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# Effects of $CO_2$-driven ocean acidification on early life stages of marine medaka (*Oryzias melastigma*)

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## Abstract

The potential effects of elevated $CO_2$ level and reduced carbonate saturation state in marine environment on fishes and other non-calcified organisms are still poorly known. In present study, we investigated the effects of ocean acidification on embryogenesis and organogenesis of newly hatched larvae of marine medaka (*Oryzias melastigma*) after 21 d exposure of eggs to different artificially acidified seawater (pH 7.6 and 7.2, respectively), and compared with those in control group (pH 8.2). Results showed that $CO_2$-driven seawater acidification (pH 7.6 and 7.2) had no detectable effect on hatching time, hatching rate, and heart rate of embryos. However, the deformity rate of larvae in pH 7.2 treatment was significantly higher than that in control treatment. The left and right sagitta areas did not differ significantly from each other in each treatment. However, the mean sagitta area of larvae in pH 7.6 treatment was significantly smaller than that in the control ($p$ = 0.024). These results suggest that although marine medaka might be more tolerant of elevated $CO_2$ than some other fishes, the effect of elevated $CO_2$ level on the calcification of otolith is likely to be the most susceptibly physiological process of pH regulation in early life stage of marine medaka.

## 1 Introduction

In the last century, the concentration of carbon dioxide ($CO_2$) released into atmosphere continued to increase as a result of fossil fuel combustion and human activities, which was in turn taken up by the ocean 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 (Sabine et al., 2004). 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 century and by 0.7 units after 300 years. Obviously, elevated $CO_2$-driven Ocean Acidification (OA) is probably more and more apparent, and its potential ecological impacts should not be ignored (Caldeira and Wickett, 2005; Field et al., 2011).





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While it is apparent that changing seawater chemistry will have severe consequences for marine environment and organisms, the effects of ocean acidification on the alteration of marine habitat, population, species competition and food web are complex and still difficult to quantify. Calcified organisms (such as coral, coccolith and mollusk) whose skeleton or shell is mainly comprised of calcite/aragonite, are particularly sensitive to OA as the reduction of carbonate ions caused by OA will make calcification process more difficult and/or require ~~calcified~~ more energy to form $CaCO_3$ (Guinotte and Fabry, 2008; Hofmann et al., 2010; Orr et al., 2005). In addition, along with the increase of dissolved $CO_2$ level and the decrease of pH, OA may also affect non-calcified organisms, such as squid and fish as well. The excessive $CO_2$ content in the ocean can lead to a drop of pH in tissue fluid of marine organisms, making them faced with pressures of decreased pH from both environment and itself (extracellular fluid), which in turn affect their growth, reproduction and survival (Orr et al., 2005). 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. The calcified organ of fish, namely otolith, mainly consisting of calcium carbonate in the form of calcite, tends to be affected by OA followed by fish functional disorders of balance, orientation and swimming (Checkley et al., 2009). At present, the impacts of OA on early life stage and calcification of fish have raised concern and are likely to be a focus in future research. A few studies have reported different OA effects on fish depending on different species and developmental stage. However, most of existing OA studies mainly focus on calcified organisms, while studies regarding the potential impact of OA on development and calcification of marine fish are still limited.

In this study, marine medaka, *Oryzias melastigma*, was selected as target organism to investigate the effects of OA on hatching rate, hatching time, deformity and otolith development of marine medaka embryos and larvae under two simulated pH conditions.





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## 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 °C, 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 New life 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 30.7 ± 0.1) was prepared by diluting sea salts (Instant Ocean, Aquarium Systems, America) 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. (2010) with some modifications. Briefly, partial pressure of $CO_2$ ($pCO_2$) was adjusted by pH modulator (aquastar pH Modul II, IKS) with SD 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, respectively. The pH control system was consisted of three parts of monitor, controller and aeration (Fig. 1). The pH meter in water monitored 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 according to the feedback of pH meter. The intake of electromagnetic valve connected to a cylinder equipped with high concentration $CO_2$ (0.1 % $CO_2$: 99.9 % air, $pCO_2$ of





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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, electromagnetic valve opened automatically to pipe concentrated $CO_2$ into the water until the pH drop 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.

