**Subject: Comment on bg-2021-34**

**Authors' responses are in italics**

Reviewer #2

1.Whilst open ocean seawater is extremely consistent in its chemical composition (at least for a given salinity), freshwater is definitely not consistent, and in fact is extremely variable, in ways that can have major consequences for physiological responses to variables such as CO2/pH. It is therefore important to report details of the freshwater chemistry, more than just the carbonate chemistry in Table 1. In particular ion concentrations that are relevant for gill ion and acid-base regulation processes (e.g. sodium and chloride), and calcium is critical for understanding and interpreting potential calcification effects of the treatments.

*The ion composition of Taipei tapwater will be added into the methods section.*

2.To help the reader the authors should provide a conversion, or direct comparison, for pCO2 values reported in Pa and uatm.

*Thank you for pointing this out. We had meant to add this conversion and will do so in the revised version*

3.Some variables were measured over a 7 day exposure period (haemolymph acid-base, oxygen consumption rate and ammonia excretion rate), or 14 days (mortality), but others (calcification and behaviour) seem to be over 6 week exposure. These different timescales are not explained in Methods section, or justified.

*Clarification will be added into the methods section justifying the timescales for each experiment. For the calcification experiment it was briefly mentioned in the results (Line 224-225).*

4.What caused mortality? In particular could this have been related to cannibalism after individual crabs had moulted? This seems likely, as is common in crustaceans in aquaculture where animals have little chance to escape their conspecifics whilst waiting for their exoskeleton to harden after moulting. There is no mention of hides or shelters being provided in the exposure tanks, so if crabs were moulting it is possible that calcification was slower in the high CO2 treatment, resulting in more crabs being prone to cannibalism whilst waiting to calcify, rather than an inability to calcify eventually (given enough time). With 6-7 crabs in a 10 litre tank cannibalism seems likely if some were moulting.

*We did have some plastic pipe tubing in the tank for shelter (This will be clarified in the methods). In regard to what caused the mortality we cannot explicitly say as the crabs had no obvious signs of disease pointing to a reason for death. We can state that it was not due to cannibalism as we were always able to recover the intact bodies of the deceased crabs. From*

*our experience death due to cannibalism usually results in recovery of just parts of the crab and sometimes just shells of a recent moult.*

5.Why not calculate the actual ammonia quotient (AQ) and include discussion of these data regarding protein utilisation, and reference the AQ values found in other species and how these numbers relate to protein utilisation.

*We did not calculate the ammonia quotient or O:N ratio as the measurements of oxygen consumption and ammonia excretion were not done on the same animals at the exact same time. In the methods it is described how we did these two measurements. Since we don't have exact paired measurement of oxygen consumption and ammonia flux we cannot do an accurate calculation to provide a quantitative number with an accurate standard error. However, we do mention in the discussion (line 335) that O:N ratio appears to decrease as we have a reduction in O2 consumption but really no change in ammonia. Unfortunately, it was a methodological issue of being able to actual run the experiment long enough to detect ammonia without over depleting O2 that prevented us from measuring both simultaneously.*

6.In the locomotory behaviour tests (and metabolic rate and ammonia excretion rate measurements) it is important to report data for the carbonate chemistry variables actually measured (at the same time) in both the experimental holding tanks the crabs were taken from, and the arena tanks the behaviour was assessed in (or respirometers). This is important because if they were different pCO2 values it could result in a rapid acid- base disturbance in the crabs transferred from one tank to another that could be the cause of behaviour differences or metabolic rate differences, rather than the actual prior high pCO2 exposure.

*The water chemistry for the experiments would be the same as that reported for the tanks. We were running a flow through system so the build up of ammonia and other wastes was negligible. Therefore, we just collected water from the experimental tanks (this assured that total alkalinity was the same) and used a separate $CO_2$ controller and $CO_2$ tank to inject $CO_2$ into the container we were placing the water to be used for the experiment. Also, before using the collected water took a portable pH meter/probe and confirmed that pH in the experimental tanks and experimental water container were the same to assure that $pCO_2$ was the same. This detail can be added into the methods section, so the reader knows we have done due diligence to assure the water parameters for the experiment were maintained as identical to the experimental tanks as possible.*

7.There is considerable discussion of the data showing a metabolic depression caused by freshwater acidification. However, if I understand the Methods accurately, metabolic rate (as oxygen consumption rate) was only measured for a single 30 minute period in each crab, and this was only after 15 minutes "acclimation" following handling and transfer to the respirometer chamber. If this is the case, then what was measured cannot be considered as the stable metabolic rate during exposure to either treatment (low or high CO2), and "metabolic depression" is not an accurate conclusion to make. Instead, what was measured is more likely to be the acute metabolic response to handling, brief air- exposure and transfer to a new environment, on top of the effects of any prior exposure to the CO2 levels used. This has not been considered but is important in interpreting the data reported.

