutionary and adaptive changes through selective pressures (Franks and Hoffmann, 2012). In the present study, the coefficients

of variation in physiological responses of limpets acclimated in simulated future conditions, including ABT, $Q_{10}$ and *hsc70* mRNA, were higher than those in the other three acclimation conditions. Crucially, this means that a subset of individuals in our experimental population might be more physiologically pre-adapted to cope with heat shock. Once acclimated to future climate change scenario (warming and ocean acidification), this variation in physiological performance increased, indicating that in a harsher environment the physiological plasticity of some individuals allows them to modify their physiological tolerance limits and increase chances for survival and reproduction (Williams et al., 2008). Under high selective pressure, these individuals would form the basis for future generations while less plastic individuals would be removed from populations. However, the results about the coefficients of variation need to be interpreted with caution, as the sample size (around 10 limpets per treatment) in the present study may affect the prediction accuracy.

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

356 appears that physiological plasticity and variability could be adaptive mechanisms in at least some
357 populations of intertidal organisms. Our research underlined the importance of physiological plasticity
358 and variability for coastal species coping with warming and ocean acidification. However, the present
359 study has only examined the physiological responses of limpets to heat stress after short-term acclimation.
360 Future studies with long-term acclimation and a larger sample size are therefore recommended in order
361 to validate our findings.
362
363 **Authors' contributions**
364 B.D.R and Y.-W.D. designed experiments. W.J. and M.-W.D. conducted experiments. Y.-W.D., B.D.R,
365 W.J. and M.-W.D. performed analyses. The manuscript was co-written by Y.-W.D., W.J. and M.-W.D.,
366 and revised by B.D.R.
367
368 **Competing interests**
369 The authors declare no conflict of interests.
370
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376
377 **References**

Angilletta, M. J., Zelic, M. H., Adrian, G. J., Hurliman, A. M., and Smith, C. D.: Heat tolerance during embryonic development has not diverged among populations of a widespread species (*Sceloporus undulatus*), Conservation Physiology, 1, cot018, 2013.

Bao, B. and Ren, G. Y.: Climatological characteristics and long-term change of SST over the marginal seas of China, Cont. Shelf Res., 77, 96-106, 2014.

~~Barry, J. P., Widdicombe, S., and Hall Spencer, J. M.: Effects of ocean acidification on marine biodiversity and ecosystem function. In: Gattuso JP & Hansson L (Eds.), Ocean Acidification, 192-209, 2011.~~

~~Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W., and Courchamp, F.: Impacts of climate change on the future of biodiversity, Ecol. Lett., 15, 365-377, 2012.~~

~~Bijma, J., Pörtner, H. O., Yesson, C., and Rogers, A. D.: Climate change and the oceans–What does the future hold?, Mar. pollut. Bull., 74, 495-505, 2013~~

Byrne, M. and Przeslawski, R.: Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories, Integr. Comp. Biol., 53, 582-596, 2013.

Cai, W. J.: Riverine inorganic carbon flux and rate of biological uptake in the Mississippi River plume, Geophys. Res. Lett., 30, 2003.

Cai, M., Liu, Y., Chen, K., Huang, D., and Yang, S.: Quantitative analysis of anthropogenic influences on coastal water–A new perspective, Ecol. Indic., 67, 673-683, 2016.

Chelazzi, G., De Pirro, M., and Williams, G. A.: Cardiac responses to abiotic factors in two tropical limpets, occurring at different levels of the shore, Mar. Biol., 139, 1079-1085, 2001.

Chevin, L. M., Lande, R., and Mace, G. M.: Adaptation, plasticity, and extinction in a changing environment: towards a predictive theory, PLoS Biol., 8, e1000357, 2010.

~~Connell, S. D. and Russell, B. D.: The direct effects of increasing CO2 and temperature on non-calcifying organisms: increasing the potential for phase shifts in kelp forests, P. Roy. Soc. Lond. B Biol. Sci., rspb20092069, 2010.~~

Denny, M. W., Miller, L. P., and Harley, C. D.: Thermal stress on intertidal limpets: long-term hindcasts and lethal limits, J. Exp. Biol., 209, 2420-2431, 2006

Deutsch, C., Ferrel, A., Seibel, B., Portner, H. O., and Huey, R. B.: Climate change tightens a metabolic constraint on marine habitats, Science, 348, 1132-1135, 2015.

Dickson, A. G.: Standard potential of the reaction: AgCl(s) + H2(g) = Ag(s) + HCl(aq), and the standard acidity constant of the ion HSO4−in synthetic sea water from 273.15 to 318.15 K, J. Chem. Thermodyn., 22, 113–127, 1990.

Dong, Y. W., Han, G. D., Ganmanee, M., and Wang, J.: Latitudinal variability of physiological responses to heat stress of the intertidal limpet *Cellana toreuma* along the Asian coast, Mar. Ecol. Progr. Ser., 529, 107-119, 2015.

Dong, Y. W., Han, G. D., and Huang, X. W.: Stress modulation of cellular metabolic sensors: interaction of stress from temperature and rainfall on the intertidal limpet *Cellana toreuma*, Mol. Ecol., 23, 4541-4554, 2014.

Dong, Y. W., Li, X. X., Choi, F. M., Williams, G. A., Somero, G. N., and Helmuth, B.: Untangling the roles of microclimate, behaviour and physiological polymorphism in governing vulnerability of intertidal snails to heat stress, Proc. R. Soc. B., 284, 2017.

