Dear Tina,

Pleased find enclosed our revised MS and the responses to the reviewer's comments below. We thank very much for your kind and positive words of our manuscript. We also would like to thank for the reviewers' pertinent comments concerning the MS. Both suggestions and criticisms are very valuable and helpful for revising and improving our MS. Below, we have addressed all of the reviewers' comments and made corresponding revisions in the MS which were marked in red. We hope that you will agree that our MS is now improved and fulfillsthe requirement for publication in Biogeosciences.

Yours sincerely,
Jingli Mu

Reviewer #1:

Comment 1. General Comments: This study investigated the effects of elevated seawater pCO_2on the embryogenesis and organogenesis of marine medaka (*Oryzias melastigma*). Newly fertilized embryos were exposed to control (pH 8.2) and reduced (pH 7.6 & 7.2) pH levels for 21 days. The experimenters found no significant differences in hatching time, hatching success, and larval heart rate between pH treatments. However, the pH 7.2 treatment was found to cause significantly more developmental abnormalities than the control; including spinal deformities, craniofacial deformities, stretched heart and pericardial edema. In addition, the researchers found slight differences in otolith development. The average areas of the left and right sagittae were significantly smaller in the pH 7.6 treatment than the control. Such an effect was absent in the pH 7.2 treatment.The study provides needed data on the effects of elevated pCO_2 on fish early life stages from a marine species. Such studies are valuable given the current uncertainty surrounding the potential effect of ocean acidification on fish early life stages, a topic that's suitable for Biogeosciences. However, I cannot recommend this manuscript for publication until inaccuracies in the description of other studies regarding ocean acidification and marine fish early life stages are corrected and uncertainties in their methodology clarified. Major Concern 1: Although it may be unintentional, the authors misstate some findings in previously published literature

Response 1: Thanks for your recognition and positive comments of our work. We realize the inaccuracies in the description of other studies regarding ocean acidification and marine fish early life stages and corrected now in the MS. We also clarified some findings in previously published literature in the MS as recommended.

Comment 2: Example 1 (P9, L222) the authors write "Based on our results, the tolerance of marine medaka to increased CO_2 level is possibly stronger than fish such as red seabream and Japanese whiting. The reason is

likely attributed to different life history and living habit of fishes. Marine medaka, which lives in estuary and adapts to differently environmental salinities, possess some ability to adjust a range of pH fluctuation, whileoffshore coral reef fish, red seabream, has a strict requirement of environmental factorssuch as salinity and DO for growth and production. Therefore, red seabream has a bad adaption to CO_2-driven pH fluctuation." The authors are referring to the findingsin Kikkawa et al. 2004 where red seabream *Pagrus major* were exposed to pH levelsof 6.2 and 5.8. These levels are not relevant in the context of future ocean acidification. Thus, suggesting red seabream have a substantially lower tolerance to CO_2 than medaka is inaccurate, the studies are not comparable due to significant differences inmethodology.

Response 2: We agree with the reviewer's comments and made corresponding revisions in the manuscript. Below are our clarifications:

In our experiments, the duration of embryonic stage, egg survival and embryonic heart rate of marine medaka were unaffected by acidification water with pH 7.6 and pH 7.2. There was a slight increase in embryonic duration of eggs, but the size effect was not different among the three pH treatments. Overall, these results suggest that the egg stage of marine medaka is relatively tolerant to elevated CO_2 and low pH level, which were consistent with the results reported by other studies on a diverse set of marine fishes. For instance, Munday et al., (2009) found the survival to hatch of orange clownfish (*Amphiprion percula*) from the Great Barrier Reef, Australia, to be nonresponsive to pCO_2 levels to 1020 ppm (pH 7.8). Similarly, Franke and Clemmesen (2012) found no significant effect of elevated pCO_2 levels from 460 to 4635 ppm (corresponding to pH 8.08 ~ pH 7.05) on survival to hatch of Atlantic herring from the western Baltic Sea. In the study of Frommel et al. (2013), the survival of embryos of Atlantic cod from the Bornholm Basin of the western Baltic Sea was not altered at pCO_2 levels up to 4000 ppm (pH 7.2). Hurst et al. (2013) also reported no effect on embryo survival of walleye pollock (*Theragra chalcogramma*), common in the temperate eastern North Pacific, at pCO_2 levels up to 1933 ppm (pH 7.4). In other cases, however, a strong effect of CO_2 was observed evident in the embryo survival of summer flounder (*Paralichthys dentatus*), an ecologically and economically important flatfish of the inshore and nearshore waters of the Mid-Atlantic Bight (Chambers et al., 2013). The relative survival of summer flounder embryos was reduced to 48% when maintained at 1808 ppm pCO_2 (pH 7.5) and to 16% when maintained at 4714 ppm pCO_2 (pH 7.1). Baumann et al. (2012) also reported a 74% reduction in survival of embryos and young larvae of inland silverside, *Menidia beryllina*, native to estuaries of the US Atlantic coast, when maintained at 1100 ppm pCO_2 compared to those held at 410 ppm pCO_2.

