Effects of CO_2-driven ocean acidification (OA) on early life stages of Marine Medaka (*Oryziasmelastigma*)

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Abstract

The potential effects of high CO_2 and associated ocean acidification (OA) in marine fishes and other non-calcified organisms are less well understood. In this study, we investigated the responses of early life stages (ELS) of marine medaka (*Oryzias melastigma*) exposed to a series of experimental manipulation of CO_2 levels. Results showed that CO_2-driven seawater acidification (pH 7.6 and pH 7.2) had no detectable effect on hatching time, hatching rate, or heart rate of embryos. However, the deformity rate of larvae in the pH 7.2 treatment was significantly higher than that in the control treatment. There is no significant difference between the left and right otolith areas in each treatment. However, the average otolith area of larvae in the pH 7.6 treatment was significantly smaller than that in the control. Such alterations in the developmental abnormalities and otolith size of marine medaka larvae due to elevated-CO_2 levels suggests that this species will be increasingly challenged by future OA. Further studies of the impacts of OA on marine fish to assess whether or not the environmental influence in one generation can affect the later life history and the phenotype of subsequent generations are needed.

Keywords: climate change, non-calcified organisms, marine fish, early life stage, otolith, calcification

1. Introduction

In the 20th century, the atmospheric carbon dioxide (CO_2) concentration continued to increase as a result of fossil fuel combustion and other human activities. It was in turn taken up by the ocean gradually through air-sea exchange. Oceanic CO_2 can hydrolyze to increase the concentration of hydrogen ions (H^+), which leads to the reduction of pH in the ocean by 0.1 units (Orr et al., 2005). Based on the amount of global CO_2 emission at present, the pH of ocean is likely to drop by 0.3 ~ 0.4 units by the end of the 21st century and by 0.7 units after 300 years. The current and predicted levels of CO_2 and acidity of seawater of nearshore, estuarine, and higher-latitude habitats are expected to be even greater and substantially more variable than those of the open ocean (Gruber et al., 2012; Zhai et al., 2014). One alarming consequence is the 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, and the nervous system of marine organisms, leading to long-term effects such as reduced growth rates and reproduction (Frommel et al., 2013a). Other direct response to ocean acidification (OA) 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 otoliths (Checkley et al., 2009; Maneja et al., 2013; Munday et al., 2011b) of marine fish larvae. 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 (ELS) and in the calcifying invertebrates such as corals, bivalves, pteropods, and echinoderms, there are 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). Moreover, marine fish exemplifies this complexity. 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 its most fragile and sensitive early life stage during its life history. In addition, in ELS 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). Munday et al. (2011a) observed no effect on spiny damselfish otolith calcification at 850 μatm, while Munday et al. (2011b) and Checkley et al. (2009) highlighted an otolith hypercalcification in white seabass (*Atractoscion nobilis*) larvae exposed at 993 μatm and 2558 μatm pCO_2 and in clownfish (*Pomacentridae*) larvae at 1721 μatm pCO_2, respectively. In case of calcification modulation, otolith morphology can be affected, which may have negative repercussions on the behavior and acoustic function of fish and decrease their survival probabilities (Bignami et al., 2013; Réveillac et al., 2015).

Marine medaka, *Oryzias melastigma* or *Oryzias javanicus*, is one of the 14 species belonging to the genus *Oryzias*, which distribute in estuarine waters from East to Southeast Asia (Koyama et al., 2008). It has been proposed as a model species in marine environmental risk assessments (Mu et al., 2014). However, few studies have addressed OA effects on the ELS of marine medaka so far. The objective of this study was to examine how CO_2-driven OA affected the embryos and newly hatched larvae of marine medaka after 21 d exposure through investigating the embryonic, larval, and otolith development.