Dong, Y., Miller, L. P., Sanders, J. G., and Somero, G. N.: Heat-shock protein 70 (Hsp70) expression in four limpets of the genus *Lottia*: interspecific variation in constitutive and inducible synthesis correlates with in situ exposure to heat stress, Biol. Bull., 215, 173-181, 2008.

Dong, Y. W., Wang, H. S., Han, G. D., Ke, C. H., Zhan, X., Nakano, T., and Williams, G. A.: The impact of Yangtze River discharge, ocean currents and historical events on the biogeographic pattern of *Cellana toreuma* along the China coast, PLoS One 7, e36178, 2012.

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

~~Dupont, S. and Pörtner, H.: Marine science: get ready for ocean acidification, Nature, 498, 429-429, 2013.~~

~~Dupont, S. and Thorndyke, M. C.: Impact of CO 2 -driven ocean acidification on invertebrates early life-history–What we know, what we need to know and what we can do, Biogeosciences Discussions, 6, 3109-3131, 2009.~~

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

Fabricius, K. E., Langdon, C., Uthicke, S., Humphrey, C., Noonan, S., De'ath, G., Okazaki, R.,

436 Muehllehner, N., Glas, M. S., and Lough, J. M.: Losers and winners in coral reefs acclimatized to
437 elevated carbon dioxide concentrations, Nat. Clim. Change., 1, 165-169, 2011.
438 Fabry, V. J., Seibel, B. A., Feely, R. A., and Orr, J. C.: Impacts of ocean acidification on marine fauna
439 and ecosystem processes, ICES J. Mar. Sci., 65, 414-432, 2008.
440 Feder, M. E. and Hofmann, G. E.: Heat-shock proteins, molecular chaperones, and the stress response:
441 evolutionary and ecological physiology, Annu. Rev. Physiol., 61, 243-282, 1999.
442 Firth, L. B. and Williams, G. A.: The influence of multiple environmental stressors on the limpet *Cellana*
443 *toreuma* during the summer monsoon season in Hong Kong, J. Exp. Mar. Biol. Ecol., 375, 70-75, 2009.
444 Franks, S. J. and Hoffmann, A. A.: Genetics of climate change adaptation, Annu. Rev. Genet., 46, 185-
445 208, 2012.
446 Gibson, R., Atkinson, R., Gordon, J., Smith, I., Hughes, D.: Impact of ocean warming and ocean
447 acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in
448 a changing ocean, Oceanogr. Mar. Biol. Annu. Rev., 49, 1-42, 2011.
449 Giomi, F., Mandaglio, C., Ganmanee, M., Han, G. D., Dong, Y. W., Williams, G. A., and Sara, G.: The
450 importance of thermal history: costs and benefits of heat exposure in a tropical, rocky shore oyster, J.
451 Exp. Biol., 219, 686-694, 2016.
452 Giomi, F. and Poertner, H. O.: A role for haemolymph oxygen capacity in heat tolerance of eurythermal
453 crabs, Front. Physiol., 4, 110, 2013.
454 ~~Hale, R., Calosi, P., McNeill, L., Mieszkowska, N., and Widdicombe, S.: Predicted levels of future ocean~~
455 ~~acidification and temperature rise could alter community structure and biodiversity in marine benthic~~
456 ~~communities, Oikos, 120, 661-674, 2011.~~
457 Gooding, R. A., Harley, C. D., and Tang, E.: Elevated water temperature and carbon dioxide
458 concentration increase the growth of a keystone echinoderm, Proc. Natl Acad. Sci., 106, 9316-9321,
459 2009.
460 Han, G. D., Zhang, S., Marshall, D. J., Ke, C. H., and Dong, Y. W. Metabolic energy sensors (AMPK
461 and SIRT1), protein carbonylation and cardiac failure as biomarkers of thermal stress in an intertidal
462 limpet: linking energetic allocation with environmental temperature during aerial emersion, J. Exp.
463 Biol., 216, 3273-3282, 2013.
464 Hansen, L. K., Houchins, J. P., and O'Leary, J. J.: Differential regulation of HSC70, HSP70, HSP90 alpha,

and HSP90 beta mRNA expression by mitogen activation and heat shock in human lymphocytes, Exp. Cell. Res., 192, 587-596, 1991.

Helmuth, B., Mieszkowska, N., Moore, P., and Hawkins, S. J.: Living on the edge of two changing worlds: forecasting the responses of rocky intertidal ecosystems to climate change, Annu. Rev. Ecol. Evol. Syst., 37, 373-404, 2006.

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Harvell, C. D., Sale, P. F., Edwards, A. J., Caldeira, K., Knowlton, N., Eakin, C. M., Iglesias-Prieto, R., Muthiga, N., Bradbury, R. H., Dubi, A., and Hatziolos, M. E.: Coral reefs under rapid climate change and ocean acidification, Science, 318, 1737-1742, 2007.

Hoffmann, A. A. and Sgro, C. M.: Climate change and evolutionary adaptation, Nature, 470, 479-485, 2011.

Hofmann, G. E. and Todgham, A. E.: 2010 Living in the now: physiological mechanisms to tolerate a rapidly changing environment, Annu. Rev. Physiol., 72, 127-145, 2010.

IPCC: Climate change 2007: The physical science basis, in: Working group I contribution to the fourth assessment report of the IPCC, edited by Solomon, S., Cambridge Uni. Press, New York, 2007.