Three replicates were setup for each pH level with 30 fish eggs in each treatment. Temperature was 27.4 ± 0.12 °C, and the exposure period 21 d. The seawater was renewed once a day during experiment. When changing the water, the pH in alternative containers was adjusted and kept at desired level before the moving in of eggs to minimize the effects of fluctuating pH on eggs. The developmental stages were examined daily and the dead individuals were picked out.

### 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 °C 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 accuracy more than ±2 μmol $kg^{-1}$, 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 ppt, ±0.1 °C and ±2 % air saturation, respectively. Aragonite saturation ($\Omega_{Ar}$) was calculated based on temperature, salinity and measured TA and DIC through $CO_2$-SYS carbonate system software (Pelletier et al., 2011). Other parameter adoption including dissociation con-





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stants of carbonic acid and sulfuric acid, saturated solubility product of $CaCO_3$ was consistent with those internationally applied (Millero et al., 2006).

### 2.4 Developmental toxicity

The effects of OA on early life stage of marine medaka were assessed through the measurements of hatching rate, deformity rate, heart rate and hatching time of exposed embryos. During exposure, embryonic eggs were randomly selected daily from each treatment and observed under an inverted fluorescence microscope (Leica DMI4000B) for possible morphological and developmental abnormities. The developmental process of marine medaka was determined based on the diagnostic features of the developing embryos of *Oryzias latipes* (Iwamatsu, 2004). Estimates of heart rate were completed by counting the number of heart beats over a 30 s period (three replicates) at day 8. From day 11, newly hatched larvae were examined for abnormity under microscope and the hatching numbers in each day were recorded until the end of exposure. Embryonic hatching rate and hatching time, larval deformity rate were then calculated accordingly.

### 2.5 Otolith measurement

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





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## 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 groups and among multi-groups. A paired *T* test was also used to compare the difference between left and right sagittal areas in each treatment. If there was a significant difference, one-way ANOVA was processed to further compare the difference between groups for left and right sagittae, respectively. If not, one-way ANOVA was performed after data combining of left and right sagittae. Results were expressed as mean ± standard deviation (SD).

# 3 Results

## 3.1 Seawater chemical parameters

Measured pH in three treatments and different chemical parameters in seawater were shown in Fig. 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, with fluctuation less than 0.05 (Fig. 3), indicating the stability of pH control system.

## 3.2 Hatching rate, deformity rate, hatching time, and heart rate

The effects of different pH treatments on early life stage of marine medaka demonstrated that in pH 7.6 and 7.2 groups, the hatching rate, hatching time, and heart rate were not significantly different from those in the control group ($p > 0.05$) (Fig. 3a, c and d). However, these two pH treatments can both cause spinal deformities, craniofacial





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deformities, stretched heart and pericardial edema of marine medaka larvae (Fig. 4). In pH 7.2 treatment, the deformity rate was significantly higher than the control group ($p < 0.05$) (Fig. 3b).

### 3.3 Otolith development

The effects of different pH treatments on sagittal size of marine medaka larvae were shown in Fig. 5. The areas of left and right sagittae were not significantly different ($p > 0.05$) in three pH treatments (Fig. 5a). In pH 7.6 group, the average area of left and right sagittae was significantly smaller than the control group ($p = 0.025$), while this difference between pH 7.2 and control groups were not statistically significant (Fig. 5b).

