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Biogeosciences Discussions



# *Interactive comment on* "The subtle effects of sea water acidification on the amphipod *Gammarus locusta*" *by* C. Hauton et al.

**Anonymous Referee #3**

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General comment:

The study "The subtle effects of seawater acidification on the amphipod Gammarus locusta"; by Chris Houton, Toby Tyrrell and John Williams addresses important questions about the consequences of ocean acidification on the resilience of the whole organism and underlying molecular mechanisms in an species, usually facing large variation in pH, salinity and other abiotic factors. Although it can be expected that these organisms have the ability to deal with the predicted future changes in seawater chemistry, studying the responses and mechanisms in tolerant species are necessary to improve our understanding about constraints and limitations in more sensitive species. Therefore, the authors reared juvenile amphipods at three different pH levels, and determined basic parameters about survival and growth rate. These factors were not significantly



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affected with water pH. Furthermore, they isolated the genes encoding a heat shock protein 70 (Hsp70) and glyceralaldehyde-3-phosphate dehydrogenase (gapdh) in this -from the molecular point- alien species. Besides the large divergence within the Hsp70 family with respect to their responsiveness to abiotic stressors, the expression of the Hsp70 isolated in the present study was found highly inducible upon heat shock within hours. However, no response of this chaperone was found in response to acidified water. Instead gapdh expression, which usually serves as endogenous control in expression studies, was found significantly increased. The author concluded shifts in oxidative metabolic processes, and furthermore that seawater pH has subtle effects on the physiology of tolerant species, which are not perceived in whole animal studies. Although I see a huge potential in using candidate expression studies as sensitive markers for assessing subtile (molecular) responses, which exert its effect on whole animal performance, the authors made no ideal choice for their candidate genes. Therefore, especially the conclusions upon the molecular data are -as the author stated in the last paragraph of the discussion- quite preliminary. Although I see a number of merits in the present study on this important issue, I have some points, which should be considered before acceptance:

Major points:

1.I really appreciate the molecular work on the isolation and characterisation of the genes in an alien marine species. The function of the Hsp70 as inducible heat shock factor is very convincing. Nevertheless, the time-course of the temperature experiment and the pH experiment are completely different. 2000fold induction of Hsp70 became apparent after only 3 hours at 30°C, whereas under acidified seawater incubation the expression was assessed after 14 and 28 days. The authors mention the divergence in the functional roles within the Hsp70 family, and concluded from the temperature experiment that the isolated Hsp70 may function as chaperone under stressful conditions in that it helps to refold denatured proteins. I can follow this conclusion. However, the reaction window of this "fireworker"; chaperone seems to be to fast to be perceived in



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the acidification eperiment. Although the authors did not mention the natural temperature window and the variation in the environment, I would think that 30°C represents a severe stress for these animals. Besides, Gammarus locusta is facing large fluctuations in pH and salinity in its natural habitat, which needs a powerful ion/pH regulatory capacity, as shown for many crustaceans. In other metazoans with high ion regulatory capacities pH recovery usually takes place within 24 hours or at least within a few days. Thus, it can be assumed that after 14 days full recovery of the extracellular pH have taken place and no cellular stress situation as in the case of the heat shock exist. Instead it could be speculated that the less inducible Hsp/Hscs would have been the better choice to assess subtle stressful steady state responses with moderate higher expression levels. The isolation protocol of the gene used by the authors prevented them to find other Hsp70s, which may be more appropriate to assess long-lasting permanent stress. However, when using the isolated isoform the authors should assess its expression during the acute response to see, whether the isolated isoform is involved. This could indeed be an sensitive molecular marker to assess disturbances upon acidification.

2. GapDH is used in many expression studies as endogenous control, since it is believed that its expression does not change under many experimental conditions. During recent years more and more studies came up, which questioned the role of gapdh as appropriate endogenous control. The results upon changes in gapdh expression are in line with these studies. For model organisms it therefore became common to test a set of several potential endogenous control genes to find the one with the least variance under the applied experimental conditions. For organisms like Gammarus locusta this solution is very difficult as it is not simple to isolate all these potential candidate genes. My impression is that the original plan of the authors was to use gapdh as endogenous control against Hsp70, but which finally led to the present results contrary to the expectations with constant expression of the stress inducible gene and variable expression of the desired endogenous control. Although these results are worth mentioning, the conclusions upon the physiological significance of the variable expression are very pre-



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liminary. One reason for taking gapdh as endogenous control is besides its presence in every tissue at reasonable expression level the limited capacity as rate-limiting enzyme in the metabolism. For assessing glycolytic or oxidative capacities and their shifts upon acclimation to environmental factors other genes and their proteins are in common use (e.g. pyruvate kinase, pyruvate DH, citrate synthase, cytochrome c oxidase). The variation in expression of gapdh prevents its use as endogenous control, but its function as equilibrium enzyme limits its use as single indicator of shifts in metabolic flux.