Comment 3: Example 2 (P10 L230) the authors write that "Inland silverside is also common in estuary;however, the survival and length of larvae are positively related with CO_2 concentrationwhich is possibly associated with its life history". Presumably, the authorsare referring to the findings of Baumann et al. 2012 that actually showed the opposite effect, survival and growth were negatively correlated with increasing CO_2 concentrations.

Response 3: Corrected. The corresponding revisions in MS are as below:

All of these studies varied in the number of parents used, the time lapse between egg fertilization and the

initiation of the CO_2 treatment, and in how and when the survival was scored. For example, the CO_2 treatments of inland silverside by Baumann et al. (2012) began at approximately 24 h post-fertilization, and the survival was scored at approximately 1 week post-hatching. The different approaches used among previous studies may preclude a fair cross-study comparison (Chambers et al., 2014), however, the overall presence of effect of elevated CO_2 environments on embryo survival is in contrast to the findings here. The habitats occupied by a species, particularly its ELS, may play a role in their sensitivities whose ELS are found in estuarine (marine medaka) or inner shelf (summer flounder) habitats, both with relatively high ambient CO_2 levels, exhibit different sensitivity to experimentally elevated-CO_2 levels which is counter to expectations and requires further attention.

Comment 4: Example 3 (P10 L 232) the authors write "In addition, research on inland silversidefound that survival and body length of larvae significantly decreased compared to thecontrol group after exposure to 1000atm CO2 for 7 days, while those of embryos werenot affected, indicating more sensitivity of larvae to CO2 than embryos. The reasonswere attributed to the self-protection of fertilized embryos and their less dependenceon external environment (Baumann et al., 2012)." This statement is confused andinaccurate. Baumann et al. 2012 found the embryonic stage to be more sensitivethan the larval stage, and never concluded that 'self-protection' of fertilized embryosincreased their CO2 tolerance.

Response 4: According to Baumann et al. (2012), when compared with present-day CO_2 levels (~ 400 ppm), exposure of Inland Silverside (*Menidia beryllina*) embryos to ~ 1000 ppm until one week post-hatch reduced average survival and length by 74% and 18%, respectively. Therefore the eggs were more vulnerable to high-CO_2 induced mortality than the post-hatch larval stage. Sorry for the confusion and we have revised the statements in manuscript as shown below:

An unexpected result of our study was that elevated levels of CO_2 affected larval development abnormalities, and the average deformity rate of marine medaka larvae (approximately one-week post-hatch) increased significantly ($p < 0.001$) by 16 % as CO_2 increased from control level (pH 8.2) to high-CO_2 level (pH 7.2). Although CO_2-induced acidification up to the high-CO_2 level (pH 7.2) had no noticeable effect on larval survival by the end of experiments (21 d), the larval development abnormalities may ultimately influence the later life consequences and therefore further reduce the productivity of fish stock in future acidified oceans. Chambers et al. (2014) found no reduction in survival with CO_2 for larvae during the first four weeks of larval life (experiment ended at 28 d post-hatching (dph)), however, the sizes, shapes, and developmental status of larvae showed initially longer and faster growing when reared at pH 7.5 and pH 7.1 levels, and the tissue damage was evident in larvae as early as 7 dph from both elevated-CO_2 levels. At present, it is unknown that how increasing CO_2 levels affect development and survival in fish early life stages. Even if fish embryos and early larvae are capable of physiological adaptation to increased CO_2 somehow, this would incur further metabolic costs and thus reduce energy available for tissue synthesis or post-hatch survival on diminished yolk reserves. As some fish eggs, including those of *Oryzias melastigma*, seem to be tolerant to low-pH conditions, the high levels of CO_2 or associated changes in carbonate chemistry may be more important to larval-fish development than hydrogen ion concentrations. (Baumann et al., 2011; Ishimatsu et al., 2008).