## 4 Discussions

Assessment of species sensitivity or tolerance to $CO_2$-driven acidification in marine environment is critical to evaluate the impact 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 early life stages of many marine invertebrates, especially calcified organisms including coral, coccolith and mollusk. 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 this study, we found that in the following 100 years, there was no significant effect of $CO_2$-driven acidification (pH 7.6) on hatching rate, hatching time, heart rate, and deformity rate of marine medaka. Only under the extremely acidic condition (pH 7.2) deformity rate of exposed medaka significantly decreased compared to the control group. Similar results were also found in several recent studies. For example, Franke and Clemmesen (2011) showed that fertilization rate, hatching rate, length, dry weight, embryo deformity rate and mortality of Atlantic herring (*Clupea harengus* L.) after exposure to 1260, 1859, 2626, 2903, and 4635 µatm $pCO_2$ (corresponding to





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pH 7.67, 7.49, 7.33, 7.28, and 7.05, respectively) were not different from the control treatment or linearly related with $pCO_2$ level significantly. Early development study of spiny damselfish (*Acanthochromis polyacanthus*) and clownfish (*Amphiprion percula*) exposed to 500–1036 µatm $pCO_2$ (pH 8.0–7.8) demonstrated no significant impact on survival, growth or development of two fishes (Nilsson et al., 2009; Munday et al., 2009). Similarly, under 1000 µatm $pCO_2$ level, embryo survival, hatching rate and larvae morphology, development of Atlantic cod (*Gadus morhua*) did not change either (Frommel et al., 2010). However, during the early life stages of inland silverside (*Menidia beryllina*), Japanese whiting (*Sillago japonica*) and red seabream (*Pagrus major*), embryo survival, hatching rate and larvae growth rate significantly decreased with increasing $CO_2$ level (400–2200 µatm, pH 8.1–7.3) and exposure duration. In addition, after exposure to high-concentrated $CO_2$ level, morphological deformities were found in these fish embryos and larvae (Baumann et al., 2012; Kikkawa et al., 2004; Widdicombe and Spicer, 2008). These results indicate that there are differences of $CO_2$-driven OA tolerance and pH regulation ability among species. 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, while offshore coral reef fish, red seabream, has a strict requirement of environmental factors such as salinity and DO for growth and production. Therefore, red seabream has a bad adaptation to $CO_2$-driven pH fluctuation. Inland silverside is also common in estuary; however, the survival and length of larvae are positively related with $CO_2$ concentration, which is possibly associated with its life history. In addition, research on inland silverside found that survival and body length of larvae significantly decreased compared to the control group after exposure to 1000 µatm $CO_2$ for 7 days, while those of embryos were not affected, indicating more sensitivity of larvae to $CO_2$ than embryos. The reasons were attributed to the self-protection of fertilized embryos and their less dependence on external environment (Baumann et al., 2012). Interest-





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ingly, our results seemed to support the above conclusion as heart rate, hatching rate and hatching time of marine medaka embryos were not significantly affected while obvious deformities were observed in newly-hatched larvae, suggesting the latter was more liable to be influenced by OA.

The pH drop driven by $CO_2$ can change concentrations of bicarbonate and non-bicarbonate ions which in turn affects saturation state of cadmium ions (Munday et al., 2008). Fish otolith is composed of aragonite structure possessing protein framework, and includes three parts namely lapillus, sagittae and asteriscus. The function of otolith includes sound detection, body orientation and acceleration. Its formation occurred during embryonic development, and any alteration of otolith size or shape is important for physical performance and individual adaptability of fish. Therefore, otolith is considered to be the most sensitive calcium structure of fish (Munday et al., 2008). In this study, we found no significant difference existing between the left and right sagittae of marine medaka under the same pH level. In pH 7.6 treatments, the areas of left and right sagittae were significantly smaller than those of control treatment while this difference was not observed in pH 7.2 treatment. This suggested that the size of otolith, instead of their symmetry, was affected by OA, and calcification of otolith was the potentially most liable physiological process in early life stage of marine medaka. The reduction of sagittal area was likely associated with reduced saturation of $CaCO_3$ which slowed down its formation. However, sagittal area tended to increase under the lowest pH level instead of continuously reducing. This 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. Similarly, 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 and 2558 ppm $CO_2$, respectively. This was thought due to the increasing $HCO_3^-$ and $CO_3^{2-}$ in endolymph resulted from changed acid-base condition by increasing $CO_2$ concentration, which in turn affected the sedimentation of aragonite and lead to the enlargement of otolith. Reversely, in the study regarding spiny damselfish and clownfish, there was no significant impact of