2. Materials and methods

2.1 Fish rearing

Marine medaka, *O.melastigma*, were provided by the Key Laboratory of Coastal Ecological Environment of State Oceanic Administration. Fish were maintained in aquatic habitats system (Aquatic Habitats, USA) with a salinity of 30 ± 2, temperature of 26 ± 1 ℃, and a photoperiod of 14 h:10 h (light:dark). All fishes were fed with nauplii of *Artemia* three times a day and synthetic food (New life spectrum thera-A formula, Made in the Newlife International, Inc, USA.) twice a day. One-tenth of the

total amount of water in the system was automatically renewed daily. To ensure developmental synchronization of embryos during experiment, all eggs were collected within 3 ~ 5 h after initiation of spawning, and fertilized and viable ones were selected under dissecting microscope.

The experimental seawater (salinity of 30.7 ± 0.1) was prepared by diluting sea salts (Instant Ocean, Aquarium Systems, USA) with deionized water. The standard NBS pH was 8.2 ± 0.004.

2.2 Seawater manipulation and experimental design

The design of seawater pH control system was based on Riebesell et al.(Riebesell, et al., 2010) with some modifications. Briefly, partial pressure of CO_2 (pCO_2) was adjusted by pH modulator (aquastar pH Modul Ⅱ, IKS) with standard deviation of ±0.01. Three pH gradients, 8.2, 7.6 and 7.2, were set according to the predicted levels upon CO_2 emission at present, after 100 and 300 years (Orr et al., 2005), respectively. The pH control system consists of three parts, namely monitor, controller and aeration (Fig. 1). The pH meter in water monitored the real-time pH changes during experiment. The controller associated with pH meter was also connected with electromagnetic valve, which opened or closed the electromagnetic valve based on the feedback of pH meter. The intake of electromagnetic valve connected to a cylinder equipped with a high concentration of CO_2 (0.1 % CO_2: 99.9 % air，$pCO_2$of 1000 ppm), and its outtake connected to silicone tube, drying tube, check valve and refiner which insert into seawater. The refiner was placed in the middle of the aquarium (10 L) bottom to make the gas bubbled into water quickly and homogeneously. When the pH in seawater was higher than the set value, the electromagnetic valve opened automatically to pipe concentrated CO_2 into the water until the pH dropped to the set value, and then the valve closed. During the exposure experiment, parameters including pH, inorganic carbon (DIC), temperature, salinity, total alkalinity (TA) and dissolved oxygen (DO) were continuously monitored and analyzed to ensure the stability of pH control system.

For each pH treatment, 90 fertilized eggs were randomly assigned to three tanks (three replicates) with 30 eggs per replicate. These tanks were monitored daily for

dead embryos, hatched larvae, and hatching time. Subsets of hatched larvae per tank were then transferred to the alternative aquariums with the same exposure conditions to start the larval exposure sub-experiment (3 CO_2 levels × 3 replicates). Larvae were monitored daily and dead ones were removed until the termination of experiment at 21 days (approximately one-week post-hatch). By the end of experiments, the survival larvae were anesthetized and photographed under microscope (Leica DMI4000B) for deformity analyses, and the otoliths were then removed and dry-stored in well-plates.

[**Figure.1**]

2.3 Determination of Water Quality Parameters

The determination of pH, TA and DIC referred to the methods of Dickson et al. (2007). In brief, samples were collected into vials without obvious bubbles by an overflow manner, and then fixed with 0.1 % saturated $HgCl_2$ solution. The pH was detected using combined electrode (Orion 8102 BN Ross) and high-precision pH meter (Thermo Orion 3-Star, USA) in 25℃water bath within 2 h after sampling. The deviation was less than 0.01. TA and DIC were detected by TA analyzer (Apollo AS-ALK2, USA) and DIC analyzer (Apollo AS-C3, USA) with an accuracy of more than ± 2 μmol/kg, respectively. Salinity, temperature and DO of seawater were detected by YSI-85 water quality monitor (YSI Inc, USA), and the accuracy of each parameter was more than ± 0.1, ± 0.1 ℃ and ± 2% air saturation, respectively. Aragonite saturation (Ω_{Ar})was calculated based on temperature, salinity and measured TA and DIC through CO_2-SYS carbonate system software (Pelletier et al., 2011). Other parameters adoption including dissociation constants of carbonic acid and sulfuric acid, saturated solubility product of $CaCO_3$ were consistent with those internationally applied (Millero et al., 2006).

