Response to review of:
The influence of food supply on the response of Olympia oyster larvae to ocean acidification

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Author response in bold italics:

We would like to thank Anonymous Reviewer 1 for his/her thoughtful review of our manuscript. The comments, questions, and suggestions raised by the Reviewer have improved our manuscript. Below are our point by point responses (in bold italics) to all issues raised by Reviewer 1. The manuscript has been revised accordingly.

Anonymous Referee #1
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This is a very nice contribution by Hettinger et al that examines the implications of variable food and CO2 levels for the performance of Olympia Oysters. They performed an experiment that examined three response variables and documented differential, significant effects on each variable measured. The experiments were performed well from a biological and chemical perspective. The results were clear and important. There are multiple good opportunities to improve this manuscript. I specifically believe some of the methods and statistical design require significant clarification. There are a series of key references what were not included. The discussion seemed too abbreviated as multiple opportunities to place the current findings in the proper perspective of current and former OA research were missed. This will be a good contribute to OA research.

Introduction and discussion: Add Gazaeu et al 2013 Marine Biology (has DOI number, not volume and pages, yet) as an excellent reference regarding general OA effects on bivalves.

The reference has been added to the introduction and discussion sections.

Intro page 3, line 6: salinity, dissolved oxygen, and patterns of primary production. There are good references for these what should be cited.

We now include references regarding each of these environmental factors (Behrenfeld et al., 2006; Harley et al., 2006; Riebesell et al., 2007; Diaz and Rosenberg, 2008).

Intro page 3, line 21: While Reisbell showed C:N ratios change, he did not examine larvae. Can you supply a references demonstrating that the C:N in Reisbell's paper are detrimental to the larvae?

We revised this section and added three references: two that specifically tested OA effects on phytoplankton (Rossoll et al., 2012; Caron and Hutchins, 2013), and one that tested effects of HAB species vs. non-HAB species on bivalve larval performance (Talmage and Gobler, 2012).

We also located a study by Reinfelder (2012) that examined elemental compositional changes as a function of elevated pCO_2 in various phytoplankton species (including Isochrysis galbana, which was the microalgal food used in our study). In this case, I. galbana C:N ratios were positively correlated with pCO_2. We are not aware of a study that explicitly tested effects of OA-induced elemental compositional changes in microalgae on larval growth.

Intro page 4, line 8: Holcomb et al., 2010, 2012; Melzner et al., 2011; Thomsen et al., 2013; Comeau et al., 2013. Talmage and Gobler 2012, MEPS, considered OA and altered food, specifically a harmful alga which is known to be a poor nutritional food source and could be considered here.

Indeed, the Talmage & Gobler (2012) study is certainly relevant and is now referred to several times in the manuscript.

End of intro, page 5: "In these laboratory experiments, we paid particular attention to the possibility that negative effects of high pCO_2 might be ameliorated under high food availability." Its seems like this should specifically references the findings of Melzner et al 2011 and Thomsen et al 2013 or that the findings of Melzner et al 2011 and Thomsen et al 2013 should be explained in some detail somewhere in the introduction which sets up this hypothesis.

We have added a discussion of this work in the introduction (Pg 5, line 3-8).

Methods, page 5, line 18: Given the subject of the paper, it was surprising the level of T-Iso fed the adults was not mentioned; please provide.

We now include the approximate concentrations of Isochrysis galbana fed daily to the adult oysters during the 72 hours they were held in the culturing cylinders.

Methods, page 5, line 22: Comment on and provide a reference to put the larval density used (1000 per 4.5L) compares to densities in the field. This is very important, as the larval density is proportional to the nutritional demand and thus this will allow your food supply rate to be placed in its proper environmental perspective.

We do not have values for the density of Olympia oyster larvae in the water column in Tomales Bay. Garcia-Peteiro et al. quantified larval densities ranging from < 10 to > 50 larvae per m^3 between June – October in Coos Bay, Oregon. The larval densities we used for laboratory rearing were based upon general rearing recommendations for invertebrate larvae (~1 larva per 2 mL seawater). Our food levels were the equivalent of daily algal cell to larva ratios of 50, 25, and 5 cells per larva per day for the high, medium, and low food levels, respectively.

Methods, page 6, line 6. 1000 μatm is also the level found in upwelled water on the US west coast (Feely et al 2008, Science). I believe this is worth pointing out perhaps while pointing out the finding of Barton et al 2012 that modern day CO_2-enriched seawater can impact bivalve larvae.