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397–1721 µatm $CO_2$ on size or shape of otolith in two fish larvae (Munday et al., 2011), nor was 1260–4635 µatm $CO_2$ on the area of lapillus and sagittae of Atlantic herring (Franke and Clemmesen, 2011). The above results indicated that acid-base condition or decrease of aragonite saturation state may affect otolith calcification rate and size instead of shape or symmetry, and their impacts on otolith were different among species. The response mode and regulation ability to different OA stresses were also different in fish. However, it is certain that impacts of OA on organ morphology of these calcified organisms (including fish) will inevitably result in their functional disorder, recession or missing, and produce negative effects on survival, development, growth and reproduction of calcified organisms (Caldeira and Wickett, 2005; Checkley et al., 2009).

Although the early life stage of marine medaka has certain tolerance or adaption to $CO_2$-driven OA in the following 100 years, its response to hypoxia and temperature is very sensitive (Yu et al., 2006; Huang et al., 2012; Mu et al., 2012). This indicates that under the conditions of climate change and coastal eutrophication, the risk of temperature rise or DO decline to early development of fish is higher than the present acidification level. Therefore, more consideration or emphasis in future studies should be placed on combined effects of multi-factors in environment as study on single pH change is difficult to predict the impact of OA on non-calcified organisms (including fish) in marine environment.

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

| $pH_{NBS}$* | DIC ($\mu mol\,kg^{-1}$) | $pCO_2$ ($\mu atm$) | $CO_2$ ($\mu mol\,kg^{-1}$) | $HCO_3^-$ ($\mu mol\,kg^{-1}$) | $CO_3^{2-}$ ($\mu mol\,kg^{-1}$) | $\Omega_{Ar}$ |
|---|---|---|---|---|---|---|
| 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 the hydrogen ion activity; NBS: National Bureau of Standard.





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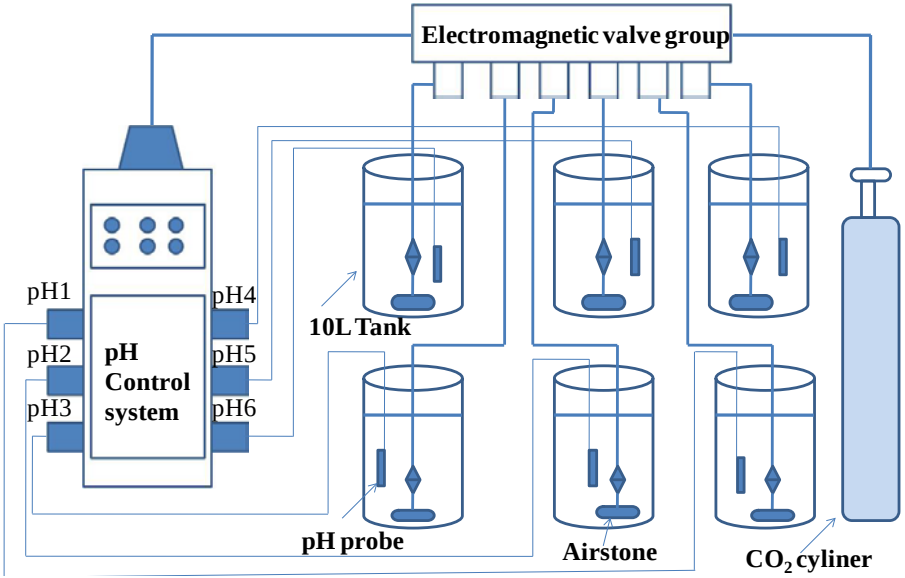