Comment 5: Example 4 The authors make conclusions regarding their results based on their inaccurate understanding of previous studies. For example, on P10 L237 the authors write “Interestingly, our results seemed to support the above conclusion as heart rate, hatchingrate and hatching time of marine medaka embryos were not significantly affected while obvious deformities were observed in newly-hatched larvae, suggesting the latterwas more liable to be influenced by OA.” Presumably the authors are suggesting their results that embryos appear unaffected by CO_2 but larvae show deformities, corroboratethe findings in Baumann et al. 2012. Again, Baumann et al. 2012 concluded thatembryos were most sensitive to CO_2.

Response 5: Based on the findings of Baumann et al. (2012), we have made revisions in manuscript (as shown in response to comment 4) .

Comment 6 : Major Concern 2: The methodology employed for the developmental toxicity may need further clarification. How was deformity rate calculated? Is it simply the proportionof larvae, which demonstrated one of the mentioned developmental deformities? Theauthors sampled both embryos and larvae for analysis. Does the calculated deformityrate include both? This is unclear. In addition, were embryo or larval samples replacedafter analysis? If so, with a rather small sample size, how did the authors take intoaccount the possibility of resampling? I worry about the conclusiveness of their toxicologyresults given the uncertainties in their methodology. Also, presumably the authorsmaintained survival data during this experiment. Such data would be extremely usefulfor other investigators and I wonder why it was not presented.

Response 6: The detailed protocols for the developmental toxicity were described in the revised manuscript according to the reviewer’s suggestion (see section 2.4). For the sample size, three replicates produced a total of 90 eggs in each pH groups during the embryonic stage, and three replicates produced a total of 66 ~75 newly hatched larvae in each CO_2 treatment level. The data of survival larvae were added in revised manuscript as recommended. The methodology employed for the developmental toxicity were shown as follows:

The numbers of embryos surviving to hatching were counted based on daily inspection of the embryos in each treatment. Hatching rate data were summed and converted to proportions of survival numbers out of 30 eggs in per replicate. After 8 days post fertilization, and 3 days before expected hatching, embryos were inspected at least twice a day to record hatching numbers. Estimates of heart rate were completed by counting the number of heart beats over a 30 s period (n = 10) at day 8. The time when ≥ 50 % of the embryos had hatched was recorded as "hatching time" (Forsgren et al., 2013). As observations of spawning and hatching were made at somewhat irregular intervals over the course of the study, spawning and hatching times were analyzed. The embryonic hatching time was calculated as the time elapsed between spawning and hatching.

On day 21, thirty larvae (10 larvae per replicate) from each CO_2 treatment were randomly selected and photographed for deformity analyses. The deformity rate were calculated based on the proportions of abnormal larvae numbers out of 10 eggs in per replicate. Survival rate of larvae was the obtained proportions through dividingthe larvae numbers remaining at termination of the experiment by the larvae numbers initially newly

hatched in per replicate.

Comment 7 : Specific Comments: (1) P3 L40 I believe it is premature to state that OA will havesevere consequences for marine organisms, for you reasons described in the second half of this sentence.

Response 7: Revised as below:

One alarming consequence is a rapid change in seawater chemistry and decrease of ocean pH, which could have great impacts on marine ecosystems, and pose a threat to marine life (Frommel et al., 2013a; Kerr, 2010). Elevated CO_2 concentrations can disturb the acid-base regulation, blood circulation, respiration, as well as the nervous system of marine organisms, leading to long-term effects such as reduced growth rates and reproduction (Frommel et al., 2013a). Other directs of ocean acidification have been found in the alteration of behavior (Dixson et al., 2010; Munday et al., 2009a), development (Frommel et al., 2012b), RNA/DNA ratio (Franke and Clemmesen, 2011), and otholiths (Checkley et al., 2009; Maneja et al., 2013; Munday et al., 2011b) of marine fish larvae.

Comment 8:P3 L50 This sentence is awkward, authors should restructure it to increase clarity**.**

Response 8: Reworded.

Comment 9: P3 L54 The authors state fish physiology will "certainly decline" during acid/base regulation induced by ocean acidification. This statement is too strong, the current literature demonstrates a variety of response, many of which are neutral or minimal.

Response 9: Revised in the MS as below:

However, the emerging picture remains intriguingly complex. While the majority of responses to high CO_2 appear to be negative (Branch et al., 2013) with highest sensitivities observed during the early life stages and in calcifying invertebrates such as corals, bivalves, pteropods, and echinoderms, there arealso substantial evidences for non-linear, neutral, or even positive reaction to increasing CO_2 conditions (Hurst et al., 2013; Munday et al., 2011b; Murray et al., 2014).