We now include a discussion of the dynamics of upwelling and pCO_2 concentrations off the northern California coast, and refer specifically to work by Barton et al. (2012) (Pg 7, line 2-5 and Pg 15, line 15-21).

Methods, page 6, lines 22-24. Provide the mean and the range of food levels found. As stated, the value provide ('can reach 24.6 mg/m^3') makes it sound like a maximum. True? This would be very high. Stating the mean and range would be of greater value to the readers.

We have revised this statement to better clarify how food levels in our cultures compare to those in field locations in our region (Tomales Bay) during the time of the year when oyster larvae are likely to be in the water column.

Page 7 lines 12-17: "These buffers were made in-house and checked against a certified TRIS buffer (A. Dickson, Scripps Institute of Oceanography, La Jolla, California)." And how did they check out? Please provide a percent similarity ± SD. Similarly, provide a level of accuracy attained via measuring Dickson's TA standard. Similarly, it would give readers confidence if they had a sense of how the measured pCO_2 levels compared with those calculated using CO2SYS using TA and pH.

We monitored pH primarily as a real-time indicator of changes in the carbonate system, not as an input parameter for CO_2 system calculations, given the limitations of potentiometric pH measurements for the purposes of CO_2 calculations. We now specify this in the manuscript. The percent similarity of the TRIS buffers was approximately 3% (±0.01) The offset between measured and certified TA values for Dickson CRM (Batches 104, 107, 111) from 10/22/10 to 9/7/11 was -0.01 (±2.81) meq/kg (n=91). This is now included in the manuscript. Due to recognized challenges in using potentiometric pH measurements as inputs for CO_2 system calculations, we feel that it is possibly misleading to include pH-based calculated pCO_2 concentrations in Table 1 and have therefore refrained from doing so. Instead, we used our check samples of DIC and TA.

Page 8 lines 1-9. Drying day old larvae would seem to alter their size (shrivel). Please comment on how drying for 24h effected the larval size in the methods.

Moisture content can certainly influence the mechanical properties (e.g., strength or stiffness) of some biomaterials, but we are unaware of data indicating significant changes in the size of calcium carbonate structures due to water loss.

Page 8, line 15: The use of AFDW on calcifying organisms can present an issue as shell can volatilize resulting in bias of the results, see (Goulletquer and Wolowicz, 1989) and salt residue on un-rinsed larvae may also contribute to measured weights (Moreno et al., 2001). Can the authors demonstrate that is not a concern in this case?

All larvae sampled for AFDW were rinsed twice with distilled water prior to drying, in order to remove salt residue on larval shells. We have clarified this in the text.

Page 8, line 20: Very clever use of the plates! I think it would be very useful to state precisely how you interpret settlers on plates. Is the assumption that those that do not settle have perished? Do any individuals settle on regions besides the plates? Is that possible?

"Settlers" include only settled and metamorphosed individuals (not pediveligers or dead larvae). The only other surface onto which larvae could settle would be the sides of the jar. All jars were thoroughly inspected for settlers, and if found, were included in the count. We have clarified these details in the manuscript.

2.5 Statistical analyses: The design here was unexpected as its not clear why headspace was a treatment factor that was considered statistically. It was not clear how the headspaces would be different, even upon re-reading the methods several times. I think the headspaces required further clarification earlier in the methods and an explanation here as to why it was evaluated statistically.

Results: Again, the headspaces does not make sense. Why would the flasks differ based on this? If this is because the details are in the author's 2012 paper, they should make the effort to add those details to the methods here, as presently, why headspaces would be different or test is not clear

We have clarified our experimental design (Pg 7, line 10-14), especially in terms of the sealed air spaces above the jars (termed "headspaces" in the manuscript). These headspaces are constructed of acrylic boxes mounted over the seawater table. Jars are screwed into the bottom of these boxes, which provide the same mixed gases and a common headspace for 5 jars. In total, there were 3 boxes ("headspaces") for each pCO_2 level (2 pCO_2 levels x 3 headspaces = 6 boxes total), and there were 5 jars screwed into the bottom of each box/headspace. Each food level was represented in each of the boxes/headspaces. This design was used to minimize off-gassing during culturing. Any biological processes occurring within each jar and any resultant effect on seawater chemistry within each jar operated independent of the box/headspace. However, because the jars were nested within the headspaces, we were interested in whether there was a significant effect of the headspace, although we did not anticipate any differences due to the box/headspace. For this reason, we include Headspace[pCO_2] as a factor in our analyses. These results are now presented in supplementary statistics tables.