3. Regarding the last point the authors used an absolute quantification for their realtime PCR data in lack of an endogenous control. This procedure is not very common and has a lot of limitations. First of all it does neither account for differences in the quality of the isolated RNA nor in the efficiency of the cDNA synthesis. Individual variation in expression may thus depend more on these factors then on the applied experimental conditions. For absolute quantification a standard curve of RNA with known copy number concentration should be taken, which should be treated in the same way as the experimental samples. Instead, in the present study plasmid DNA was used, so the given copy number in figure 4 is at the best an equivalent concentration of the plasmid copy number. Unfortunately, the authors did not give any information about testing the integrity of the RNA by means of denatured RNA gel electrophoresis or bioanalyser. Especially in invertebrates, RNA extraction may be complicated by the tissue structure, endogenous RNases, other cell constituents and many unknown factors so that the extraction protocols designed for mammals may or may not work. The quantitative extraction of RNA at high integrity is the absolute prerequisite for meaningful expression studies.

4. The presentation of the expression data is not well done. I am not sure whether a nested ANOVA is the better choice instead of doing two factorial ANOVA on the different batches. However, the presentation of the statistic data may be correct but is quite unusual for presentation of expression data. Why do the authors not show the changes in copy numbers as they did in figure 4. What was the xfold increase in



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expression in acidified seawater? If the batch has a significant effect on the expression, how did the expression pattern differ between the two batches? Any ideas about the reason for this difference (season, diet, etc.)? I would furthermore suggest adding the Hsp70 expression data, which were taken from the same RNA samples, into the graphs to get an idea about the sample variance (with special emphasis on point 3).

5. The interpretation in the abstract ("disruption to oxidative metabolic processes") and the last part of the discussion about higher energy demand for pH regulation is highly speculative. Capacities of -for instance- the Na+/K+ ATPase, the main driver for many ion regulatory processes, was not measured, the expression of gapdh is a weak measure of metabolic shifts, and no functional evidence (protein number, enzymatic capacities) of the observed response at the RNA level are presented to substantiate this interpretation. These parts should be rewritten with more care.

6. I appreciate the usage of predicted CO2 concentrations in this study, where it may be difficult to see any effects in higher metazoans with high regulatory potential. At these pCO2 concentrations the design of the incubation system (microcosms) and monitoring of the water chemistry is of great importance. In line with the comment of Sam Dupont and Mike Thorndyke besides pH the carbonate chemistry should be assessed in the system. This seems to be even more important, if a primary producer like macroalgae were added to the aquarium tanks, as it was the case in this study. Did the authors measured total alkalinity or DIC in their system?

Minor points:

7. For a meaningful interpretation of the increased survival at pH 7.8, information about the natural pH scenario in relation to the developmental stages should be included, as it was already extensively commented by Sam Dupont and Mike Thorndyke.

8. Figure 2: Do the authors have any explanation for the large drop in the control group at day 10? Also, how does the moulting frequency fits into this picture? Please give an estimate about the number of moulting during the experiments.



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9. Figure 1 would be much more meaningful if other relevant factors like DIC or alkalinity would have been added. I would believe that salinity and temperature could be kept within certain limits without presenting these data in a graph.

10. Discussion: The paragraph about is a little bit confusing. According to their own data the Hsp70 is a highly inducible heat shock factors (2000fold expression), in other cases in the literature proteins with a twofold induction are nominated as inducible. A definite assignment of the gene according to the sequence is with this inconsistent nomination of Hsc and Hsp in the literature difficult. I would suggest to keep this section simple and clear as the induction of the isolated gene is convincing.

Very minor:

11. Page 12: The three letter code for isoleucine is ile not isoleu, otherwise use the full name for every amino acid

12. Page 16: Amphiura instead of Amphura

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Interactive comment on Biogeosciences Discuss., 6, 919, 2009.