IPCC: Climate change 2013: The physical science basis, in: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Clilmate Change, edited by: Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex, V., and Midgley, P. M., Cambridge Univ. Press, New York, 2013.

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, G. M., and Gattuso, J. P.: Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming, Glob. Chang. Biol., 19, 1884-1896, 2013.

Kwiatkowski, L., Gaylord, B., Hill, T., Hosfelt, J., Kroeker, K. J., Nebuchina, Y., Ninokawa, A., Russell, A. D., Rivest, E. B., and Sesboüé, M.: Nighttime dissolution in a temperate coastal ocean ecosystem increases under acidification, Sci. Rep., UK 6, 2016.

Lee, K., Kim, T. W., Byrne, R. H., Millero, F. J., Feely, R. A., and Liu, Y. M.: The universal ratio of boron to chlorinity for the North Pacific and North Atlantic oceans, Geochim Cosmochim. Ac., 74, 1801-1811, 2010.

Millero, F. J., Graham, T. B., Huang, F., Bustos-Serrano, H., and Pierrot, D.: Dissociation constants of

carbonic acid in seawater as a function of salinity and temperature, Mar. Chem., 100, 80-94, 2006.

Morton, B. S., and Morton, J. E.: The seashore ecology of Hong Kong. Hong Kong University Press, Hong Kong, 1983.

Munday, P. L., Crawley, N. E., and Nilsson, G. E.: Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes, Mar. Ecol. Prog. Ser, 388, 235-242, 2009.

Oleksiak, M. F., Churchill, G. A., and Crawford, D. L.: Variation in gene expression within and among natural populations, Nat. Genet., 32, 261-266, 2002.

Parmesan, C. and Yohe, G.: A globally coherent fingerprint of climate change impacts across natural systems, Nature, 421, 37-42, 2003.

~~Perry, A. L., Low, P. J., Ellis, J. R., Reynolds, and J. D.: Climate change and distribution shifts in marine fishes, Science, 308, 1912-1915, 2005.~~

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., and Maintainer, R.: Package 'nlme', 2013.

~~Pörtner, H. O., Peck, L. S., and Somero, G. N.: Mechanisms defining thermal limits and adaptation in marine ectotherms: an integrative view, Antarctic Ecosystems: An Extreme Environment in a Changing World, 379-416, 2012.~~

Prosser, C. L.: Physiological Variation in Animals, Biol. Rev., 30, 229-261, 1955.

Reed, G. F., Lynn, F., and Meade, B. D.: Use of coefficient of variation in assessing variability of quantitative assays, Clinical and diagnostic laboratory immunology, 9, 1235-1239, 2002.

Robbins, L. L., Hansen, M. E., Kleypas, J. A., and Meylan, S. C.: CO2calc: A user-friendly seawater carbon calculator for Windows, Mac OS X, and iOS (iPhone), US Geological Survey, 2012.

Rodolfo-Metalpa, R., Houlbreque, F., Tambutte, E., Boisson, F., Baggini, C., Patti, F. P., Jeffree, R., Fine, M., Foggo, A., Gattuso, J. P., and Hall-Spencer, J. M.: Coral and mollusc resistance to ocean acidification adversely affected by warming, Nat. Clim. Change, 1, 308-312, 2011.

Ruppert, E. E., Fox, R. D., Ruppert, R. S., Fox, R. S., and Barnes, R. D.: Invertebrate zoology: a functional evolutionary approach, No. 592 RUPi, 2004.

Sanford, E., and Kelly, M. W.: Local adaptation in marine invertebrates, Ann. Rev. Mar. Sci., 3, 509-535, 2011.

~~Scheffers, B. R., De Meester, L., Bridge, T. C., Hoffmann, A. A., Pandolfi, J. M., Corlett, R. T., Butchart,~~

S.H., Pearce-Kelly, P., Kovacs, K.M., Dudgeon, D., and Pacifici, M.: The broad footprint of climate change from genes to biomes to people, Science, 354, aaf7671, 2016.

Seebacher, F., White, C. R., and Franklin, C. E.: Physiological plasticity increases resilience of ectothermic animals to climate change, Nat. Clim. Change, 5, 61-66, 2015.

Sokolova, I. M. and Berger, V. J.: Physiological variation related to shell colour polymorphism in White Sea *Littorina saxatilis*, J. Exp. Mar. Biol. Ecol., 245, 1-23, 2000.

Somero, G. N.: The physiology of global change: linking patterns to mechanisms, Annu. Rev. Mar. Sci. 4, 39-61, 2012.

Somero, G. N., Lockwood, B. L., and Tomanek, L.: Biochemical adaptation: response to environmental challenges, from life's origins to the Anthropocene. Sinauer Associates, 2016.

Sunday, J. M., Bates, A. E., and Dulvy, N. K.: Thermal tolerance and the global redistribution of animals, Nat. Clim. Change., 2, 686-690, 2012.

Sunday, J. M., Calosi, P., Dupont, S., Munday, P. L., Stillman, J. H., and Reusch, T. B.: Evolution in an acidifying ocean, Trends Ecol. Evol., 29, 117-125, 2014.

Team, R. C.: R: A language and environment for statistical computing, Vienna, Austria: R Foundation for Statistical Computing, 2014.

Tomanek, L.: The heat-shock response: its variation, regulation and ecological importance in intertidal gastropods (genus *Tegula*), Integr. Comp. Biol., 42, 797-807, 2002.