2.4 Developmental toxicity

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 and hatching numbers were recorded. Heart rates were estimated 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 the "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 was 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 dividing the larvae numbers remaining at termination of the experiment by the larvae numbers initially newly hatched in per replicate.

2.5 Otolith measurement

The measurement of marine medaka otolith was based on the method of Franke and Clemmesen (2011). Briefly, the left and right otoliths were removed from 16 fish larvae randomly selected from each CO_2 treatment. Each otolith was observed and photographed under microscope (Leica DMI4000B). Digital pictures of otolith were taken at 1000 × magnification using the microscope equipped with Leica DFC420C Digital Camera. Otolith area (μm^2) was calculated through Image-Pro Plus 5.0 software after calibration and gray-scale processing of photos.

2.6 Statistical Analyses

Data analyses were performed using SPSS ver.16.0 (Chicago, IL) software. All data were tested for normal distribution using the Kolmogorov-Smirnov test. Non-normally distributed data were log-transformed. The difference between measured and nominal pH was analyzed by T-test. For heart rate, hatching rate, hatching time, and deformity rate, one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests were applied to test the differences between and among groups. An independent sample test was used to compare the difference of otolith

areas between left and right sides in each treatment. If there was a significant difference, one-way ANOVA was used to further compare the difference between treatments for left and right sides, respectively. If not, one-way ANOVA was performed after data combining of left and right sides. Results were expressed as means ± standard deviation (SD).

3. Results

3.1 Seawater chemical parameters

Measured pH in three treatments and different chemical parameters in seawater were shown in Figure 2 and Table 1, respectively. During the 21 d of exposure, measured pH in pH 8.2, 7.6 and 7.2 groups were 8.22 ± 0.004, 7.63 ± 0.007 and 7.22 ± 0.002, respectively. The fluctuation was less than 0.05 (Fig.2), indicating the stability of pH control system.

[**Figure 2**.]

[**Table 1**.]

3.2 Embryonic development

Three replicates produced a total of 90 eggs in each CO_2 treatment. The hatching times were extended with decreasing pH level, but there was no significant difference among the three pH treatments ($F_{2, 6}$ = 5.8, p= 0.066) (Fig. 3A). On average, 83 percent of eggs in three pH treatments survived to hatch, and the hatching rate of eggs was not significantly different among the three pH treatments ($F_{2, 6}$ = 1.1, p= 0.4) (Fig. 3B). For the heart rates of embryos, pH 7.6 and 7.2 groups were not significantly different from those in the control group ($F_{2,28}$= 1.7, p = 0.7) (Fig. 3C).

[**Figure 3.**]

3.3 Larval development

Three replicates produced a total of 66 ~75 newly hatched larvae in each CO_2 treatment level. By the end of experiment, larvae survival rate was highly variable but did not differ significantly between the control and acidified water groups ($F_{2, 6}$ = 0.3, p= 0.7) (Fig. 4B).

However, the two lower pH treatments (pH 7.6 and pH 7.2) can both cause spinal deformities, craniofacial deformities, stretched heart and pericardial edema of marine medaka larvae (Fig.5). Furthermore, in pH 7.2 treatment, the deformity rate was significantly higher than that of control group ($F_{1, 4} = 32$, $p = 0.005$) (Fig. 4A).

[**Figure 4.**]

[**Figure 5.**]

3.4 Otolith development of larvae

The effects of different pH treatments on otolith size of marine medaka larvae were shown in Figure 6. There was no statistically significant difference between the areas of left and right sides in each pH treatment (pH 8.2: $F_{1, 59} = 0.092$, $p = 0.76$; pH 7.6: $F_{1, 67} = 0.045$, $p = 0.83$; pH 7.2: $F_{1, 68} = 0.005$, $p = 0.95$, respectively) (Fig. 6A). In pH 7.6 treatment, the average areas of left and right sides were significantly smaller than those of the control treatment ($F_{1, 128} = 8.8$, $p = 0.013$) (Fig. 6B).

