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Interactive comment on "Ocean acidification challenges copepod reproductive plasticity" *by* A. Vehmaa et al.

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We thank Referee #2 for the comments on our manuscript. We have considered all comments and suggestions. Please see response below:

Anonymous Referee #2 Received and published: 9 February 2016

The manuscript investigates the effects of ocean acidification reproduction of a Baltic Sea copepod and a potential role of the production of antioxidants for a better quality of the offspring. The effects of changing pH on the performance of zooplankton are at present in focus of the scientific community, and a large number of publications – mostly laboratory studies on reproduction– have been published in recent years. Although focusing on a timely topic, the manuscript is seriously flawed. As can be seen

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from the many comments below, I have problems with the strong focus on adaptation/plasticity in the introduction/discussion for which barely relevant data is presented and some conclusions which are not supported by careful interpretation of data. Most relevant, however, is the relatively weak experimental quality of the study which is below the requirements of Biogeosciences. Replication is lacking in most experiments, egg hatching and development is based on a low number of observations. While the flawed interpretation of results might be corrected (see details below), the methodological issues cannot. Therefore, I the MS does not have the quality to be published in Biogeosciences.

Author response: We thank the reviewer for the comments. It is unfortunate that our focus on maternal effects as a possible reason for the high buffering capacity of copepods in the face of ocean acidification did not convince the reviewer. The methodology used in the egg transplant experiment is published (Vehmaa et al., 2012) and we have conducted several laboratory-based OA studies using Acartia bifilosa as a study object (Vehmaa et al., 2012; 2013; Engström-Öst et al., 2014). The current study offered us a great opportunity to test previous results in a more natural environment in mesocosms. Until now, we had observed that A. bifilosa egg production rate is not affected by pH decrease predicted for the next century, but egg development might be. The egg transplant experiment used in this study enabled the possibility to separate the effect of OA on the reproducing female and on the developing egg. At least in this species, it seems to make a difference for the offspring development whether the eggs are laid in the same environment where they are developing (i.e., transgenerational plasticity). Also, when testing survival and reproductive success of a contemporary population in the ”future conditions”, we are testing plasticity of the animals.

The experimental set-up of the whole mesocosm campaign was planned in such a manner that there was an array of fCO2 treatments that were sampled repeatedly. The set-up for this copepod experiment followed the overall sampling schedule. This was not an ANOVA-type of study, so the criticism of the lack of replication is not valid.

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Our studies were replicated in time and were analysed statistically using appropriate nested-designs.

Introduction: p. 188543, line 4: I don't understand the context of plasticity and rapid change postu- lated here. Research has generally shown that oceanic copepods living in less variable environments have a large plasticity to pH beyond that of year 2100 scenarios (there are now several reviews available on this topic which should be cited; the few studies highlighted for negative effects in a later rather exceptions than the rule). This suggests that there might not be a significant selective pressure for a larger plastic- ity towards pH as suggested by the authors. In addition, I wonder why the focus is primarily on plasticity. The results presented here do not relate much to this or the underlying mechanisms (physiological, genetic). Finally, I miss the justification of the study in the seasonal context. In the seasonal variable environment the MCs were located, pH is driven up by the biological activity in spring, followed by the increase in production of heterotrophs. I therefore wonder about the pH conditions likely experienced by copepods in different climate change scenarios. Certainly, they will not experience equilibrium conditions. I miss a few words on this in the introduction. The only rapid and unusual change experienced by the species is the one associated with the very rapid decrease in pH at the beginning.

Author response: We have a small dilemma here since the first reviewer recommends us to add references showing negative effects of OA on copepods, and the second reviewer thinks that the studies showing negative effects are rather exceptions than the rule.

Unfortunately, the reviewer did not mention any of the reviews that should be cited. Despite a thorough literature search, we are not sure which reviews should be added. The claim that research has generally shown that oceanic copepods living in less variable environments have a large plasticity to pH beyond that of the year 2100 is new to us, because our impression on the matter is the opposite. For example, reviews by Whiteley (2011) and Halsband & Kurihara (2013) state that species that are currently

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inhabiting fluctuating environments are likely to be more tolerant to ocean acidification than those adapted to stable conditions.