*Your interpretation of the methods is correct. We do acknowledge that this is not the perfect way of doing measurements of oxygen consumption but is a widely published approach used for crustaceans. From our experience on other crustaceans using intermittent flow respirometry crustaceans do not typically require super long rest times for metabolic rate to stabilize. However, as we cannot explicitly say that is the case for our study, we will mention in the manuscript that the handling stress and brief air exposure are caveats for the readers to consider when interpreting the results and conclusions we make. We are all for transparency in our research and believe that there is still value in these results. We also believe this does show a metabolic depression as both animals were treated the same but with the caveat that the handling stress and brief air exposure must be considered when assessing what our results indicate.*

8.The manuscript often refers to "calcification" being measured, but this implies the rate of calcification which was not actually measured. Instead, carapace calcium content was measured at a few timepoints, which has been used to imply "calcification rate", but that is not strictly true. See also comments above about moulting, immediately after which is when the greatest rates of calcification occur.

*We apologise for the confusion. This issue basically comes down to how one interprets calcification. By definition it is simply the build up of calcium salts on a tissue and not necessarily a rate. As we have measured the calcium content in the carapace we have indeed measured calcification but not a calcification rate. I have double checked the manuscript and can confirm we never state that we are measuring a calcification rate. In the methods line 140 we state that we are assessing carapace calcium content as a proxy of calcification. Based on this information we do not believe any of our statements about calcification measurement is false.*

9.L.77-78 – The description of how pH/CO2 was controlled is not sufficiently detailed to provide a full explanation. Presumably this was done using 4 pH electrodes permanently recording the pH in each of the 4 individual experimental tanks, and the signals received from each electrode by 4 separate pH controllers was used to regulate the flow of CO2 via air stones into these individual tanks? Please provide enough details to clarify this issue.

*Additional details will be provided in text. Essentially your description is accurate and there were multiple CO2 controllers each with their own pH probe and mini CO2 tank that was regulating a single 10L aquaria.*

10. l.86 – Given that the CO2sys program requires salinity as an input variable to calculate carbonate chemistry, how was salinity measured, and what value(s) were used in these calculations?

*The CO2sys program has a freshwater function where they essentially count salinity as 0. This function was used for the calculations.*

11. Table 1 – It is not clear what these data are reporting, i.e. what timepoints do these data represent? From which experiments (the 7 day, 14 day or 6 week experiments?), and how were

the means calculated with respect to the four different replicate tanks per treatment? More details are needed. It would seem appropriate to report data separately for the different duration experiments (7 day, 14 day or 6 week).

*Clarification of this will be provided in the revised manuscript.*

12. l.115 – How were the crabs selected "randomly"? Unless a truly random method was used, this usually means the first animals that were able to be caught be experiments, which can result in a bias based on behavioural traits of the animals.

*We will change the wording as this was more of a haphazardly selecting crabs from the four tanks.*

13. l.119 – All units should be separated from their number by a space. So the 200mL should be 200 mL. This comment also applies throughout the whole manuscript.

*We will check for this and make the change throughout*

14. The control values for haemolymph pH are very high (pH >8.1) for an aquatic animal at the temperature used (23 degrees C). The haemolymph bicarbonate is also surprisingly high (13-14 mM) in the control conditions (time zero for both treatments). Studies on other crustaceans suggest haemolymph pH at this temperature would be closer to 7.6-7.8 and bicarbonate closer to 3-6 mM, and usually only reach values this high if the animals were already exposed to very high CO2 (e.g. >10,000 uatm) and had accumulated bicarbonate to compensate pH. Perhaps there is a precedent for such high bicarbonate and pH in this species, or other crab species, that I am not aware of, but the authors provide no discussion or comment on this discrepancy.

*This might be something specific to this species as it is quite a high pH and bicarbonate level compared to other crustaceans we have come across although those are mostly marine. Nevertheless, the values we measured in our study are comparable to what has been previously measured in adult E. sinensis (See Truchot 1992 Resp Physiol 87 419-427). A mention of this abnormal levels can be added into the text if deemed necessary although this is not the central focus point of the study.*