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# Effect of ocean acidification on otolith development in larvae of a tropical marine fish

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## Abstract

Calcification in many invertebrate species is predicted to decline due to ocean acidification. The potential effects of elevated  $p\text{CO}_2$  and reduced carbonate saturation state on other species, such as fish, are less well understood. Fish otoliths (earbones) are composed of aragonite, and thus, might be susceptible to either the reduced availability of carbonate ions in seawater at low pH, or to changes in extracellular concentrations of bicarbonate and carbonate ions caused by acid-base regulation in fish exposed to high  $p\text{CO}_2$ . We reared larvae of the clownfish *Amphiprion percula* from hatching to settlement at three  $\text{pH}_{\text{NBS}}$  and  $p\text{CO}_2$  levels (control: pH 8.15 and 404  $\mu\text{atm}$   $\text{CO}_2$ ; intermediate: pH 7.8 and 1050  $\mu\text{atm}$   $\text{CO}_2$ ; extreme: pH 7.6 and 1721  $\mu\text{atm}$   $\text{CO}_2$ ) to test the possible effects of ocean acidification on otolith development. There was no effect of the intermediate treatment (pH 7.8 and 1050  $\mu\text{atm}$   $\text{CO}_2$ ) on otolith size, shape, symmetry between left and right otoliths, or otolith elemental chemistry, compared with controls. However, in the more extreme treatment (pH 7.6 and 1721  $\mu\text{atm}$   $\text{CO}_2$ ) otolith area and maximum length were larger than controls, although no other traits were affected. Our results support the hypothesis that pH regulation in the otolith endolymph of fish exposed to elevated  $p\text{CO}_2$  can lead to increased precipitation of  $\text{CaCO}_3$  in otoliths of larval fish, as proposed by an earlier study, however, our results also show that sensitivity varies considerably among species. Importantly, our results suggest that otolith development in clownfishes is robust to even the more pessimistic changes in ocean chemistry predicted to occur by 2100.

## 1 Introduction

Absorption of additional carbon dioxide ( $\text{CO}_2$ ) from the atmosphere is causing ocean pH to decline and is reducing the availability of carbonate ions required by many marine species to form calcium carbonate ( $\text{CaCO}_3$ ) shells and skeletons (Feely et al., 2004; Orr et al., 2005). As the saturation states of aragonite and calcite decline, the

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the precipitation of  $\text{CaCO}_3$  in the otolith (Payan et al., 1997). Indeed, one recent study reported that otoliths were larger in larval fishes exposed to elevated  $\text{CO}_2$ , possibly because pH regulation caused carbonate concentrations to increase within the endolymph (Checkley et al., 2009). Maintenance of a steady pH within the endolymph, despite an influx of  $\text{CO}_2$ , would increase the abundance of bicarbonate and carbonate ions in the endolymph, both of which could potentially be used in aragonite precipitation. Finally, physiological stress can also affect otolith size, shape, and the symmetry between left and right otoliths (Gagliano and McCormick, 2004; Payan et al., 2004). Thus, even if acid-base regulation or declining aragonite saturation state do not directly affect otolith development, increased stress caused by elevated  $p\text{CO}_2$  could potentially influence otolith shape and symmetry.

Changes to ocean chemistry associated with declining pH, or changes in extracellular dissolved inorganic carbon (DIC) concentration due to acid-base regulation, could also affect otolith chemistry. The chemical composition of otoliths responds to environmental variation, including concentrations of elements in ambient water, temperature, and salinity (Campana and Thorrold, 2001). In many instances ambient water chemistry is the primary control on the incorporation of elements in otoliths, as evidenced by high correlations between the ratio of these elements to Ca at the site of otolith deposition and their concentration in the external environment (e.g. Bath et al., 2000; Martin and Thorrold, 2005). Bicarbonate ion concentration and pH has been shown to influence Sr, but not Mg, incorporation in calcitic foraminifera (Dissard et al., 2010). However, the effect of changes in aqueous carbonate chemistry on fish otolith composition remains unknown.