Tomanek, L. and Sanford, E.: Heat-shock protein 70 (Hsp70) as a biochemical stress indicator: An experimental field test in two congeneric intertidal gastropods (Genus: *Tegula*), Biol. Bull., 205, 276-284, 2003.

Tomanek, L., and Somero, G. N.: Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography, J. Exp. Biol., 202, 2925-2936, 1999.

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

Walther, K., Sartoris, F. J., Bock, C., and Pörtner, H. O.: Impact of anthropogenic ocean acidification on thermal tolerance of the spider crab *Hyas araneus*, Biogeosciences, 6, 2207-2215, 2009.

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

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

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

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

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

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

565

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

566 [1]Temperature coefficients ($Q_{10}$) were calculated using heart rate from 24 to 33 °C

567 [2]After acclimated at different $CO_2$ and temperature for one week, limpets (n = 8-10) from each acclimation treatment

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

569 acclimation treatments were used for calculating coefficients of variation.

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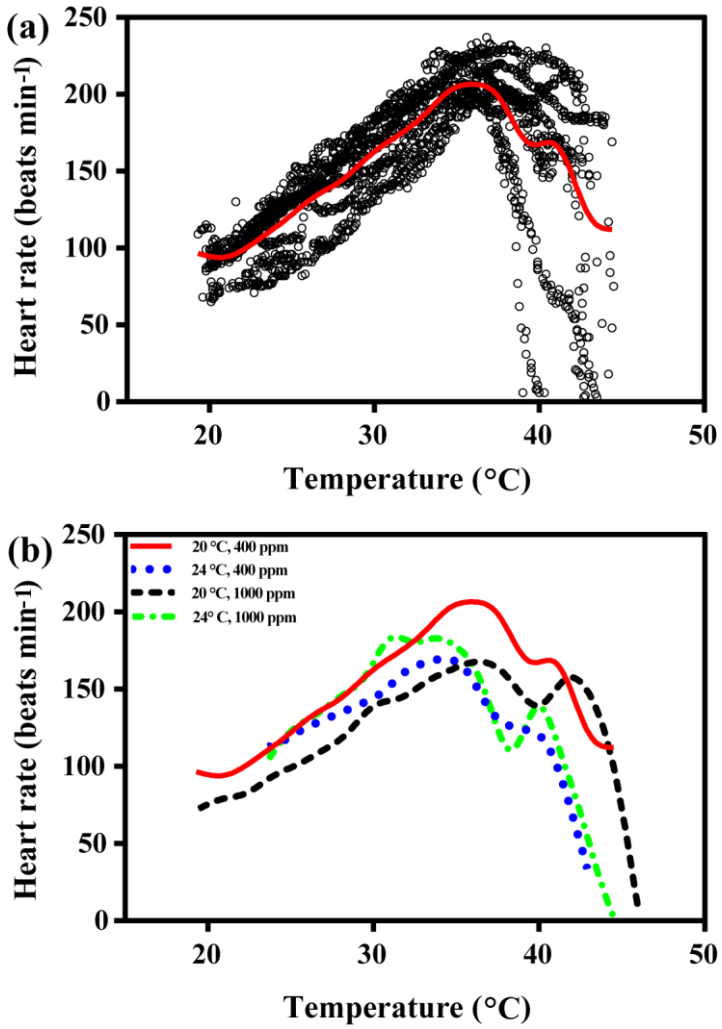


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574 **Figure 1.** (a) Heart rates of all limpets acclimated to 24 °C and 400ppm, presented as an example of HR calculation

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

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

577 different experimental temperature and $CO_2$ conditions.

