Biogeosciences Discuss.,
https://doi.org/10.5194/bg-2017-47-AC2, 2017

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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Received and published: 2 July 2017

Responses to comments of anonymous referee #2 "Ocean acidification increases the sensitivity and variability of physiological responses of an intertidal limpet to thermal stress"

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, CTmin, CTmax, 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 CTmax 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 need to state that: "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. CO_2 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 Q10 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 (Q10) 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.

Q4: The authors should make it clear if the limpets were kept in a chamber with constant CO_2 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., tem-

perature or CO_2) ..." is not sufficiently clear to me.

Response to Q5: This sentence is rephrased to make it clear. “For limpets acclimated under HTLC and LTHC (i.e., only temperature or CO_2 condition changed in comparison to 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), which may reflect divergent adaptive strategy to the environment. Some studies 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). In the present study, 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). For limpets acclimated under HTLC and LTHC (i.e., only temperature or CO2 condition changed in comparison with the LTLC treatment), there was significant upregulation of hsc70 mRNA when the heat shock temperatures were beyond 30 °C. These results indicate that expression of hsc70 mRNA is relatively constitutive. That is, the upregulation of hsc70 mRNA in response to heat shock represents an increasing capability for coping with the enhanced protein denaturation and more energy allocated into the somatic maintenance after being exposed to either warming or high CO2 environment. However, the absence of significant upregulation of hsc70 mRNA in limpets acclimated to future conditions (warming and elevated CO2) might 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 days). Yet, the combination of elevated temperature and CO2 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-CO2 conditions. Therefore, while elevated CO2 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 (several months) and a larger sample size are therefore recommended in order to validate our findings."

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