Fish ears detect sound, body orientation and acceleration from the position of the otoliths in the inner ear and movement of the otoliths over sensory hair cells (Helfman et al., 1997). Any substantial change to the size, shape, or symmetry of otoliths could have serious implications for individual performance and survival (Gagliano et al., 2008). To date, two studies have examined the potential effects of ocean acidification on otolith growth and development. Checkley et al. (2009) found that otolith size

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increased in larval seabass exposed to  $\sim$ 1000  $\mu\text{atm}$   $\text{CO}_2$  for eight days. In contrast, Munday et al. (2011) detected no effects of  $\sim$ 850  $\mu\text{atm}$   $\text{CO}_2$  on size, shape or symmetry of otoliths on juvenile spiny damselfish, a species without a larval phase. No studies have investigated the possible effects of ocean acidification on otolith chemistry. We reared larvae of a model species, the clownfish *Amphiprion percula*, through their entire larval phase at two ocean acidification scenarios to test if exposure to elevated  $p\text{CO}_2$  and reduced carbonate ion concentrations affects otolith size, shape, symmetry (between left and right otoliths) and otolith chemistry compared with current-day controls. The experimental conditions are consistent with more extreme scenarios for the years 2100 (pH 7.8 and 1050  $\mu\text{atm}$   $p\text{CO}_2$ ) and 2200–2300 (pH 7.6, 1721  $\mu\text{atm}$   $p\text{CO}_2$ ) based on a business-as-usual trajectory of  $\text{CO}_2$  emissions (Caldeira and Wickett, 2005; Meehl et al., 2007). This combination of treatments allowed us to test the susceptibility of otolith development in clownfish to changes in seawater chemistry and to investigate potential mechanisms involved.

## 2 Materials and methods

### 2.1 Larval rearing

Clownfish were reared in a 70 000 l recirculating seawater system at James Cook University's experimental marine aquarium facility. Adult breeding pairs were kept in separate 70 l aquaria supplied with a continuous flow of filtered seawater at 18 l  $\text{h}^{-1}$ . Breeding pairs laid eggs on the underside of a terracotta pot placed in their aquarium. Pots were checked each morning for the presence of eggs. On discovering a new clutch of egg, the parental aquarium was assigned to one of three  $\text{pH}_{\text{NBS}}$  levels (8.15 (control), 7.8, 7.6) and pH adjusted as described below. On the night of hatching (6–8 days) the pot was removed from the parental aquarium and transferred to a 100 l larval rearing aquarium set to the same pH as the parental aquarium. Larvae were reared in a semi-closed system, where each aerated aquarium had no water flow during the

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day and was then slowly flushed with filtered seawater each night. This daily cycle ensured that larvae could feed ad-libitum throughout daylight hours and that any unconsumed food was removed each night. Larvae were fed rotifers (*Brachionus* sp.) at 5 individuals ml<sup>-1</sup> each morning for the first three days. *Artemia nauplii* were added at 1 individual ml<sup>-1</sup> each morning from day three. The ratio of *Artemia nauplii* to rotifers was increased each day until larvae were only fed 5 *Artemia nauplii* m<sup>-1</sup> from 8 days post-hatching. A summer light cycle of 13 h light/11 h dark was simulated with fluorescent lights. Larvae were reared to the end of their larval phase (11-d post hatching), at which time behaviour consistent with competency to settle (attraction to the sides of the rearing aquarium) and the appearance of benthic colouration was observed.

## 2.2 Seawater manipulation

Seawater pH<sub>NBS</sub> in the parental aquaria and larval rearing tanks that had been allocated to the 7.8 or 7.6 treatments was adjusted by CO<sub>2</sub> injection. A separate pH-controller (Tunze Aquarientechnik, Germany) was attached to each aquarium to maintain pH at the desired level. The pH controller was connected to a laboratory-grade glass pH probe in the aquarium and to an electronic solenoid connected to a cylinder of CO<sub>2</sub>. The solenoid injected a slow stream of CO<sub>2</sub> into a diffuser (Red Sea Reactor 500) at the bottom of the aquarium whenever the pH of the aquarium seawater rose above the set point. A precision needle valve inserted before the solenoid was adjusted to ensure a slow, steady, delivery of CO<sub>2</sub> into the diffuser. Using this method it was possible to constantly maintain pH within  $\pm 0.05$  units of the desired level and there was no detectable gradient in seawater pH within the aquarium. A diffuser without CO<sub>2</sub> injection was placed in each aquarium and rearing tank assigned to control conditions. The pH<sub>NBS</sub> of each aquarium was independently checked each day using a WP80 pH meter (TPS, Australia) calibrated with fresh pH buffers (Merk, Germany). CO<sub>2</sub> was only injected into aquaria to adjust pH when eggs or larvae were present. All water returned to a 60 000 l sump where it was degassed by stirring and using a 1000 l algal bio-remediation tank. Water temperature was maintained at 30°C  $\pm$  0.6

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(SD) using electric heaters. Oxygen saturation was checked regularly with an Oxi 340i oxygen probe (WTW, Germany) and was always above 90%.