[**Figure 6.**]

4. Discussions

Assessment of species sensitivity or tolerance to CO_2-driven acidification in marine environment is critical to evaluate the impacts of OA on marine biodiversity and ecosystem function (Fabry et al., 2008;Melzner et al., 2009). A number of studies found that CO_2-driven acidification had obvious influences on ELS of many marine invertebrates, especially calcified organisms including coral (Doropoulos et al., 2012; Fabricius et al., 2011), coccolithophores (Berry et al., 2002), and mollusk (Kroeker et al., 2013; Thomsen et al., 2013; Waldbusser et al., 2011). OA was predicted to potentially affect individual behavior such as development, growth, survival and swimming particularly during the early life stage of marine organisms (Munday et al., 2008). 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 the embryonic duration of the 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 on 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. All of these studies varied in the number of parents used, the time lapse between egg fertilization and initiation of CO_2 treatment, and how and when 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 in previous studies may preclude a fair cross-study comparison (Chambers et al., 2014); however, the overall present of effect of elevated CO_2 environments on embryo survival is in contrast to the findings here. Habitats occupied of species, particularly in the their ELS, may play a role in their sensitivities.

It is counter to expectations and requires further attention that species in their ELS are found in estuarine (marine medaka) and inner shelf (summer flounder) habitats, both with relatively high ambient CO_2 levels, but exhibit different sensitivities to experimentally elevated-CO_2 levels.

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 (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 ELS. 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 *O. 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).

The pH drop driven by CO_2 can change concentrations of bicarbonate and non-bicarbonate ions during which elevated CO_2 affects saturation states of calcium ions carbonate polymorphs (Munday et al., 2008). Otoliths are bony structures of fish to sense orientation and acceleration and consist of aragonite-protein bilayers, which document fish age and growth (Checkley et al., 2009). Its formation starts during

embryonic development, and any alteration of otolith size or shape is important for physical performance and individual adaptability of fish. Therefore, any substantial change in the size, shape, or symmetry of otoliths could have serious implications for individual performance and survival (Munday et al., 2011a; Munday et al., 2008). In this study, we found no significant difference existing between the left and right sides of marine medaka larval otolith under the same pH level. However, otolith area of larval fish exposed to the intermediate-CO_2 level (pH 7.6) was smaller than that of control. Results suggested that there was no significant pCO_2 effect on otolith symmetry of marine medaka, defined as the difference between the right and left sides. However, the otolith area was significantly affected. The trend of reduction in otolith area of marine medaka larvae exposed to elevated CO_2 environments found here has not been reported in most previous studies focusing on other marine fishes. For instance, Checkley et al. (2009) found that otolith area of white seabass (*Atractoscion nobilis*) larvae increased by 7% ~ 9% and 10% ~ 14% after exposure to 993 ppm and 2558 ppm CO_2, respectively. Munday et al. (2011b) found that the size, shape, and symmetry of otoliths in larval clowfish was unaffected by exposure to simulated levels of OA (pH 7.8 and 1050 μatm CO_2); however, in a more extreme treatment (pH 7.6 and 1721 μatm CO_2) otolith area and maximum length were larger than those of control otoliths. Maneja et al.(2013) found that elevated CO_2 had no significant effect on the shape of the otoliths nor was there any trend in the fluctuating asymmetry, while increased otolith growth was observed in 7 to 46 d post hatch cod larvae in two pCO_2 treatments of 1800 μatm and 4200 μatm. In contrast, Munday et al. (2011a) did not detect any effect of elevated CO_2 on otolith size of juvenile spiny damselfish, *Acanthochromis polyacanthus*, which were reared for 3 weeks in treatments up to 841 μatm CO_2. Our results seemed to support the hypothesis that otoliths of larvae reared in seawater with elevated CO_2 would grow more slowly than they do in seawater with normal CO_2. The reduction of otolith area was likely associated with reduced $CaCO_3$ saturation which slowed down its formation. We do not know whether smaller otoliths have a deleterious effect, although we do know that asymmetry between otoliths can be harmful (Checkley et al., 2009).The difference between our results and other