According to our understanding, plasticity is the ability of an individual or a population to alter its physiological state, appearance or behaviour in response to the environment (West-Eberhard, 2003). In the current study, one population of copepods was divided into mesocosms subjected to different CO2 treatments. We followed copepods for several weeks and measured their physiological state (reproduction and antioxidant balance) and appearance (prosome length).

With respect to the seasonal context, the study was intended to cover the late spring/early summer period. For reasons outlined in the overview paper by Paul et al. (2015), this season was chosen to focus on the low productivity, i.e., the post-spring bloom period. Because of the low productivity during this time the pCO2 in the enclosed and surrounding waters were comparatively stable over time (see Paul et al., 2015 for detailed information on the carbonate chemistry in the mesocosms and the surrounding waters). Over the annual cycle, pCO2 and pH vary substantially at the study site as a result of biological activity and mixing/upwelling of CO2-enriched deep water. There are also strong spatial gradients in seawater pCO2/pH, most prominently between the surface layer and the CO2-rich deeper waters. Thus, the copepods in the study area are likely to experience strong changes in seawater carbonate chemistry, both seasonally and during their diurnal migration. As outlined above, one might therefore expect the plankton community in the study area to exhibit comparatively high plasticity and low sensitivity. As requested by the referee, we will add some information on this issue in the introduction.

p. 188543, line 26: Bron et al. might not be the original source for the information provided here, as well as Beaugrand et al. 2003 certainly does not provide original evidence of the diets of several important fish species. Whether zooplankton control harmful blooms is also disputable, the lack of grazing is more often inferred as a reason for bloom formation. Again, original literature should be cited.

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Author response: We have done our best to find original citations.

p. 188544, line 26: I don't understand how transferring the eggs from one mesocosms to the outside conditions contribute to the characterization of plasticity.

Author response: With transgenerational plasticity we mean maternal and paternal effects. We will clarify the sentence.

p. 188545, line 1: The first hypothesis needs more explanation; the preceding paragraphs do not provide evidence for this. And how is this related to the evaluation of plasticity in different environments postulated in these paragraphs? Little background is also provided for the second hypothesis.

Author response: We will aim to clarify the origin of these hypotheses even more in the revised manuscript.

Material and methods: p. 188545, line 9: More background knowledge is needed to understand the set-up. What pH had the water before the pH was adjusted to different levels? Timing of the experiment related to the seasonal phase of the system? Was the pH kept constant over the 45 days?

Author response: All mesocosms had a similar pH of around 8.0 before the pH adjustments using CO2 saturated seawater additions. A second addition of CO2 was made on Day 15 in the upper 7 m to counteract pronounced outgassing. Otherwise pH was allowed to fluctuate naturally.

We will add some more information; however, more detailed information on the set-up, adjustment of the pH levels, as well as stability of pH over the whole study period can be found in overview paper by Paul et al. (2015). www.biogeosciences.net/12/6181/2015/.

p. 188545, line 17: from day 24 to 45 sampling was not weekly.

Author response: We will rephrase the sentence.

p. 188546, line 10: Was the pH measured after the incubation? pH should increase

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due to low light conditions and heterotrophic activity.

Author response: pH was measured before and after the bottle incubations. We will add a table presenting these measured values as supplementary material.

p. 188546, line 10: Why were no samples taken to count eggs already present in the incubation water? The procedures described to account for this in a later step are not convincing because egg development time at 10 degrees is likely longer than 24 hours.

Author response: We did not count the eggs already present in the incubation water, because there were no suitable methods for this. Eggs are not evenly distributed in the 55 m3 mesocosms, so counting the eggs from an extra litre of water would unfortunately not have given us accurate information on the number of eggs in the incubation water. Handling of the incubation water, on the other hand, was restricted in order to keep the natural plankton community and fCO2 conditions as stable as possible. Please see also the responses below.