Comment 10: P8 L194 The sentence that starts as "A number of studies found: " needs additionaland more appropriate citations.

Response 10: Revised as recommended.

Comment 11: P9 L202 This sentence is in contradiction tothe results presented in Figure 3, which shows deformity rate increased, rather than decreased, under pH 7.2.

Response 11: Sorry for the confusion and we have clarified this sentence in revised MS (see below).

An unexpected result of our study was that elevated levels of CO_2 affected larval development abnormalities, and the average deformity rate of marine medaka larvae (approximately one-week post-hatch) increased significantly by 16 % as CO_2 increased from control level to high-CO_2 level (pH 7.2).

Comment 12: P10 L255 A more detailed discussion on why elevated CO_2 decreased otolith area rather than increase (as seen in many other studies) isneeded. The appearance of this effect at pH 7.6 and not 7.2 also requires further explanation.

Response 12: We have made corresponding revisions in manuscript as required.

Reviewer #2:

Comment 1: For the abstract, larval deformity was also mentioned. Thus, it is not only the calcification of otoliths, which were affected.

Response 1: Clarified in revised MS.

Comment 2:P3, Line 3- "Coccolith" should be "coccolithophores"

Response 2: Revised.

Comment 3:P3, line 17- The otolith is not the only calcified organ of fish. Bones are also calcified.

Response 3: Revised.

Comment 4: P3, line 18-It is not calcite. Rather, it is aragonite.

Response 4: Revised.

Comment 5: P3, line 19-There are more recent studies showing increase otolith calcification but has no effect on fish larval swimming behavior of Atlantic cod and herring. Might consider adding new reports.

Response 5: Added.

Comment6: P3, line 19-Include introduction on how or why embryogenesis and organogenesis will be affected by OA.

Response 6: Added corresponding introduction in MS as suggested (see below).

Decades of empirical data suggest that juvenile and adult fish possess sufficient acid-base and osmoregulatory capabilities for the toleration of very high metabolic and ambient CO_2 levels (> 2000 μatm) (Murray et al., 2014). Although fish possesses the ability of acid-base balance regulation, its physiological function will certainly decline under such regulation for a long time from the perspective of energetics, especially in the most fragile and sensitive early life stage during its life history. In addition, in early life stages of multiple taxa including fish, elevated CO_2 was shown to affect calcification of shells and skeletons due to a drop in the carbonate availability (Riebesell, et al., 2010).

Comment 7: P4, line 26-Provide references for the CO_2 scenarios used.

Response 7: Added.

Comment 8: P5, line 10-30 fish eggs per treatment or replicate?

Response 8: 30 fishes per replicate, and 3 replicates per treatment (a total of 90 fish).

Comment 9: P6, line 7-Were the eggs returned back to the experimental tanks after each observation? Were there any side effects on the eggs because of the method? If eggs were not returned, how many were left until the observation of hatching? and P4, line 14 - How did you calculate the larval deformity rate? Should it be called "proportion of larvae with deformities"?

Response 9: The eggs were returned back to the experimental tanks after each observation. The sampling was gentle and the observation was accomplished within a couple of minutes for each replicate, in which process the side effect on the eggswas negligible in such short time compared to their exposure duration.

As for the larval deformity rate, on day 21, thirty larvae (10 larvae per replicate) from each CO_2 treatment were randomly selected and photographed for deformity analyses. The deformity rate were calculated based on the proportions of abnormal larvae numbers out of 10 eggs in per replicate. Larval deformity rate is a common phrase which is also generally used in other literature.

Comment 10: P6, line 18- How was the correct identification of the position of the otoliths ensured during extraction? Were adhering tissue materials removed from the otoliths?

Response 10: Body transparent is one of the advantages of marine medaka recommended as a model fish, and we can see the position of the otoliths under microscope (see the figure below). Before the measurement, adhering tissue materials has been removed from otolith to ensure the accuracy of the measurement.