Total alkalinity ( $A_T$ ) of seawater at each pH level was measured weekly by titration. Average  $pCO_2$ , bicarbonate and carbonate ion concentrations were then estimated from pH and  $A_T$  in the program CO2SYS, using the constants of Millero et al. (2006). All seawater chemistry parameters are shown in Table 1. Average  $pCO_2$  for the 8.15 (control), 7.8 and 7.6 pH treatments was estimated to be 404, 1050 and 1721  $\mu\text{atm}$ , respectively.

### 2.3 Otolith morphometrics and Fourier Shape analysis

Settlement stage larvae (11-d post hatching) were sacrificed with a lethal dose of anaesthetic and stored in 75% ethanol. When required, fish were removed from the preservative, blotted dry, weighed (nearest mg) and photographed in a lateral position under a stereomicroscope. Standard length (SL) to nearest 0.01 mm was estimated for each fish from the digital photograph using image analysis (Optimas 6.5, Media Cybernetics). The sagittal otoliths were then removed and stored dry in well-plates. The left and right otolith of each individual was photographed to produce a calibrated, grey-scale image. Morphometric measurements (otolith area,  $\mu\text{m}^2$ ; maximum length,  $\mu\text{m}$ ; maximum breadth,  $\mu\text{m}$ ; rectangularity; circularity) and Fast Fourier descriptor were obtained from the images using Optimas. An automatic pixel gradient traced anti-clockwise around the silhouette of the otolith, starting from a common landmark (distal edge of the rostrum). Fourier analysis reproduces the outline of a shape by considering it to be an aggregate of simple wave forms, describable by a series of sine and cosine curves (Younker and Ehrlich 1977). Optimas provided a complex ( $a + b_i$ ) Cartesian fast Fourier transformation of the x-y coordinates of 128 equidistant points around the otolith outline. The complex Fourier descriptors were converted ( $\sqrt{a^2 + b^2}$ ) to an absolute value for each harmonic (Lestrel, 1997). The images of the right otoliths were flipped horizontally so that the 128 sampling points were measuring the same part of the otolith for left and right otoliths.

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To remove any confounding effect of otolith image position or size from the data, the Fourier descriptors were standardized for differences in otolith position by setting the zeroth harmonic ( $H_0$ ) to  $0 + 0i$ , and for differences in otolith size by dividing all successive harmonics by the first harmonic ( $H_1$ ). Greater than 97.0% of the variance in otolith shape was accounted for by the first 19 standardized harmonics ( $H_2$ – $H_{20}$ ) in all instances (>99.0% in most instances). Consequently, we restricted our analysis to these standardized harmonics.

Morphometric measurements were calculated for 16 randomly selected individuals from each treatment and Fast Fourier coefficients were calculated for 19 randomly selected individuals from each treatment (Table 2). Individuals selected in each treatment were representative of at least two different parental genotypes.

## 2.4 Otolith chemistry

Elemental chemistry of otoliths was quantified using laser ablation inductively coupled plasma mass spectrometry. One otolith chosen at random from each fish was mounted on microscope slides with a cyanoacrylic glue, ground to the midplane using 3  $\mu\text{m}$   $\text{Al}_2\text{O}_3$  lapping film, sonified in ultrapure water for 2 min and then dried under a laminar flow hood in a class 100 cleanroom. Cleaned otoliths were then remounted onto a petrographic slide (8 otoliths per slide) using double-sided tape and stored in plastic bags for transportation to the ICP-MS facility at the Woods Hole Oceanographic Institution. Sectioned otoliths were analyzed with a 193nm excimer laser coupled with a Thermo Finnigan *Element2* high resolution ICP-MS. We sampled a 80  $\mu\text{m}$  spot centered approximately 50  $\mu\text{m}$  outside of the nucleus using a laser repetition rate of 5 Hz at 70% power and a dwell time of 60 s. A He gas stream carried ablated material from the ablation chamber to the ICP-MS where it was mixed with an Ar sample gas and a wet aerosol (2%  $\text{HNO}_3$ ) supplied by a self-aspirating (20  $\mu\text{min}^{-1}$ ) PFA nebulizer in the concentric region of the quartz dual inlet spray chamber. Initial testing found that isotopes from six elements ( $^7\text{Li}$ ,  $^{25}\text{Mg}$ ,  $^{43}\text{Ca}$ ,  $^{55}\text{Mn}$ ,  $^{88}\text{Sr}$  and  $^{138}\text{Ba}$ ) had count rates significantly higher than blank values and were free of isobaric interferences. Instrument blanks