We do not consider the extra eggs in the incubation water to have caused a notable error in our results because the adult copepods perform diel vertical migrations, and stay below the surface layers during the time of our sampling (8:00-12:00) (Almén et al., 2014). Also, Acartia eggs sink (Katajisto et al., 1998; Katajisto, 2003), so the water sampler that took the integrated samples from 17 m to the surface would not have caught a large proportion of eggs laid during the sampling or some hours before.

p. 188546, line 14: The copepods are small, likely due to shrinkage in RNAlater. This is critical as no information is available whether this is affects all specimen in the same way (several preservatives due not). Anyways, females should have measured before preservation, as no biomass estimates are possible. EP should have been normalized to the strong variation in size, which is not possible anymore.

Author response: The referee is correct that RNAlater can affect the size measurements (e.g. Foley et al., 2010). The effect depends on the number of segments in the

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animal, i.e., the more segments the larger effect. Shrinkage is approximately 15% for copepods (Prof. Elena Gorokhova, Stockholm University, personal communication). As all the measured copepods were adult females, we assume the shrinkage to be in proportion similar for all individuals, which means that our results are quite conservative, and the size differences could actually have been larger before preservation.

Please notice that there was a typo in the title of y-axes in Fig. 1b. Naturally, it should be Prosome length (mm).

p. 188546, line 17: How many eggs were incubated per treatment? EP was very low during large parts of the experiment suggesting that only few eggs were incubated per treatment because of they needed to be divided between pH and outside MCs treatments. The number of eggs appears to be by far too low for reliable estimates of hatching.

Author response: The median number of eggs incubated per petri dish was 49, and varied between 11 and 158. We agree that 11 eggs might not be an ideal number to make reliable estimations of hatching success. Therefore we are pleased that only on the Day 24 in the MC 7 both of the hatching conditions (MC and common garden) included less than 20 eggs. Further, a low number of eggs is not such a big problem when using GLMM with binomial error structure for data analysis. Even though the hatching results are presented as percentages, we did not use the percentage data in the model. Instead, we had two columns with unhatched eggs in one and hatched nauplii in the other. R accounts for sample size and the logit link function to ensure linearity (Crawley, 2009). We have confirmed this with the departmental statistician (Åbo Akademi University).

p. 188546, line 19: I am not convinced that these are common garden conditions, as it is expected that outside conditions were closest to the low CO2 treatments; in consequence, transfer stress is largest for eggs transferred from high CO2 into outside conditions, which potentially bias the results. Parafilm is not airtight, consequently pH

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conditions were not constant during egg hatching incubation.

Author response: The difference between pH treatments was maintained in the Parafilm sealed petri dishes during the hatching/nauplii development incubations. We will add a supplementary table of the pH measured from the petri dishes before and after the hatching incubation. This will also allow the readers to notify the fluctuating condition in the common garden treatments.

p. 188547, line 1: This procedure is not convincing as it assumes that hatching time is shorter than 24 hours. This is not the case.

Author response: Not to filter the water even though we might get carry-over individuals or eggs with the water was a compromise we had to do. Filtering of water affects the gas balance (Riebesell et al., 2010) and filtering would have thus affected the fCO2 conditions of the water. We decided that we cannot risk the treatment condition in favour of no extra individuals or eggs in the incubations. We managed to figure out ways how to deal with these possible source of errors as described in the text. However, the reviewer is right that if extra eggs ended up in the egg production incubation bottles and if they did not hatch during the incubation, we could not separate them from the ones that were actually produced in the bottles. Anyway as mentioned already above, during the sampling the copepods are migrated deep and eggs broadcasted then are also deeper down in the water column, whereas eggs that are broadcasted in the surface layers at night have had hours to sink before sampling takes place (Holllilland et al., 2012; Almén et al., 2014). In that sense, sampling schedule was nearly optimal for our purpose and sampling occurred when egg number was probably at its lowest in the water column on a 24 h schedule.

p. 188547, line 10: The lack of replication is seriously critical especially in the development experiment, but also for EP and EH. In addition, estimates of EH are based on small numbers, as are those of 'development'. Considering the bias due to introduced eggs and nauplii with the incubation water, this is not state of the art and below the

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experimental quality required for a journal with high impact factor.