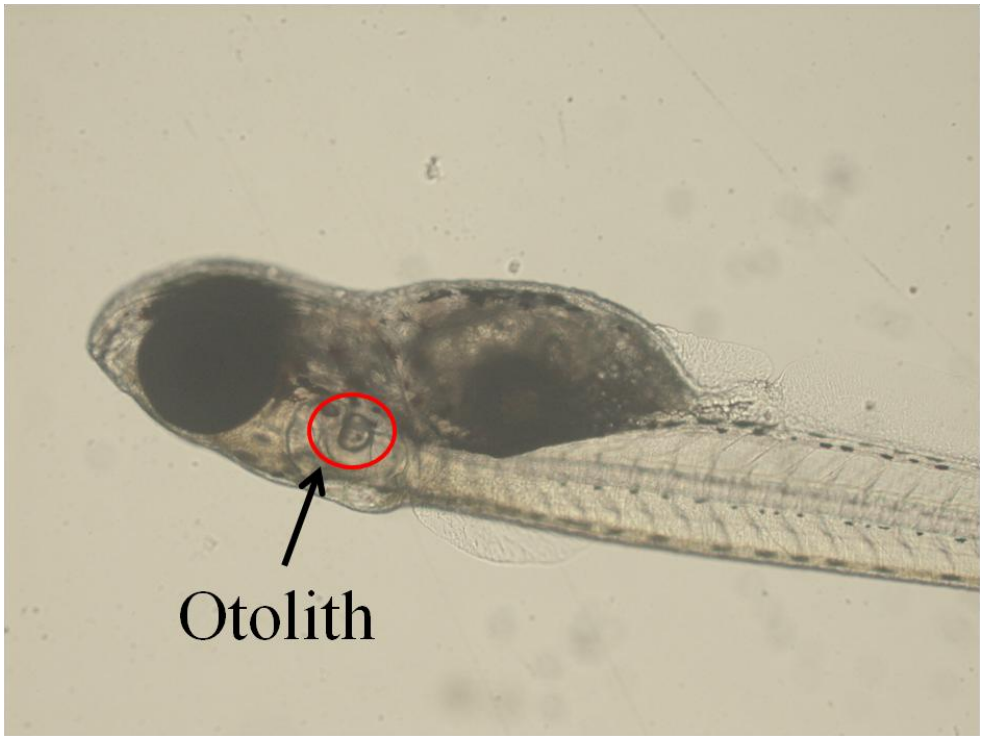

Fig. 1 The position of otolith in marine medaka larvae

Comment 11: P6, line 18- year of Franke and Clemmesen?

Response 11:Revised.

Comment 12: P7, line 5- How was the rate calculated for deformities?

Response 12:Please see the Response 9.

Comment 13: P7, line 5- So far, no hypothesis or principle on why comparison between the left and right side otoliths was made.

Response 13: Any substantial change inthe size, shape, or symmetry of otoliths could have serious implications for individual performance and survival (Gagliano et al., 2008, Dispersal without errors: symmetrical ears tune into the right frequency for survival. Proc. R. Soc. B., 275: 527-534.). **Munday et al. (2011) found that mean area of otoliths in the pH 7.6 treatment was larger than that of control for left otoliths, but not right otoliths. Therefore, it is very necessary to compare the difference between the left and right otoliths. (**Munday et al., 20011, Effect of ocean acidification on otolith development in larvae of a tropical marine fish, Biogeosciences 8, 1631-1641.)

Comment 14: P7, line 9- Change "proceed" with "used".

Response 14:Changed.

Comment 15: P7, Results - How was the size (standard length) of the fish larvae affected by OA? Otolith size is influenced by fish size and growth rate. Thus, it must be taken into account when analyzing the effects of OA on otolith growth.

Response 15: Thanks for your suggest. In order to ensure the synchronization of the experimental eggs, all eggs were collected within 3–5 h after spawning and screened with a stereoscope to ensure normal fertilization and development of each egg. As the reviewer mentioned, the size of fish larvae is an important parameter for assessing the effects of OA to otoliths. It is a pity that the size of fish larvae was not measured in this study. However, we would like to adopt the reviewer's suggestions and take the size of fish larvae into accountin our future researches.

Comment 16: P7, line 9- 'Seawater chemical parameters' Transfer to Methods section.

Response 16: For the study of OA effects on fishes, we considered that the seawater chemical parameters were an important part of results to ensure the stability of pH control system, therefore we prefer to retain 'Seawater chemical parameters' in Results section.

Comment 17: P7, line 23-add F statistics and sample sizes. Is the p-value for all the three parameters?

Response 17:Added.

Comment 18: P8, line 21- "decrease" should be "increase".

Response 18:Revised.

Comment 19: P9, line 24- Is this from Baumann et al., 2012 also? Is it negatively or positively related? In the next sentence, it is stated that survival and length significantly decreased at 1000uatm, which means negatively correlated.