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## 2.5.2 Size difference within otolith pairs

To investigate directional asymmetry of the otoliths (i.e. is the right or left otolith usually larger), signed differences in otolith morphometrics were obtained by subtracting the value for the left otolith from that of the right otolith (R–L) for otolith area, maximum length, and maximum breadth. This was not conducted for rectangularity and circularity because it is the absolute values of these parameters that have meaning. The frequency of positive versus negative scores among the pH treatments was then compared with a chi-square test of independence for each measure of otolith size.

To determine if pH treatment affected the magnitude of otolith asymmetry with respect to otolith area, maximum length, and maximum breadth, we used ANOVA to compare unsigned differences between left and right otoliths for these traits. ANCOVA was not required as there was no relationship between SL and unsigned differences for any of these traits. Unsigned differences in area and maximum length were log transformed to meet assumptions of homogeneity of variances.

## 2.5.3 Fourier analysis of otolith asymmetry

To investigate differences in the degree of asymmetry among treatments, unsigned differences between right and left otoliths in the standardised harmonic amplitude were obtained for each harmonic number. The data were analysed in two ways. First, a Kruskal-Wallis test was performed on the unsigned differences in standardised harmonic amplitudes for each harmonic number (shape descriptors). Parametric tests were not used because transformation of data did not produce normality. Second, a one-way between-groups multivariate analysis of variance (MANOVA) was performed using the unsigned differences between right and left otoliths in harmonic numbers  $H_2$  to  $H_{20}$  (shape descriptors) as dependent variables. The assumption of multivariate normality was checked by confirming normality for each of the dependent variables. The multivariate data were visualised using non-metric multidimensional scaling (MDS),

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which is presented with a stress coefficient that reflects how well the data have been reduced to two dimensions.

## 2.5.4 Otolith chemistry

A one-way MANOVA was used to compare multivariate elemental signatures among 5 treatments. Assumptions of multivariate normality and homogeneity of covariance matrices were confirmed as above.

## 3 Results

### 3.1 Otolith Morphometrics

#### 3.1.1 Otolith size and shape

10 There was a significant difference among pH treatments in mean otolith area (left otoliths only) and otolith maximum length (left and right otoliths) (Table 3; Fig. 1). One-tailed t-tests (assuming unequal variance) revealed that mean area of otoliths in the 7.6 treatment was larger than controls for left otoliths ( $P = 0.02$ ), but not right otoliths ( $P = 0.11$ ) and that maximum length of otoliths in the 7.6 treatment was larger than 15 controls for both left ( $P = 0.01$ ) and right otoliths ( $P = 0.03$ ). On average otoliths in the 7.6 treatment had a 15% greater area and were 10% longer than otoliths from control fish. There was also an apparent trend for otolith breadth to be larger in the 7.6 treatment (Fig. 1), however, this was not statistically significant (Table 3). In contrast to the 20 7.6 treatment, there were no differences in average size of otoliths in the 7.8 treatment compared with controls (Fig. 1;  $P > 0.1$  for all statistical comparisons).

There was a statistically significant effect of pH treatment on otolith rectangularity for right otoliths only (Table 3), with a lower rectangularity value in the pH 7.8 treatment compared with controls ( $P = 0.003$ ). However, the difference in the two values (0.76 and 0.73 for control and 7.8 respectively) represents a negligible difference in otolith

shape, and there was no difference in shape between the pH 7.6 treatment and the controls. Therefore, this significant result does not appear to be causally related to the pH treatment. There was no significant effect of pH treatment on otolith circularity (Table 3), although there was a trend for decreased circularity for both left and right otoliths in the 7.6 treatment (Fig. 1).

5 Variation around the mean was lower in the pH 7.6 group for otolith area, maximum length and circularity (with one outlying value excluded) for both left and right otoliths (Fig. 1). This suggests that otoliths in this treatment were a more consistent size and shape than otoliths in the control and 7.8 groups.