studies may be related to: (1) different pCO_2 levels; (2) different life histories; or (3) different exposure duration (Munday et al., 2011a). However, another interesting result from the present study was that the otolith area of marine medaka larvae under the extreme CO_2 level (pH 7.2) tended to increase instead of continuously reducing. We should not ignore its own acid-base regulation ability that increased the available amount of carbonate by compensation mechanism for otolith to intensify the calcification process under such acidic condition (Checkley et al., 2009). Calcium incorporation into the otolith was modulated by the seawater pH. This question the stability of the elemental: Ca ratio under environmental hypercapnia. During the biomineralization of the otolith, chemical elements such as metals and metalloids are supposed to substitute for calcium (Réveillac et al., 2015). The changes of pH and seawater chemistry caused by increased CO_2 can modify the speciation of metals and their subsequent bioavailability to organisms (Millero et al., 2006). The physiological response of fish to hypercapnia might in turn stimulate processes to compensate for acidosis based on the key role of ion transporters. In present study, OA may interfere with trace element uptake and body concentrations and therefore could affect otolith growth and microchemical constituent. Further studies are thus needed to investigate the possibility that OA impacts on the trace metals properties, molecular-binding affinities and incorporation pathway into the otolith.

In conclusion, this study demonstrated that, under projected near-future pCO_2 levels, the ELS of marine medaka exhibited a dramatic increase of larval developmental 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 which on later life history and phenotype of subsequent generations should be concerned. As the otolith is an essential tool used in reconstructing fish life history in terms of age, somatic growth and attended habitats, further studies should investigate the process of otolith biomineralization. Finally, we emphasize that there is considerable variation among species in their sensitivities to elevated CO_2 and reduced pH. Determining the traits that render some species more susceptible than

others will be helpful and valuable to predict the long-term and ecological effects of OA.

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Table list

Table 1. Summary of chemical parameters in control and acidic seawater (*n* = 3)

pH_{NBS}*	DIC (μmol/kg)	pCO_2 (μatm)	CO_2 (μmol/kg)	HCO_3^- (μmol/kg)	CO_3^{2-} (μmol/kg)	Ω_{Ar}
8.22 ±0.004	2645.1 ±28.5	495.9 ±2.2	14.4 ±0.1	2380.3 ±10.1	280.4 ±3.9	4.5 ±0.06
7.63 ±0.007	3014.2±74.3	2372.6 ±52.3	68.7 ±1.4	2861.0 ±20.7	84.5 ±0.3	1.4 ±0.006
7.22 ±0.002	3202.7 ±18.5	6165.7 ±56.4	178.4 ±1.8	2988.8 ±9.3	35.5 ±0.6	0.6 ±0.01

* pH_{NBS}: The fundamental definition of pH in terms of thehydrogen ion activity; NBS: National Bureau of Standard.

Figures and captions

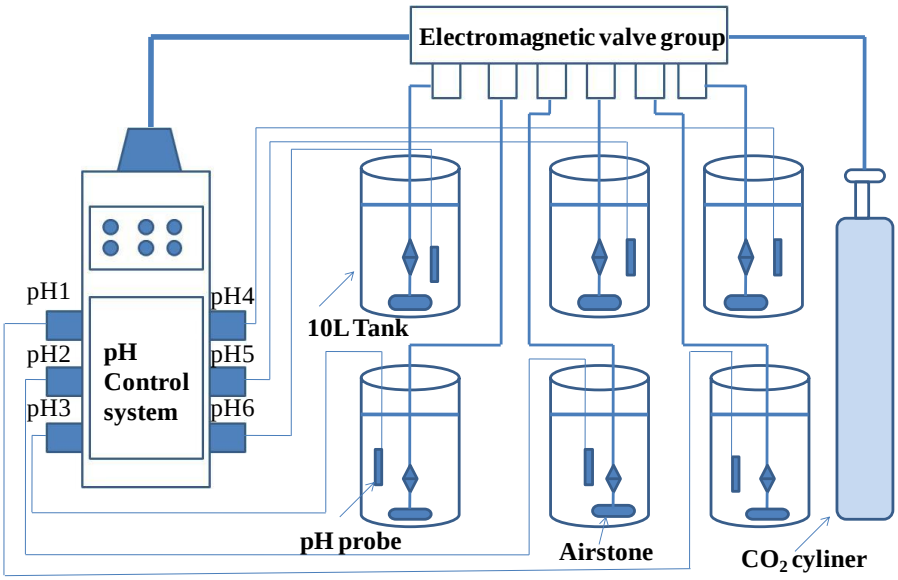

Figure 1. Schematic illustration of the pH control system applied in exposure experiment (For details refer to the text).