Response19:Yes it is from Baumann et al., 2012. We clarified the sentence as below:

Baumann et al. (2012) reported a 74% reduction in survival of embryos and young larvae of inland silverside, *Menidiaberyllina*, native to estuaries of the US Atlantic coast, when maintained at 1100 ppm pCO_2 compared to those held at 410 ppm pCO_2.

Comment 20: P10, line 3- There was no mention of the procedure that analysis of deformities was done in the embryos. Thus, on the basis of deformities observed in newly-hatched larvae alone, one cannot make comparison of the vulnerability between embryo and larvae. Also, heart rate was not

monitored in the larvae.

Response 20:We added the procedure regarding the analysis of deformity in revised MS.

Comment 21: P10, line 3-What is the role of Cadmium ions in this context?

Response 21: Sorry, "Cadmium" should be "calcium".

Comment 22: P10, line 9- These are not parts of the otolith. These are three types or pairs of otoliths.

Response 22: Revised.

Comment 23: P10, line 9-Its formation starts during...

Response 23: Revised.

Comment 24: P10, line 10-important? Maybe you meant alteration of otolith size or shape has implications on physical performance... Not "important for..."

Response 24: Revised and further described the procedure of the analysis of deformity. Revisions in MS is as below:

Therefore, any substantial change to the size, shape, or symmetry of otoliths could have serious implications for individual performance and survival (Munday et al., 2008, 2011).

Comment 25: P10, line 10-should be "calcium carbonate structure"

Response 25: Revised.

Comment 26: P10, line 14- There is a natural fluctuation of sizes between the left and right otoliths, without preference to any side. This applies on the individual level. If you compare the two sides by combining all the data from each side from all the individuals, the inherent natural fluctuation between the sides is masked. A comparison of the magnitude of absolute differences between the left and right sides (Fluctuating Asymmetry) can be made among the treatments.

Response 26:The difference between the right and left side was compared only under the same CO_2 treatment, but did not among three CO_2 treatments. There was no systematic pattern of deviation from the normal fluctuating asymmetry, and these findings support the earlier report by Munday et al. (2011) on the maintenance of otolith symmetry.

Comment 27: P10, line 15-Kindly double check the analysis because the standard deviations in the Figure 5B overlaps among the treatments. Also, check the influence of fish sizes on the sizes of the otoliths.

Response 27: In pH 7.6 treatment, the average area of left and right sides was significantly smaller than otoliths from control treatment ($F_{1, 128} = 8.8$, $p = 0.013$) (Fig. 6B).

Comment 28: P10, line 18- Perhaps, the deformities observed have more significant implications on the survival of the larvae compared to the otolith calcification. Please add references on hypercalcified larval fish otoliths with corresponding no negative impacts on the swimming behavior. There are other studies showing that even absence of 1 or more otoliths, behavior of fish larvae was not affected (e.g. zebrafish).

Response 28: The relevant reference was added as recommended in revised MS.

Comment 29: P10, line 20- This discussion on acid-base regulation ability of the fish larvae is not clearly supported by Figure 5B of the paper.

Response29:We have made corresponding revisions in manuscript as recommended.

Comment 30: P11, line11- There seems to be clear impact of elevated pCO_2 on the formation of deformities on the larvae. However, this was not well discussed in the discussion. Instead, there was a bias towards expounding the issue of otolith calcification. Less emphasis was also placed on the non-significant effects on hatch rate, hatching time and heart rates. These data could point to the relative resilience of the species towards elevated pCO_2 scenarios. And thus, could be further discussed in the paper.

Response 30:Revised as below:

In conclusion, this study demonstrates that, even under projected near-future pCO_2 levels, the early life stages of marine medaka exhibited a dramatic increase of their larval development deformity and otolith calcification while their survival was not affected. Importantly, the observed CO_2-induced abnormal development of larvae might have predictably negative consequences on the recruitment of fish population, the effects of whichon later life history and the phenotype of subsequent generations of ocean acidification on marine fish should be concerned.

Comment 31: P19 The types of deformities can be further described in the paper. If possible, provide higher magnification picture to show the deformities.

Response 31: Further descriptions of deformities were added and higher magnification pictureshowing the deformities were also provided in MS.

Comment 32: P20- Asterisk is not very informative. Is pH 7.6 significantly different from pH 7.2 as well?explanation for panels A and B

Response 32: Explained. Asterisk indicates that the values of pH 7.6 differed significantly from the control (pH 8.2).