Author response: The set-up was built so that we had replication in time and it was analysed as such. That means that the gained results are reliable. We strongly disagree with the reviewer that state-of-the-art studies should always be replicated, ANOVA-kind of set ups. Also, as noted already above, modern statistical analysis, such as GLMM, can take into account the varying sample sizes. Of course it would be great to have many replicates but there are limiting factors concerning how many replicates are possible to handle. In this case, also the number of animals that can be caught weekly was limited as the populations had to remain abundant in the closed mesocosms until the end of the seven weeks long experiment.

p. 188548, line 8: TPC is a poor predictor for feeding conditions of copepods, which feed generally on food larger than 10 μm.

Author response: Food larger than 10 μm is included in the TPC fraction of <55 μm.

Results: p. 188550, line 6: Error bars are missing in all figures; methods should give more details on the number of eggs incubated for hatching. Is the increase from day 3 to 10 significant? When size varied, EP should be normalized. In Table 3 units are missing. The table needs explanation as it contains only limited information on the variation of environmental factors. What does 'since start' mean? A graph giving their temporal variation would be much more interesting. May be I am wrong, but must there not be 3 days averages for each time egg production was measured? What about changing food composition in terms of size and species composition. Food > 10 μm is usually a much better predictor of egg production than < 55 μm. How do the authors else explain the variation in egg production with the low variation observed in Chla? Acartia species are known for their omnivory, and heterotrophic food is not included in quantitative estimates of food abundance. This might very well influence and bias any statistical analyses.

Author response: Please notice that the average values in Figure 1 are averages per

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bottle i.e., a) the total number of eggs produced divided by the number of females (∼17) per bottle per day, b) average prosome length of ∼15 females, c) hatching success (%) calculated using all the eggs on the petri dish, d) measurement based on a sample of ∼30 females. Therefore no standard deviation can be applied here.

Our linear mixed model (LMM) did not test the difference between days. However, we tested the difference in EPR between Day 3 and Day 10 separately, and can therefore conclude that the difference is statistically significant (paired student's t-test: t = -5.115, df = 5, p = 0.0037). There are also studies stating that in A. bifilosa female size and egg production rate do not necessarily co-vary (Koski and Kuosa, 1999). Therefore, we wanted to present size and egg production results separately.

We will add more information to Table 3. Unfortunately we cannot add a graph showing the variation in fCO2, TPC (<55 μm) and C:N (<55 μm) during the study because those results are presented in Paul et al. (2015) www.biogeosciences.net/12/6181/2015/.

Please notice that total particulate matter larger than 10 μm is included in the fraction smaller than 55 μm. We are aware of that the fraction chosen to indicate the quantity of food is not perfect but it is the best one available. The other options would have been chlorophyll a, TPC (<10 μm) or biomass' of different phytoplankton taxa. As mentioned also by the reviewer, Acartia is omnivorous, and chl a or a selection of separate phytoplankton taxa would not describe its possible diet in a satisfactory manner. The used TPC fraction includes all particles that are smaller than 55 μm and contain carbon.

p. 188550, line 11: Again, error bars are missing. A major reason for changing size is the maturation of new females. The size increase seems to be delayed in the MCs with lower pH and therefore Information on the pH prior to pH adjustment must be provided. Regarding EH the authors should be careful not to emphasize differences of a few percent, especially considering that no information about the number of eggs is provided and no replication was done.

Author response: FCO2 in the mesocosms prior to the pH adjustments was 237±9

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μatm and pH ~8. We will add this information to the manuscript. Please note that Day 3 was not included in the prosome length analysis.

As already mentioned above, we had replication in time, and the median number of eggs incubated per petri dish was 49. We will pay more attention to the strength of the effects; however, if the differences are statistically significant it is our duty to report them as such.

p. 18551, line 4: I have some doubts whether these are common garden conditions. Based on the provided information on the set-up of the experiments (which is poor), one would expect from the natural seasonal variation of pH in the coastal Baltic that the common garden conditions are close to the lowest CO2 treatment. These are not common garden conditions. Anyways, environmental conditions in the common garden must be presented.