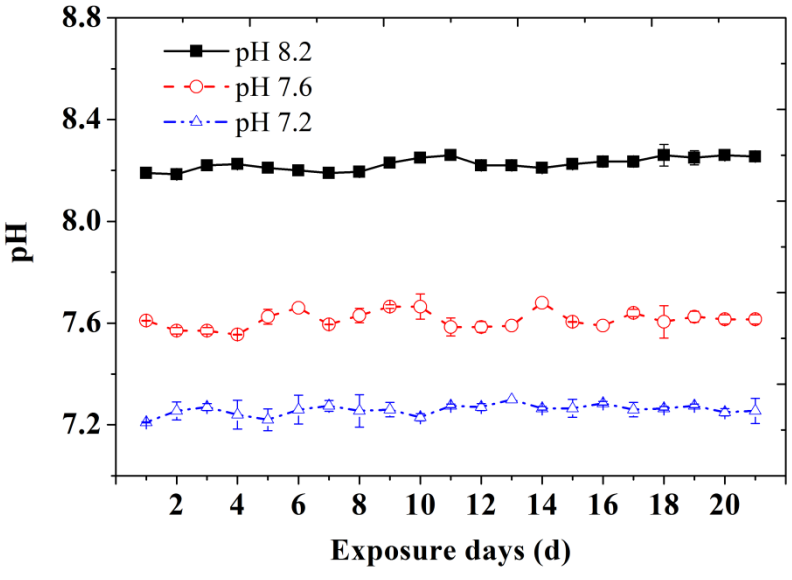

Figure 2. Measured mean pH_{NBS} of seawater in three pH treatments during 21 d of exposure (n = 3).