578

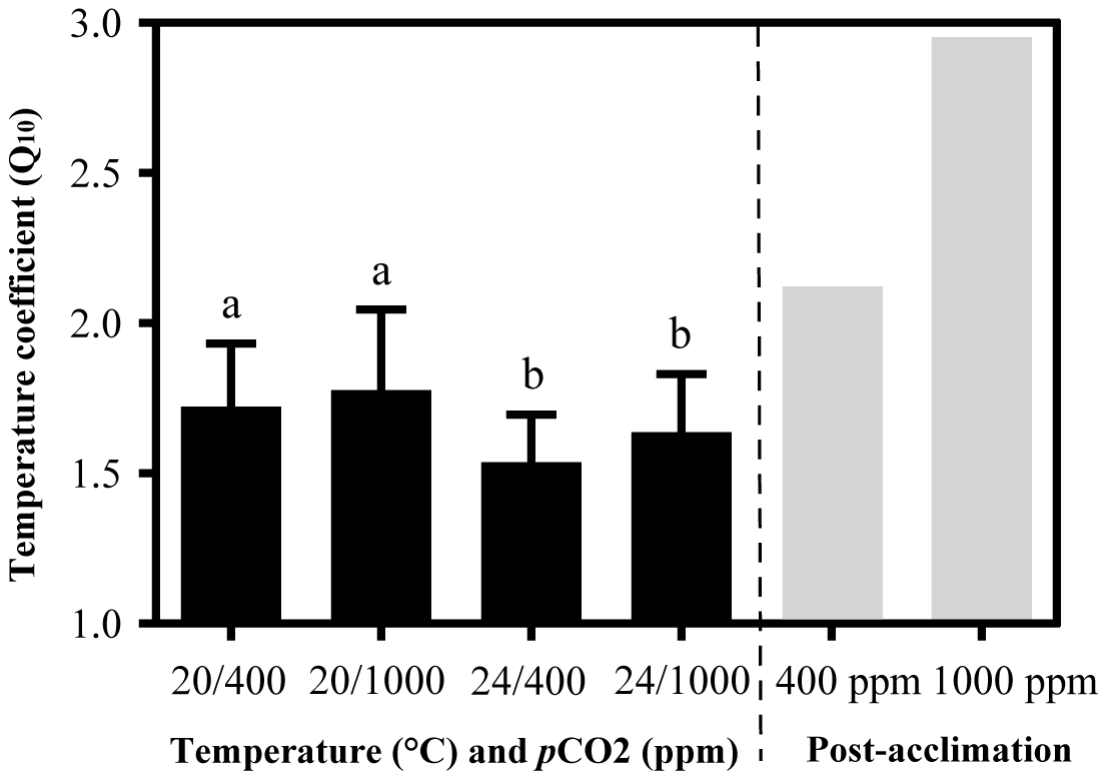
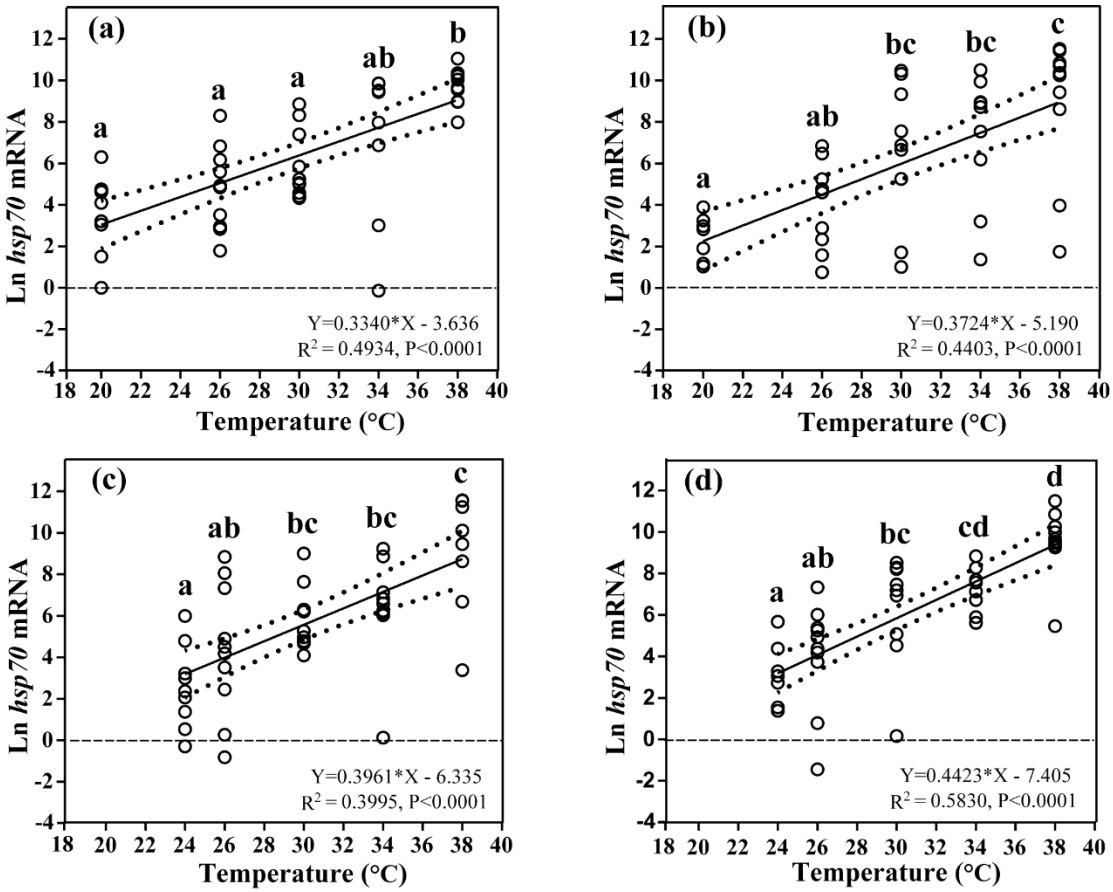



**Figure 2.** Temperature coefficients (Q10) of limpets acclimated at different temperatures (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). The temperature coefficient (Q10) values were calculated for all limpets using heart rate data from 24 to 33°C. Post-acclimation temperature sensitivity was calculated between individuals acclimated at 20 and 24°C (grey bars; *sensu* Seebacher et al., 2015) for each $CO_2$ concentration, where higher thermal sensitivity indicates less acclimation to thermal stress. The calculation of post-acclimation $Q_{10}$ is done for the mean response of all individuals as the same individual are not used at each acclimation temperature. Therefore, there was no calculation of variation or error for post-acclimation. Different letters represent significant differences in the $Q_{10}$ among different acclimation treatments.

