Subject: Comment on bg-2021-34
General Comments:
We would like to thank the three reviewers as the in-depth critical comments have vastly improved the analysis and overall quality of this manuscript. Detailed response to all concerns is provided below.

Reviewer #1

1. Length of each experiment has been included in the methods section

2. We respectfully disagree with the reviewer on the inclusion of David et al. 2020 study within our manuscript. We would like to point out that we stand by the statement that there are no **comprehensive** studies that use **realistic future** CO_2 levels. While other studies have investigated elevated CO_2 in freshwater animals the CO_2 levels employed are typically far beyond what could realistically be expected for near future FW acidification. In the David et al. 2020 study mentioned by the reviewer the effects of CO_2 induced acidification on shell growth rates of a freshwater gastropod were measured. In that study, the acidification treatments were based on a CO_2 induced pH drop from pH 7.3 to pH 6.8 and 6.3. Unfortunately, this manuscript does not provide key water parameter data (water alkalinity, water total carbon or water pCO_2 levels) that allows any reader to accurately quantify whether the treatments used are within a range that could realistically be seen in future FW systems. In addition, since the treatment pCO2 levels are unknown and sufficient data has not been provided for the reader to make the calculation themselves it is difficult to then take this study and make a direct comparison to our study as it is crucial in making comparisons between freshwater/ocean acidification studies to know the CO_2 levels employed.

3. We have restructured the final paragraph of the introduction to clarify why the Chinese mitten crab was selected as our model freshwater crustacean.

4. As mentioned above we have restructured the introduction to include our reasoning for using Chinese mitten crab and their invasive status/importance in aquaculture in Asia. In terms of trying to extend our results into the implications of this study on management and control of invasive populations we would more likely want to refrain from making too bold of claims as this is really a first probing study into the effects of freshwater acidification and our results here do not necessarily translate into how future populations will actually be affected as we are not considering multiple factors such as generational adaptation to changing environments.

5. We have amended the methods to let the reader know that these are purchased wild caught crabs.

6. Chinese mitten crab are opportunistic omnivores that in early life mainly eat plant material but become more carnivorous as they grow. Studies have essentially shown they eat plant material, small invertebrates (including bivalves), injured/dying/dead fish. We chose oatmeal due to personal communication for our aquaculture source that this is something they do feed. We have added a citation supporting their dietary behaviour.

7. Unfortunately, due to logistic reasons we were not able to fit a growth experiment into this study. Most of our experiments were not done over an extended period and as crustaceans must molt to grow the length of our experiment would unlikely allow for an accurate analysis of growth rates. We did perform the carapace calcium content experiment over 6 weeks but as we were periodically sampling, we would have only had a sample size of roughly 8 crabs that made it to the 6 week point which we feel would be too small of a sample size for proper growth experiments.

8. Sample size for each experiment is present in the figure or table captions.

Reviewer #2

1. The ion composition of Taipei tap water has been added into the methods section as measured by chloride assay and atomic absorption spectrophotometer.

2. The conversion between Pascals and micro atmospheres has been provided in the figure caption where hemolymph acid-base status is presented and in text of the results section where hemolymph acid-base status is first presented.

3. Clarification of the timescale for each experiment was added into the methods section.

4. We have added in our methods section that PVC pipes were present for shelter in all tanks. 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. We have added mention in the discussion section that the mortalities in our study were unlikely due to disease or cannibalism as there were no obvious signs pointing to these explanations. That being said we can never 100% rule out disease as a reason.

5. 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 XXX) 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. The water chemistry for the experiments would be the same as that reported for the tanks. We were taking water from the same source that was feeding our experimental tanks and then acidifying them with a pH controller. Using a portable pH probe we then measured the water being used for the experiment to the tanks to make sure we were maintaining the exact same CO_2 tension.

7. Your interpretation of the methods is correct. We do acknowledge that this is not the perfect way of doing measurements of oxygen consumption and we would have benefited from an intermittent flow respirometry approach. However, due to our time course approach and the number of animals we had to process in that time this was the most experimentally feasible approach. We believe the best course of action in this case is to add a comment in the methods acknowledging the methodological limitations of our approach and refraining from calling our result a metabolic depression but instead refer to it as a reduced oxygen consumption rate. While our close system respirometry approach is not ideal we would like to note that we have preliminary data on lobster, crayfish, and green crabs using an intermittent flow approach showing that at least in these crustaceans oxygen consumption rates level off after 30 minutes in the experimental chamber. These experiments on crayfish, lobster, and crab were done by transferring animals from their holding tank into respirometry chamber and recorded over a 24-hour period.

8. 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. Additional details have been provided in text regarding our aquarium CO_2 regulating setup. Essentially there were multiple CO2 controllers each with their own pH probe and mini CO_2 tank that was regulating a single 10L aquaria.

10. The CO2SYS program has a freshwater function where salinity is counted as 0. This function was used for the calculations and does not require a measurement of salinity.

11. Thank you for pointing out this mistake. Our table should have included the water parameters for the 7-day, 14-day and 42-day (6 week) experiment. It was our mistake that we had only added the data for the 7-day experiments but have now amended the table to include the water parameters for 14-day and 6-week experiments. We also made sure to recalculate all the water parameters in CO2SYS to assure accuracy in the reported values and the new table values have been confirmed to be accurate. It should also be noted that the previously reported tank temperature was mean +/- SD but we have now changed to +/- SEM.

12. We have changed the wording from randomly selecting crabs to haphazardly as there was no set randomization to the selection but crabs were just selected alternating between tanks.

13. Spacing between numbers and units have been changed throughout the text.

14. The hemolymph pH and HCO_3^- levels measured in this study are on the higher end of that normally seen in crustaceans which we would place around pH 7.6-7.9 and 3-9mM. That being said there are many reported cases of pH in the range we have detected (see book chapter Fehsenfeld and Weihrauch 2017). 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). As these values are not unusual for this species we haven't really gone into that as this is not really the central topic of this study.

Reviewer #3

1. We have restructured the paper so that it focuses more on what our results mean for Chinese mitten crab and avoid trying to overstretch our conclusions to try and encompass freshwater crustaceans in general
2. We appreciate your detailed review of our statistical analysis and have taken into consideration your concerns. We did not run the analysis as repeated measures because it was not a true repeated measure. The 4 different tanks were repeatedly measured but we cannot be certain that the same individuals were selected at each sampling point so avoided a repeated measures approach. Regarding the use of MANOVAs, we consulted with a colleague more familiar with this type of analysis. We found that our data violates the assumption of absence of co-linearity among dependent variables and the multiple normality test did not pass despite each dependent variable being normally distributed. For these reasons the MANOVA approach was avoided. In the end we took your advice to use a two-way ANOVA approach with a post hoc Dunnett's test as we are aiming to compare to how each group of crabs changes relative to the zero-day time point.
3. Suggested technical corrections have been addressed.