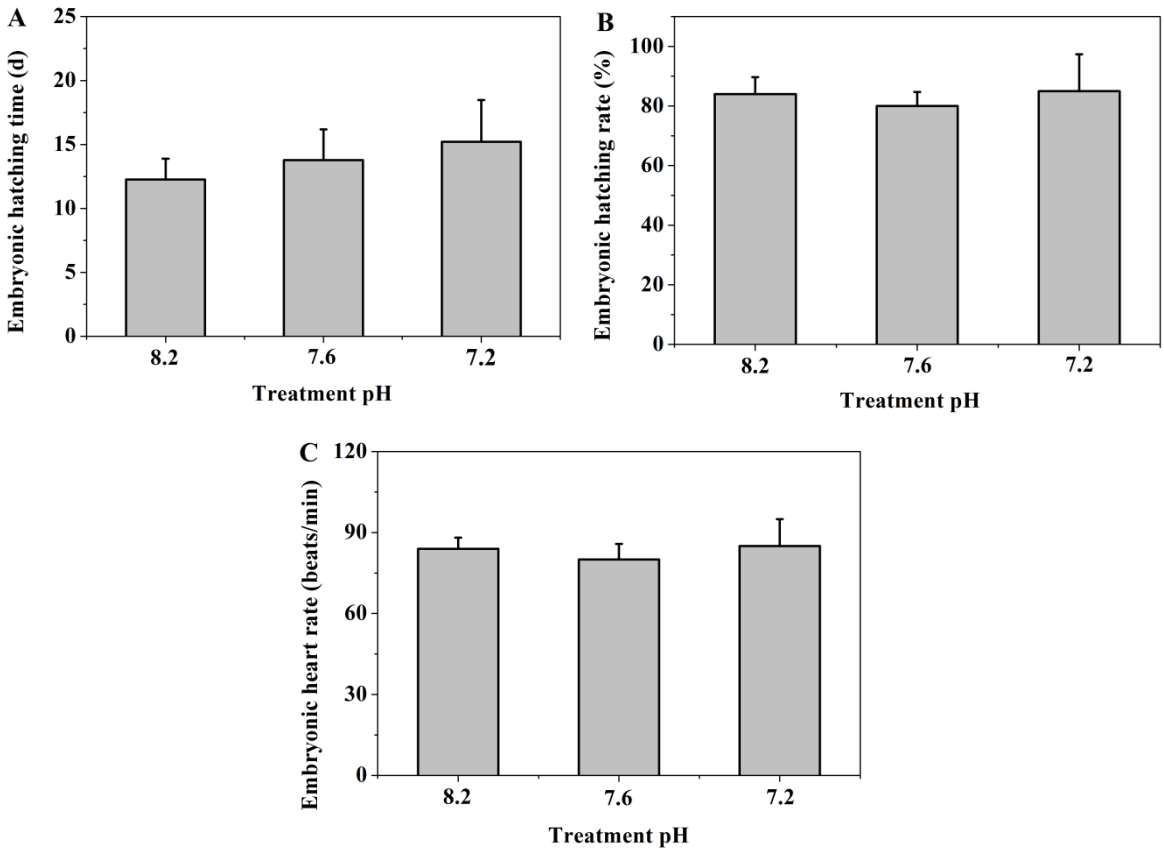

Figure 3. The hatching time, hatching rate, and heart rate of marine medaka embryos exposed to three pH levels. (A) Hatching time; (B) Hatching rate; (C) Heart rate.

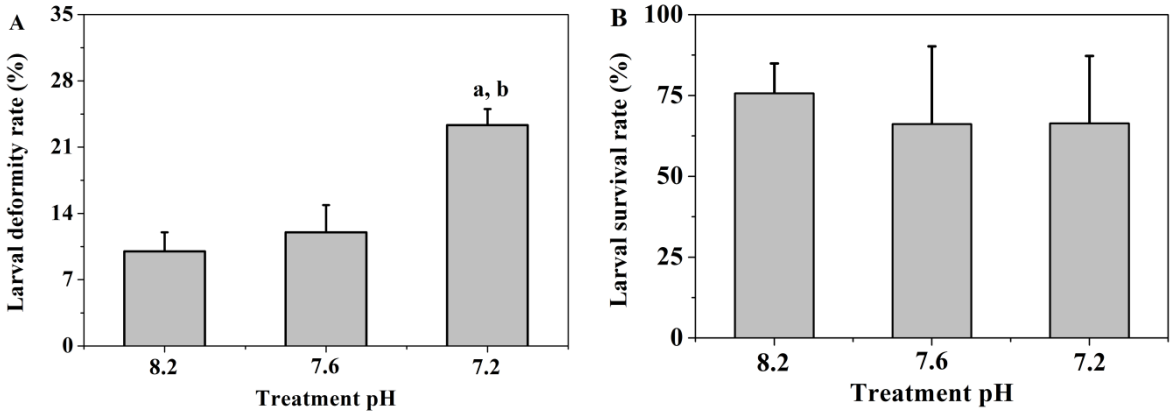

Figure 4. The deformity and survival rates of larvae exposed to three pH levels. (A)Deformity rate; (B) Survival rate. The a indicates that the value in pH 7.2 or in pH 7.6 differs significantly from that in the control (pH 8.2), and b indicates that the value in pH 7.2 differs significantly from that in pH 7.6.