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

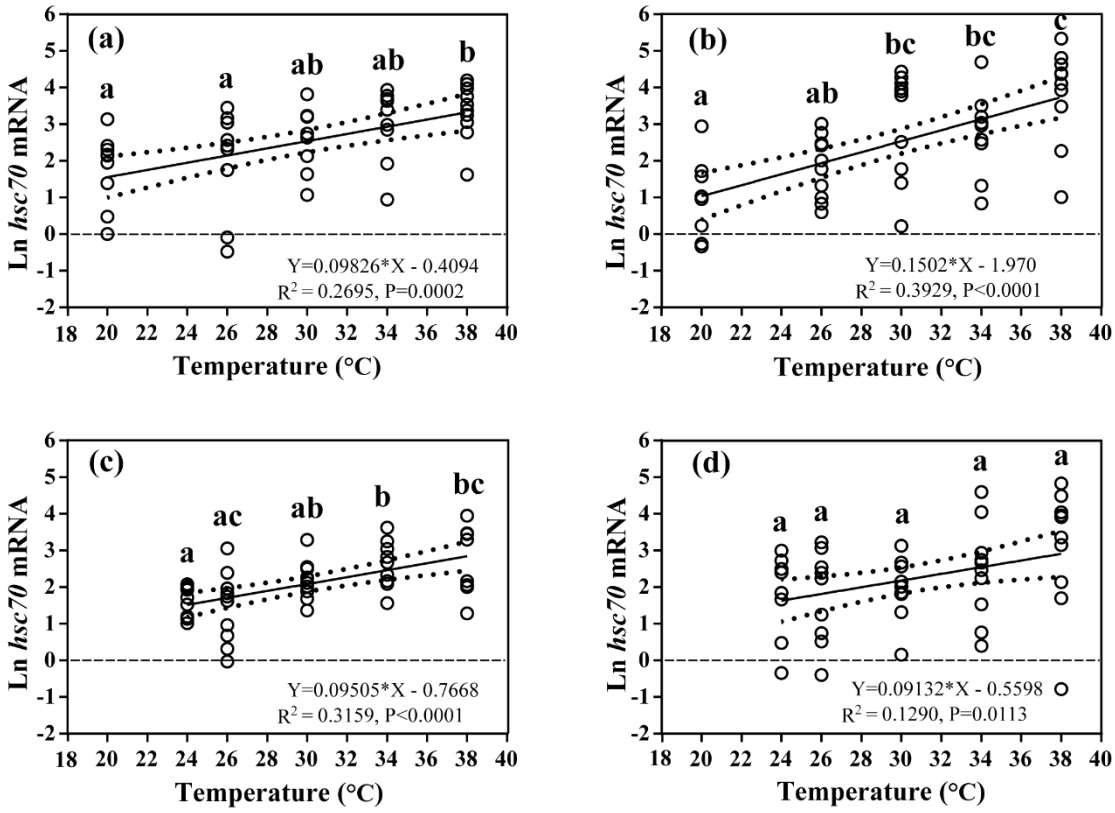
593 and 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

596 among different heat-shock temperatures.

597

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599

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

601 400 ppm, (b) 20°C and 1000 ppm, (c) 24°C and 400 ppm, and (d) 24°C and 1000 ppm. The relationship between

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

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

604 among different heat-shock temperatures.

605

**Appendix:**

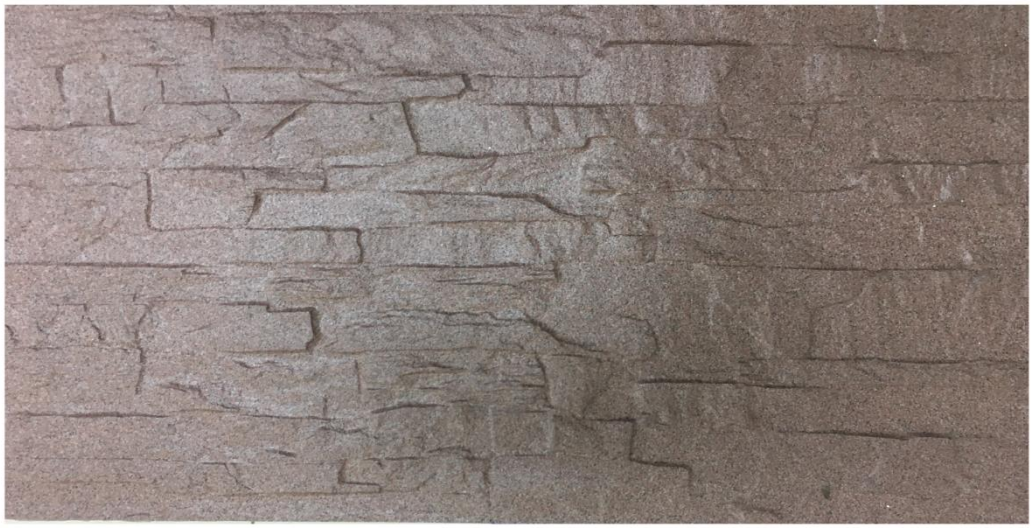


**Figure A1.** The photo of artificial rock (60 cm length × 30 cm width). Limpets were placed on artificial rock and heated to the designated temperate.