Author response: The common garden conditions are common conditions for the animals originating from the mesocosms at a certain time point. The conditions outside the mesocosms fluctuated more than inside them during the study, but they were anyway the same each week for all treatments. The analysis takes this into account by comparing hatching and nauplii development measured at the same time point. Nevertheless, we agree that using the name common garden can be misleading and we will change it to Baltic water. We will add a supplementary table presenting the measured pH values before and after the incubation and including also pH values of the Baltic water.

p. 18551, line 14: Which adaptive maternal effects are meant here, and why adaptive? As outlined above females in the high CO2 treatments were likely exposed to largest differences between start of pH lowering and first experiments. I would conclude that acclimation time to a drastic decline in pH was too short, but as soon as next G developed effects vanished. This has nothing to do with adaptation. Anyways, results should not be interpreted at this point.

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Author response: We expected eggs to have higher hatching success and develop more rapidly in the mesocosm water compared to common garden (Baltic Sea) water because mothers are able to adjust their eggs to prevailing conditions (maternal effect). Moreover, the effect is adaptive because it increases the fitness of the offspring. Nevertheless, we will delete this sentence and interpret the results in the Discussion.

72-hour acclimatisation time have been used for CO2-treatments even higher than the high treatments in this study (Cripps et al., 2014a; 2014b). Also, based on our previous experience with Acartia sp., three days is enough for this species to acclimatise to changed CO2-conditions (Vehmaa et al., 2012).

Discussion: p. 18552, line 2: T has a strong influence on the efficiency with which food is utilized by copepods, particularly, when food resources are limiting as in the MCs. Although T did not vary among the MCs, it increased over the first two weeks from 9 to 15 and, therefore, has an interactive influence on the efficiency of food utilization together with food conditions in each of the MCs. Thus, T needs to be included in the analysis. Why 'phenotypic buffering'?

Author response: Please notice that we have explained what we mean by phenotypic buffering in the Introduction, and also in the sentence in question in the Discussion.

We agree that temperature influences copepods a great deal, and analysing temperature-food interactions would certainly be highly interesting. However, with a restricted data set, only the most interesting and justifiable variables should be tested to avoid over-parameterisation of the model (Babyak, 2004).

p. 18552, line 6: I wonder how much of the significance of pH effects on hatching and size is influenced by day 3 measurements. The MC set-up introduced likely some strong, artificially rapid decrease in the pH in those MCs with very high CO2 (the authors must report the initial pH before acidification) during the first days. This has to be taken into account when comparing responses of copepods. Any delay induced in the development of a cohort due to the rapid change (which took place because size

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increased in all mesocosms!) has therefore a strong influence on the interpretation of the results at particular days and needs to be taken into account; and cannot be interpreted as threshold. Again food conditions and T interact in influencing also size of females, making the analysis of the influence of T, food and pH difficult. After 10 days size and hatching (which was in all MC > 90%) was rather similar, pointing to no strong pH effects as claimed here. In this context, I would like additionally to emphasize the methodological limitations of the study that make interpretation also difficult (see above, e.g., the lacking replication, low number off eggs, inappropriate development experiments). Anyways, the artificial rate of change in the beginning needs to be taken into account. In my opinion, the conclusion of pH effects on size and hatching and lacking adaptive maternal effects is not supported by evidence.

Author response: Please notice that Day 3 was not included in the prosome length analysis (Table 1). The significant negative effects of fCO2 and TPC on copepod size were gained without data from that day. For egg production and hatching success, three days acclimatisation period was considered to be sufficient (e.g., Cripps et al., 2014a; 2014b; Vehmaa et al., 2012).

Again, we agree that analysing temperature-food interactions would be highly interesting, however impossible in order to avoid over-parametrization of the model. Hopefully we are able to test these effects in a future study.

We consider this to be a very successful study because of the high egg hatching success in all treatments. This indicates that the copepods were doing fine, and that the differences between the treatments were actually because of the treatment conditions, and not due to stressful lab conditions, bottle effects, or bad food etc.

Please see also the replies above concerning replication and the number of eggs in the egg hatching/development incubations.

p. 18552, line 9: Here I miss an evaluation whether food < 55 μm actually can show what the authors wanted to show. This size choice is against many other studies that

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show for instance a much better predictive power of food estimates > 10-20 μm. How is the increase in EP by a factor of 3 explained?