**Figure 1.** Schematic illustration of the pH control system applied in exposure experiment (for details see text).





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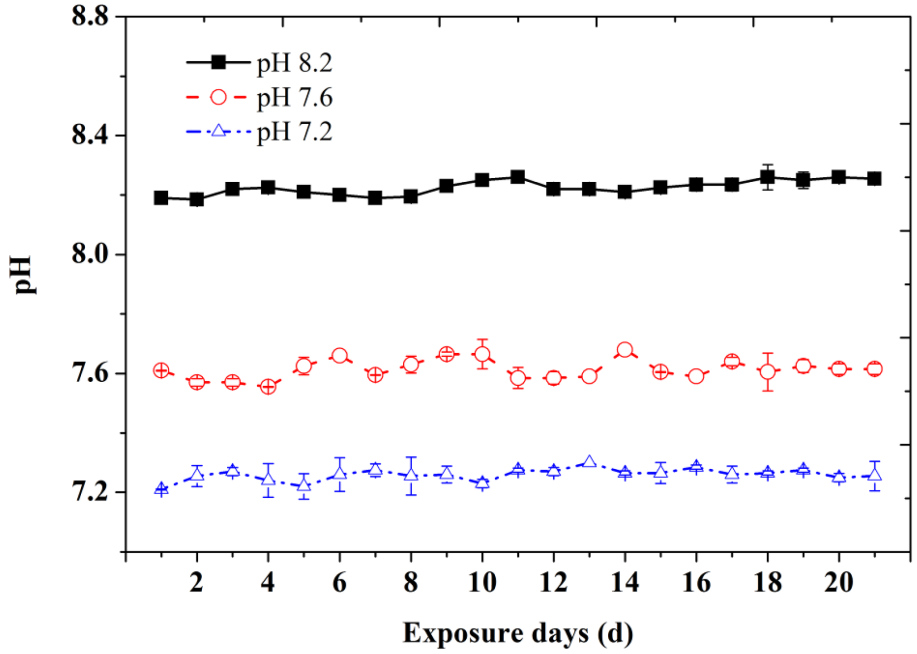


**Figure 2.** Measured mean $pH_{NBS}$ of seawater in different treatments over the 21 d of exposure ($n$ = 3).





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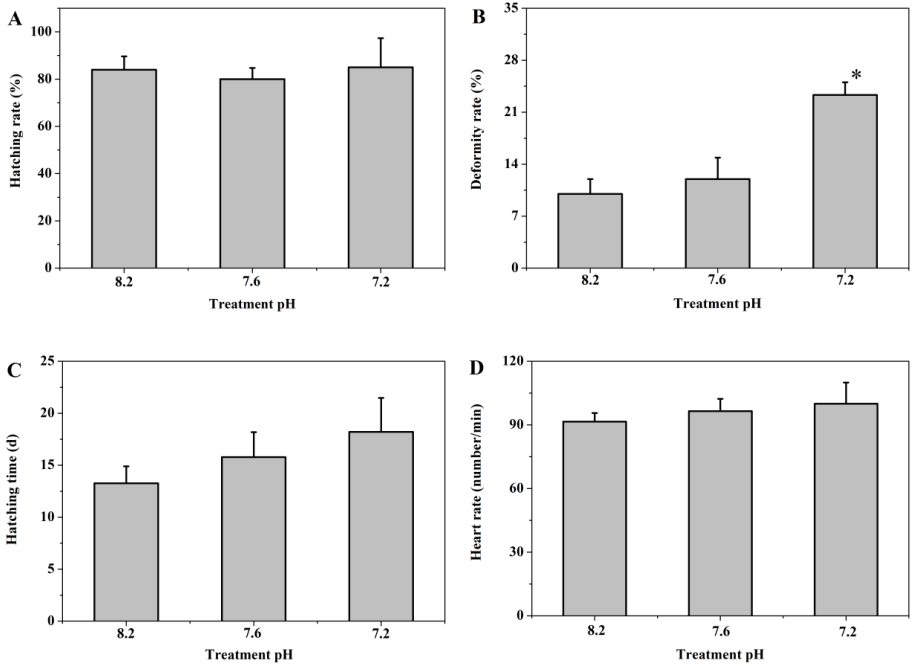


**Figure 3.** The effects of different pH levels on marine medaka embryos and larvae. **(a)** Embryonic hatching rate, $n$ = 30 eggs per replicate; **(b)** larval deformity rate, $n$ = 20–27 larval per replicate; **(c)** embryonic hatching time, $n$ = 20–27 eggs per replicate; **(d)** embryonic heart rate, $n$ = 15 eggs per replicate.