10 Mean SL (mm) of larvae was not significantly different among treatments (ANOVA:  $F_{2,45} = 2.667$ ,  $P = 0.08$ ), however there was some variation in SL within treatments (Table 2). There was a highly significant effect of SL on otolith circularity for both left and right otoliths (Table 3). Regression analysis revealed a positive relationship between otolith circularity and fish standard length (left otoliths:  $y = 1.457x + 6.507$ ;  $r^2 = 0.517$ ; right otoliths:  $y = 1.060x + 8.880$ ;  $r^2 = 0.522$ ), indicating a shift from a square-shaped boundary in smaller fish towards a more triangular shape in larger fish.

### 3.1.2 Size difference within otolith pairs

There was no evidence of directional asymmetry, with either the left or the right otolith being the largest of a pair, and no difference in the distribution of positive and negative asymmetry among treatments for any of the otolith measurements (Chi-square  $P > 0.15$  in all cases). Similarly, there was no significant difference among pH treatments in the magnitude of otolith asymmetry with respect to otolith area ( $P = 0.24$ ), maximum length ( $P = 0.79$ ), or maximum breadth ( $P = 0.79$ ), although there was a tendency for the magnitude of difference between left and right otoliths be less in the 7.6 treatment compared to the controls and 7.8 treatment (Fig. 2). As observed above in the morphometric analysis, variation around the mean value was lower in the pH 7.6 group compared to the controls and pH 7.8 group.

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### 3.1.3 Fourier analyses of otolith asymmetry

There were no significant differences among pH treatments in the Fourier descriptors of otolith shape, either when harmonic numbers were analysed individually (Kruskal-Wallis test  $P > 0.05$  in all cases, except  $H_{13}$ ) or in the multivariate analysis of all 19 harmonic numbers (MANOVA  $F_{38,72} = 1.10$ ,  $P = 0.357$ , Wilks' Lambda = 0.4). The similarity among treatments, based on the combination of all harmonic numbers, was clearly evident in the MDS plot (Fig. 3). For  $H_{13}$  there was a significant difference in median harmonic amplitude between the control and pH 7.8 treatment (Mann-Whitney U test  $P = 0.02$ ), but no difference in any other pairwise comparison.

## 10 3.2 Otolith chemistry

Minor and trace element chemistry of larval clownfish otoliths appeared unaffected by the pH of ambient water in which they were reared. A one-way MANOVA found no significant differences in elemental signatures of otoliths among treatments (MANOVA  $F_{10,22} = 1.07$ ,  $P = 0.42$ , Wilks' Lambda = 0.45). Similarly, there were no consistent patterns in any of the individual elemental ratios as a function of pH (Fig. 4).

## 15 4 Discussion

We found that the size, shape, symmetry and elemental chemistry of otoliths in larval clownfish was unaffected by exposure to simulated levels of ocean acidification that could occur at the end of this century under a business-as-usual scenario of CO<sub>2</sub> emissions (pH 7.8 and 1050 µatm pCO<sub>2</sub>). However, in a more extreme treatment (pH 7.6 and 1721 µatm pCO<sub>2</sub>) otolith area and maximum length were larger than control otoliths. Two other recent studies have examined potential effects of ocean acidification on fish otoliths. Checkley et al. (2009) detected increased otolith size in larval seabass, *Atractoscion nobilis*, exposed to elevated CO<sub>2</sub> during the egg stage and up to 8 days post-hatching. Otoliths area was 7–9% and 15–17% larger than controls for

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fish reared at 993 and 2558  $\mu\text{atm}$   $\text{CO}_2$ , respectively. In contrast, Munday et al. (2011) did not detect any effect of elevated  $\text{CO}_2$  on otolith size of juvenile spiny damselfish, *Acanthochromis polyacanthus*, reared for 3 weeks in treatments up to 841  $\mu\text{atm}$   $\text{CO}_2$ . The different results of these two studies could have been due to: (1) the higher  $p\text{CO}_2$  levels used by Checkley et al. (2009), (2) the absence of a larval phase in the spiny damselfish, which could make them less susceptible to elevated  $\text{CO}_2$ , or (3) different durations of the two experiments (Munday et al., 2011). Our results presented here suggest that otolith size can indeed be affected by reduced pH and elevated  $p\text{CO}_2$ , as proposed by Checkley et al. (2009), however, sensitivity varies among species. 5  
Clownfish otoliths were not affected at  $\sim 1000 \mu\text{atm}$   $\text{CO}_2$ , whereas seabass otoliths were significantly larger at this concentration, but otoliths of both species were larger at higher  $p\text{CO}_2$  concentrations. Thus, elevated  $p\text{CO}_2$  appears to affect otolith development in both species, but larval clownfishes are more tolerant of elevated  $\text{CO}_2$  than larval seabass. There is increasing evidence that ocean acidification affects different 10  
calcifying species in very different ways (Langer et al., 2009; Ries et al., 2009; Dupont et al., 2010; Kroeker et al., 2010) and these results indicate that the same types of variation might be observed among species without carbonate skeletons. 15