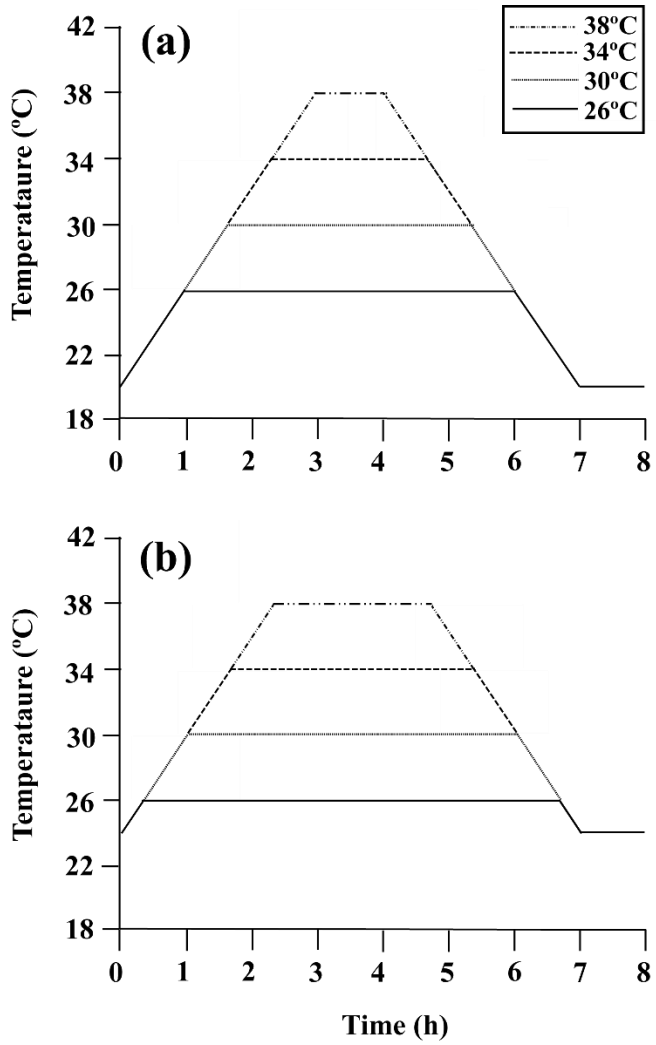



**Figure ~~A1~~A2.** Diagram of the heating protocol for (a) limpets acclimated at 20 °C and (b) limpets acclimated at 24 °C. Limpets were heated at a rate of 6°C per hour from acclimation temperatures (20 or 24 °C) to designated temperatures (26, 30, 34 and 38 °C) for simulating a natural heating rate in summer. After achieving the target temperature, the temperature was held at the designated level for the allotted time, and then decreased to acclimated temperatures (20 or 24 °C) at a rate of 6 °C per hour, for a total exposure time of 7 h. After recovery in 20 or 24 °C seawater for 1 h, limpets (n = 8-10) in each treatment were immediately collected and stored at -80 °C for gene expression measurement.

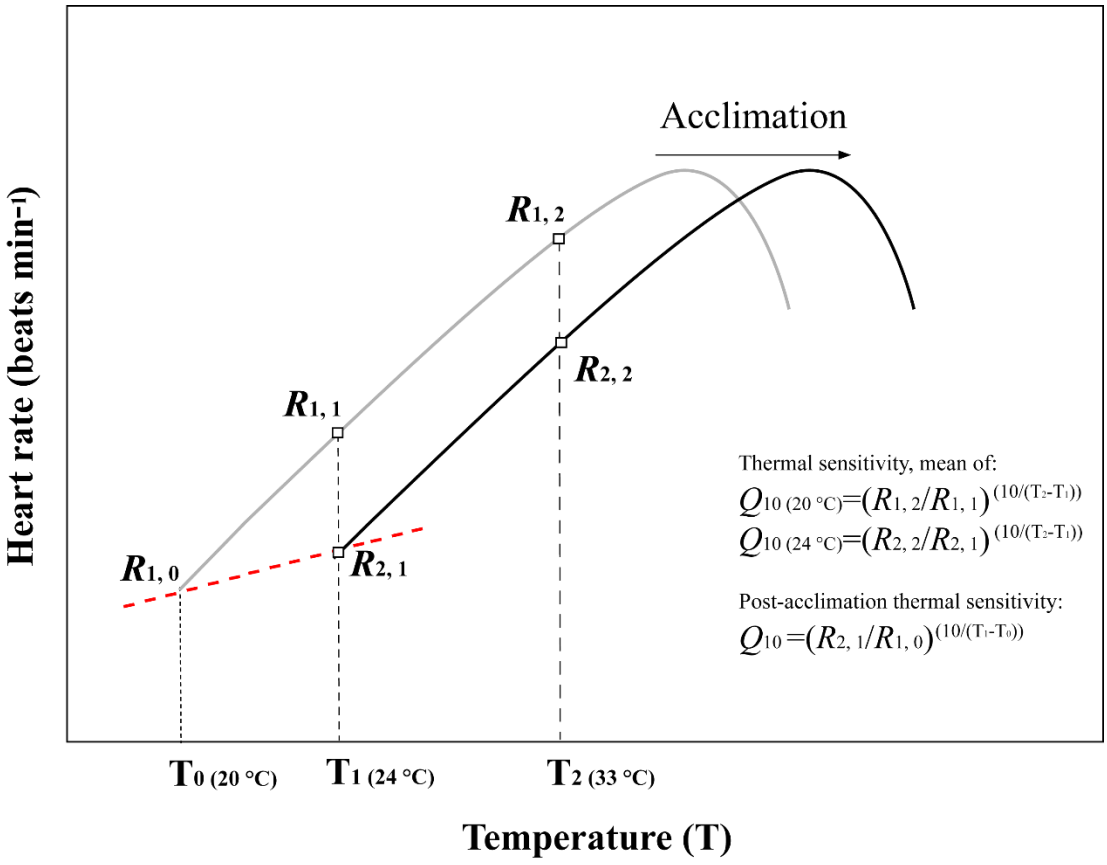
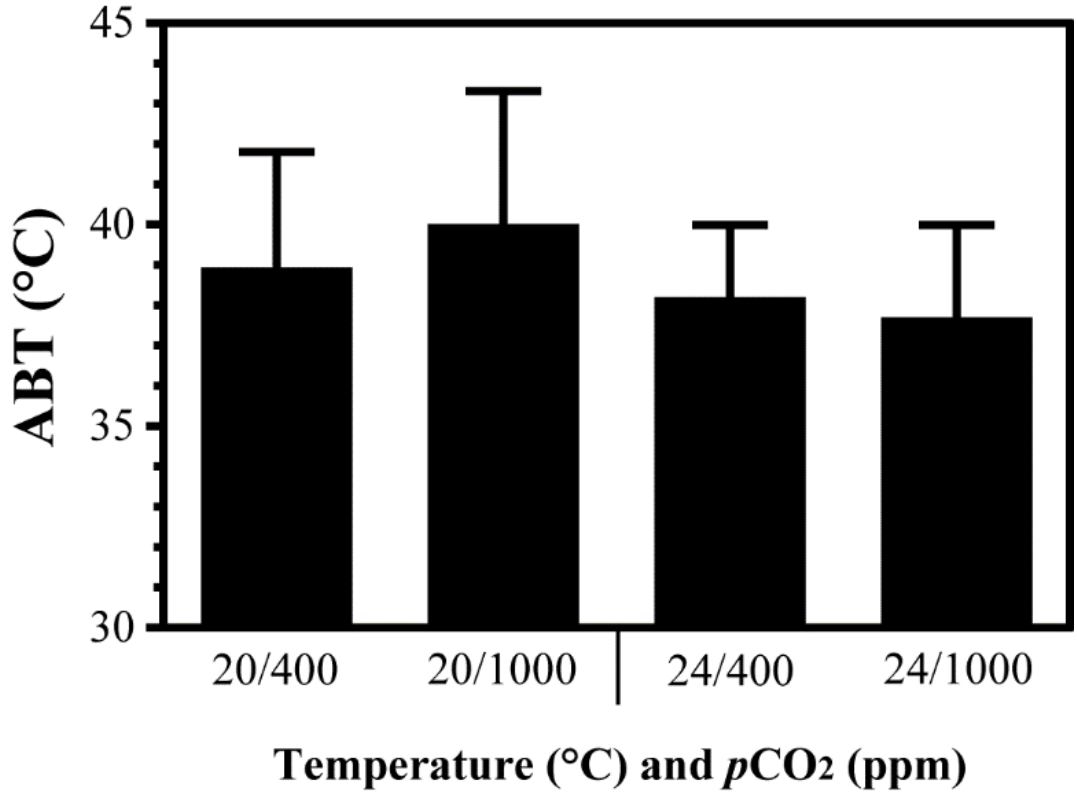



