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Interactive comment on "Ocean acidification increases the sensitivity and variability of physiological responses of an intertidal limpet to thermal stress" *by* Jie Wang et al.

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Responses to comments of M. Byrne (Referee) "Ocean acidification increases the sensitivity and variability of physiological responses of an intertidal limpet to thermal stress"

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. 43-46. This sentence is changed to: "The interaction between global warming and ocean acidification may not only reduce an organism's resistance to environmental change (Munday et al., 2009), but subsequently affect population dynamics (Fabry et al., 2008; Hoegh-Guldberg et al., 2007; Kroeker et al., 2013; Rodolfo-Metalpa et al., 2011)."

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. 72-94. 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. 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. 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). 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 pCO2 concentrations (400 ppm, 1000 ppm) and temperatures (20 °C, 24 °C). We hypothesize that (1) limpets will increase thermal physiological plasticity under elevated pCO2 and temperatures; (2) limpets acclimatized at different pCO2 concentrations and temperatures can change their heat shock response, and then related energy consumption. This study provides novel information concerning the combined effects of increased temperature and pCO2 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. Re-

cent 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 pCO2 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 CO2 (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. 10, L. 193-195: “However, post-acclimation thermal sensitivity should be considered with caution, as the present study was conducted during a short-term acclimation (7 days).”

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 cm2 in each acclimation treatment. As the density in the acclimation treatment is similar to that under field conditions (our field investigation), 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. 5-6, 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 acclimated for 7 d (i.e. short-term acclimation) in different pCO2 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) in climate chambers (RXZ280A, Jiangnan Instrument Company, Ningbo, China), which can control the pCO2 concentration. There were about 100 indiv. per acclimation treatment, and the density was ~ 1 limpet per 10 cm2 in each acclimation treatment. The density in the acclimation treatment is similar to that under field conditions (our field investigation). 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 treatments (n = 10 indiv. per acclimation treatment) were randomly sampled and frozen at -80 °C as non-heated control samples. For each acclimation treatment, 10 limpets were randomly selected from different containers, transferred to an artificial rock (see Fig. A1) and heated at a rate of 6 °C per hour (a natural heating rate, Han et al., 2013) to a designated temperature. There were four 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 were immediately collected and stored at -80 °C for gene expression quantification." (P. 7, L. 125-134)

Q5: Show a photo of the artificial rock.

Response to Q5: The photo of the artificial rock (60 cm length × 30 cm width) was 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. 10-11, L. 202-206: "The coefficient of variation (CV) of ABT, Q10 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."

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. 11, L. 210-221). "The maximal heart rate was $\sim$ 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 pCO2 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, P = 0.075) and pCO2 (Two-way ANOVA, P = 0.733) both had non-significant effects on ABTs, and there was a non-significant interaction between temperature and pCO2 (Two-way ANOVA, P = 0.347) (Table A4; Fig. A4). Temperature coefficients (Q10 rates) were higher for limpets acclimated at 20 °C than at 24 °C (Two-way ANOVA, P = 0.021), but there was no significant difference for acclimation to different pCO2 concentrations and for the interaction between temperature and pCO2 (Two-way ANOVA, P > 0.05) (Table A4; Fig. 2). The post-acclimation thermal sensitivity of limpets acclimated at low CO2 (2.12) was lower than limpets at high CO2 (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 Q10 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. 22, L. 502-504). "The calculation of post-acclimation Q10 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: "Higher thermal sensitivity to the predicted future pCO2 (1000 ppm) was indicated by increased heart rate. 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. 13-14, L. 254-279. The hsp text is expanded by comparing present study with previous researches on intertidal molluscs as follows. "Increased temperature and CO2 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 CO2 (HTHC) were significantly higher than those of limpets acclimated at the other three acclimation conditions. As a molecular chaperon, Hsp70 plays crucial roles in maintaining protein stability with the expense of a large amount of energy (Feder and Hofmann, 1999; Tomanek and Sanford, 2003). Usually, the expression of hsp70 of less thermal-tolerant species is

more sensitive to increases in temperature (Dong et al., 2008; Tomanek, 2002). Increasing evidence show that organisms from environments with much stress have a different or increased stress response compared with organisms from environments with less stress. For example, higher intertidal gastropods involved higher heat shock protein expression in response to thermal stress than their lower intertidal counterparts (higher versus lower intertidal: snail Tegula funebralis versus T. brunnea and T. montereyi, Tomanek, 2002, Tomanek and Sanford, 2003; limpet Lottia scabra and L. austrodigitalis versus L. scutum, Dong et al., 2008; limpet C. grata versus C. toreuma, Dong and Williams, 2011). Similar patterns have been observed in hsp70 gene expression for limpet Patella (higher versus lower intertidal: P. rustica versus P. caerulea and P. ulyssiponensis, Prusina et al., 2014). Peck and his colleagues (Peck et al., 2014) found that Atlantic and tropical marine ectotherms are poor in their ability to acclimate physiology to elevated temperature when compared with species from temperate zone. In the present study, the rapid upregulation of hsp70 mRNA in limpets exposed to future conditions (i.e. much stress) 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. 21. It is changed to: "... (20 °C, 24 °C) regimes in a short-term period (7 days)."

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.

Q17: L 46-49 – This is a general sentence – one ref will suffice

Response to Q17: P. 3, L. 47-49. 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)."

Q18: L. 98-99 can delete much of this detail (eg falling high tide)

Response to Q18: P. 5-6, 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."

Q19: L. 367 – this is a discussion paper – not fully peer review – delete

Response to Q19: This reference is deleted.

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Fig. 1. The photo of artificial rock (60 cm length $\times$ 30 cm width). Limpets were placed on artificial rock and heated to the designated temperate.