Author response: Please notice that the fraction of particulate carbon larger than 10-20 μm is included in the used fraction of smaller than 55 μm. As already mentioned above, TPC (<55 μm) was the best available estimate of copepod food quantity.

p. 18552, line 16: I find this confusing: Table 3 shows low concentrations and small ranges in TPC. This obviously contrasts the statement here that there was a sharp decline in Chl a.

Author response: Unfortunately, Table 3 shows only the range over the whole experiment, and the decline in primary production after Day 17 is not therefore visible in the table. Also, TPC and chlorophyll a do not necessarily co-vary since organic matter of heterotrophic origin is also included in the TPC analyses. For more detailed results of e.g., Chl a, TPC and fCO2, please check Paul et al. (2015). www.biogeosciences.net/12/6181/2015/

p. 18552, line 19: Hatching of eggs was > 85% in the majority of the incubations over a variation in ORAC by a factor of three. In addition, there are many other factors influencing egg hatching success, particularly composition and quality of food. I am not convinced that ORAC in females is the main factor influencing egg hatching. The authors postulate a threshold around 800-1000 μatm; still hatching was > 90%, and again the problem of lacking replication and estimates of variability exist in addition to the considerable low numbers of eggs that were used in experiments.

Author response: Please notice that we are not at any point suggesting that ORAC in females would be the main factor influencing egg hatching success. We did test the effect of food quantity and quality on egg hatching success, and ORAC was not included in that generalized linear mixed model (GLMM). The measurements of female copepod antioxidant balance were done in order to provide possible additional information of the maternal provisioning on the offspring. We do not have proof that this would be a case

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of cause and effect, and that is exactly why we tested their correlation. We will clarify this even more in the revised manuscript.

p. 18552, line 22: Results on development should be shown, and details on the number of nauplii examined should be provided.

Author response: We will add a supplementary table showing the total number of incubated eggs, as well as the number of hatched nauplii.

p. 18553, line 1: The authors are analyzing here differences in egg hatching of a few percent based on estimates that are seriously flawed by the experimental quality. I am not convinced.

Author response: We consider it to be our duty to report statistically significant results and trust on them. As already mentioned several times above, we do not agree with the reviewer concerning the reputed flawed experimental quality.

p. 18553, line 22: The relevance of transgenerational effects for interpreting the present results needs explanation. In addition, what is the potential influence of changing T over time for the interpretation of the observation in comparison to other studies?

Author response: We will clarify the connection between our results and transgenerational effect in the Discussion. In addition, we will add a sentence concerning the effects of temperature on copepod food requirements to the Discussion.

p. 18555, line 1: Again the interpretation of the cause of the effects on size suffers from adequate measurements of food quantity available to copepods. In addition, an evaluation of the variability in size is lacking, and the measurements are based on insufficiently low female numbers. There is also some variability in the estimates (e.g., MC6). Any suggestions? Moreover, the generalization to 'high' CO2 is not supported by data, as at 1000 ppm, size doesn't seem to be influenced much. In addition, the problem exists that due to potential delays in development caused by an initial pH 'shock', the conditions for cohort development (food, T) differ among MCs. For instance, a delay

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in MC 8 might have caused a cohort of copepods to develop at suboptimal food conditions at a different T (as indicated by EP). Thus, results are not directly comparable with regard to pH.

Author response: Here the reviewer is asking about the food quantity, which we have already shown to match the preference of the reviewer. It would be very helpful if the reviewer could have provided an estimate of a sufficient number of animals for prosome length analysis. We realise that the needed number depends on the size of the effect. We managed to find a significant negative fCO2 effect with 462 adult Acartia-females measured. We will add the possibility of delay in cohort development as a potential reason for the detected effects to the Discussion.

p. 18557: Conclusions: The generalization from effects of mineral composition (C/N) to food quality is doubtful.

Author response: We agree that C:N is not the best, or at least not an all-inclusive estimate of copepod food quality. We will add references and this information to the revised manuscript, as well as tone down conclusions based on it.

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