**Figure ~~A2~~A3.** Schematic diagram of temperature coefficients ($Q_{10}$) and post-acclimation $Q_{10}$ calculations. This figure was modified from Seebacher et al. (2015). Black line and grey line showed the heart rate of limpets at the warm-acclimated temperature (24 °C) and the cold-acclimated temperature (20 °C), respectively. $Q_{10}$ values for thermal sensitivities were calculated from data for limpets kept at an acclimation treatment in which heart rate were measured at two different temperatures. $Q_{10}$ value for post-acclimation thermal sensitivities was calculated across two temperature acclimation conditions under the same $pCO_2$ condition.

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630

631 **Figure ~~A3~~A4.** Arrhenius break point temperature of heart rate (ABT) of limpets acclimated at different temperatures

632 (20 or 24 °C) and CO2 concentrations (400 or 1000 ppm). After acclimation in different conditions, limpets were

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

634 rates were recorded and ABTs were calculated.

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636

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

638 experimental period[1]

639

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

640 [1]Seawater temperature, salinity, pH and total dissolved inorganic carbon ($C_T$) were monitored every 6 h. Total

641 alkalinity ($A_T$), $pCO_2$, $CO_3^{2-}$ and $\Omega_{cal}$ were calculated using CO2SYS software. Results were pooled and averaged

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

643

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

645

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

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647

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

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

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

**Table A4.** Two-way ANOVA to investigate the effects of temperature (20 °C and 24 °C) and $pCO_2$ (400 ppm and 1000 ppm) on Arrhenius break point temperature of heart rate (ABT) and temperature coefficients ($Q_{10}$) on *Cellana toreuma*

| Source of variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| **Two-way ANOVA for ABT** | | | | | |
| Temperature | 1 | 22.580 | 22.580 | 3.375 | 0.075 |
| *p*CO2 | 1 | 0.790 | 0.790 | 0.118 | 0.733 |
| Temperature × *p*CO2 | 1 | 6.076 | 6.076 | 0.908 | 0.347 |
| Residual | 35 | 234.200 | 6.692 | | |
| **Two-way ANOVA for $Q_{10}$** | | | | | |
| Temperature | 1 | 0.257 | 0.257 | 5.878 | 0.021 |
| *p*CO2 | 1 | 0.058 | 0.058 | 1.332 | 0.256 |
| Temperature × *p*CO2 | 1 | 0.005 | 0.005 | 0.1135 | 0.738 |
| Residual | 35 | 1.527 | 0.0436 | | |