Also, can the authors defend the use of their nested ANOVA multiple times (multiple days) as opposed to using a repeated measures ANOVA for the course of the experiment?

We agree that there are other approaches could conceivably be employed for the analyses: a split-split plot (in which the second split is day) or a repeated measures analysis. We chose to use separate tests at each time point rather than a repeated measures approach because we measured different individuals at each time point. In this case, the replicates at time 1 (day 5) are independent of those at time 2 (day 5) and time 3 (day 11), and thus we felt that there was not a need to account for any non-independence across time.

Between the pH rising and the alkalinity not changing (= no uptake of nitrate or phosphate by algae), this suggests the cultures were in stationary phase growth which, in and of itself, alters the nutritional content of the algae. As such, a greater effort should be made in the methods to describe the growth phase of the algal culture (exponential vs stationary phase growth). To be clear, this is not nit-picking but rather plays directly to the entire theme of the paper: Algal food quality changes as a function of the growth phase and condition of the algal cells. For example, the C:N ratio change through the growth cycle can be larger than the C:N ratio the authors referenced in the introduction in the Riesebell et al 2007 paper.

We agree that not only high pCO_2, but also the growth phase can potentially impact phytoplankton, but we believe that our water change/feeding schedule minimized any potential effects. We have added an acknowledgement of these factors in the manuscript, and explain how our methods likely minimized any such confounding effects on larvae (P 8, line 7-17).

Please describe the number of settlers in terms of percentage of total in the figure and/or results.

We have recalculated our "number of settlers" data to represent percent metamorphosis. All text, figures, and figure captions have been edited appropriately.

Discussion: Overall, the discussion was surprisingly brief. To the point is good, but some good opportunities were missed here. The authors should consider some of the following points:

1. The effects of CO_2 on growth here were tiny (10%) compared to the very large effects on settlement (70%). Why would this be? What are the implications for early life history oysters?

This is an excellent point, and we have added a discussion of the causes and implications of these patterns to the discussion.

2. Upwelling of nutrients enhances primary production and thus counteracts acidification effects. This was discussed, but I think the paper would benefit from some further detail on this. How long to the low vs high pH / CO_2 periods last during upwelling events? How does the timing and duration of upwelling compare to the larval stages for Olympia oysters in this region?

We have added these details to the discussion.

3. How do the precise physiological effects of less than ideal food described here compare to the findings of Holcomb et al., 2010, 2012; Melzner et al., 2011; Talmage and Gobler, 2012; Thomsen et al., 2013? Given this number of studies, can we now drawn some larger conclusions about food level and CO_2 or are we getting different answers for different organisms? The experimental design and results here are quite similar to Melzner et al., 2011 and Thomsen et al., 2013, so I believe expanding on the parallels there is warranted.

We have added details in the discussion on studies that have examined ocean acidification and varying food levels in bivalves. There are few comparable studies within single life history stages. Melzner et al. (2011) studied adult mussels, and Thomsen et al. (2013) studied juvenile mussels. Talmage and Gobler (2012) is perhaps the best study for comparison since larval

oyster and scallop larvae were the focus, and one of the food sources used was the same as in our study (I. galbana). However, this study was focused primarily on the effects of a HAB species on larvae as compared to food density (i.e., supply) effects.

Page 12, line 9: "in the absence of high seawater pCO_2" Given you talk about high CO_2 water next, it would seem better to use the term low CO_2 here instead of "in the absence of high seawater pCO_2"

We have reworded this sentence.

Page 12: Seems appropriate to make the linkage to the upwelling / oyster larvae hatchery paper by Barton et al., 2012 in this portion of the discussion.

It was an oversight on our part to not include discussion of Barton et al. (2012) in this section. We thank the reviewer for pointing this out, and have added a brief discussion of the study to this section.

Page 13: "Our results suggest that Olympia oyster larvae do not demonstrate the ability to counteract exposure to elevated pCO_2 conditions in high food environments." This statement is not reflective of the data in the paper. Better to state "Our results suggest that Olympia oyster larvae do not demonstrate the ability to fully counteract exposure to elevated pCO_2 conditions in high food environments.

We have made this change by adding the word "fully" to the sentence.