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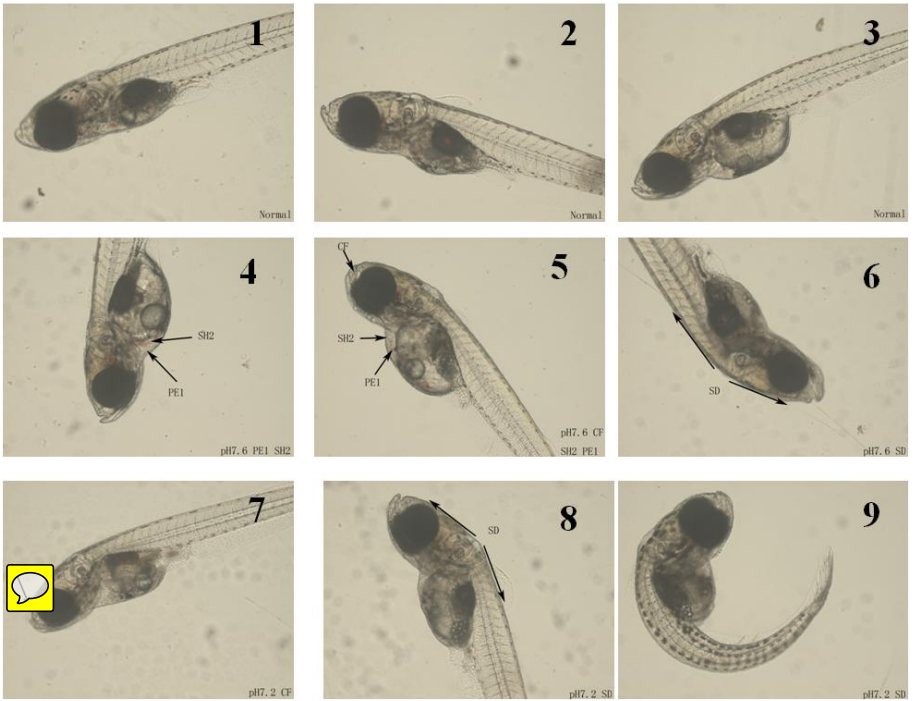


**Figure 4.** Morphological changes of medaka larvae under different pH levels. **(1–3)** control group (pH 8.2); **(4–6)** pH 7.6 treatment; **(7–9)** pH 7.2 treatment. SD: spinal deformities; CF: craniofacial deformities; PE: pericardial edema; SH: stretched heart.





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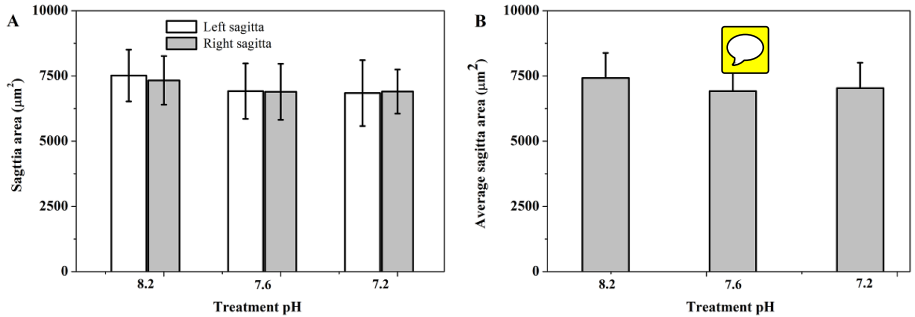


**Figure 5.** The effects of different pH levels on the sagittal area of marine medaka larvae after 21 d of exposure, $n$ = 16 larvae per replicate.





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