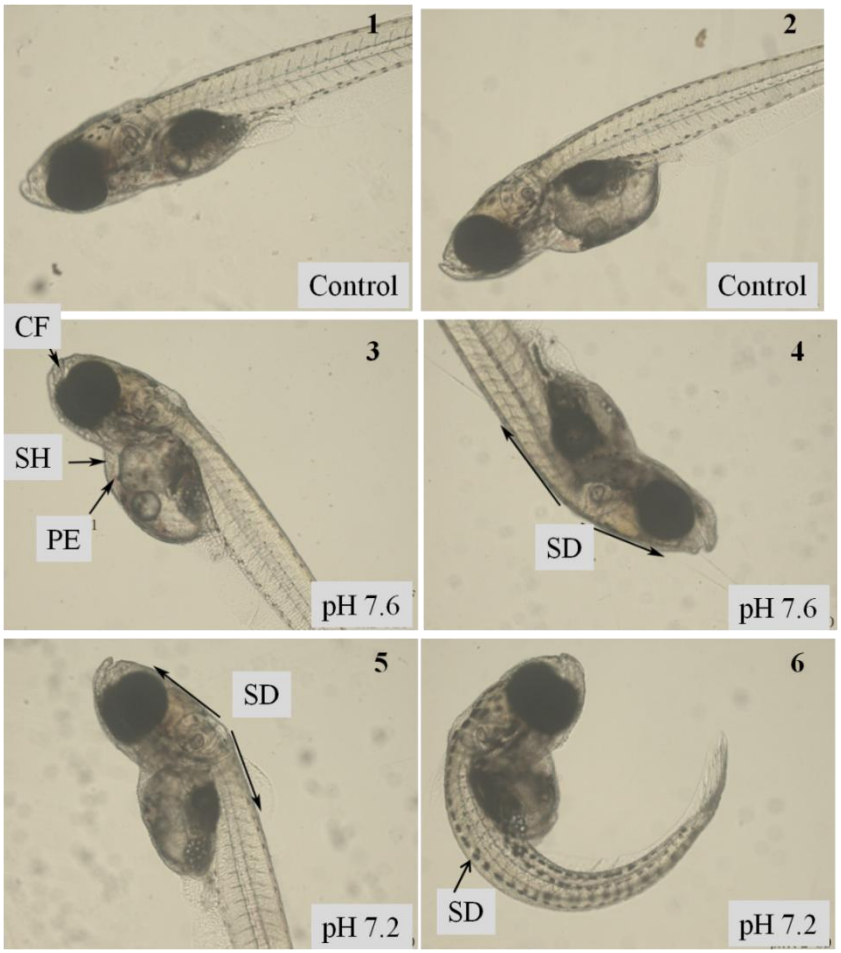

Figure 5. Morphological changes of medaka larvae exposed to three pH levels. 1~2: Control：Normal (pH 8.2); 3~4: pH 7.6 treatment; 5~6: pH 7.2 treatment. SD: Spinal deformities; CF: Craniofacial deformities; PE: Pericardial edema; SH: Stretched heart.

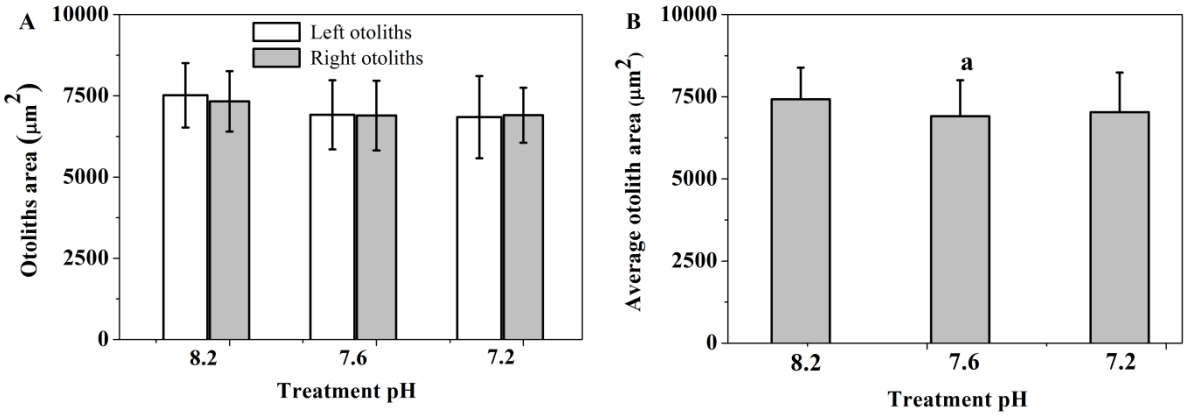

Figure 6. The effects of different pH levels on the otolith area of marine medaka larvae after 21 d of exposure. The a indicates that the value in pH 7.2 or in pH 7.6 differs significantly from that in the control (pH 8.2).