The variable responses observed among the three species studied to date may be related to their life histories. The spiny damselfish, *Acanthochromis polyacanthus*, 20  
studied by Munday et al. (2010) has direct developing juveniles that remain on the reef after hatching. Juveniles shelter with their parents in small caves, where  $\text{CO}_2$  levels are likely to become elevated due to respiration. Consequently, newly hatched *A. polyacanthus* may be adapted to periods of high ambient  $\text{CO}_2$ . The clownfish, *Amphiprion percula*, has pelagic larvae, but is a demersal spawner. Benthic eggs are likely to experience significant fluxes in ambient  $\text{CO}_2$  due to consumption of  $\text{CO}_2$  by photosynthesis 25  
during the day and release of  $\text{CO}_2$  by respiration of reef organisms at night (Munday et al., 2008). Hatching from benthic eggs may precondition larval clownfishes to moderate increases in ambient  $\text{CO}_2$ . In contrast, the seabass *Atractoscion nobilis* is a pelagic spawner, so both eggs and larvae are likely adapted to a low  $\text{CO}_2$  environment, and

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may be more susceptible to elevated CO<sub>2</sub>. Further studies on a range of benthic and pelagic spawners are required to test this hypothesis.

If reduced seawater carbonate ion (CO<sub>3</sub><sup>2-</sup>) concentrations affected the rate of otolith precipitation we would expect to see a decline in otolith size in acidified seawater. Instead there was a trend for otolith size to increase in these conditions. Fish actively regulate their acid-base balance through bicarbonate (HCO<sub>3</sub><sup>-</sup>) accumulation and ion exchange across the gills (Claiborne et al., 2002; Evans et al., 2005; Brauner and Baker, 2009) and, thus, are able to compensate for intra- and extra-cellular acidosis caused by increased pCO<sub>2</sub>. Changes to extracellular HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> concentrations caused by acid-base regulation at high pCO<sub>2</sub> could potentially influence the precipitation of CaCO<sub>3</sub> in structures such as otoliths (Payan et al., 1997). Checkley et al. (2009) proposed that larval fish controlled the concentration of H<sup>+</sup> and Ca<sup>2+</sup> ions in the blood plasma and endolymph (the fluid surrounding an otolith), but not the neutral molecule CO<sub>2</sub>. Consequently, maintenance of a constant pH in the endolymph despite elevated extracellular pCO<sub>2</sub> would lead to increased CO<sub>3</sub><sup>2-</sup> concentrations in the endolymph due to diffusion of CO<sub>2</sub> from blood plasma across the endolyphatic membrane. Increased CO<sub>3</sub><sup>2-</sup> concentrations in the endolympathic fluid may, in turn, increase the rate of aragonite precipitation at the otolith surface. However, CO<sub>2</sub> concentrations are generally higher in endolympathic fluid than in blood plasma (Takagi, 2002), perhaps related to the removal of H<sup>+</sup> ions produced during disassociation of HCO<sub>3</sub><sup>-</sup> to CO<sub>3</sub><sup>2-</sup> at the time of aragonite precipitation (Payan et al., 1999). Under this scenario there would be net diffusion of CO<sub>2</sub> out of, not into, the endolymph. Alternatively, HCO<sub>3</sub><sup>-</sup> accumulation as a result of acid-base compensation, either in the plasma or endolymph, may promote otolith accretion if HCO<sub>3</sub><sup>-</sup> is the preferred dissolved inorganic carbon species for calcification (Allemand et al., 2007; Herfort et al., 2008). Consistent with this hypothesis, increased calcification of cuttlebone is also associated with increased extracellular HCO<sub>3</sub><sup>-</sup> in cephalopods exposed to elevated pCO<sub>2</sub> (Gutowska et al., 2010). The precise mechanisms involved in acid-base regulation in larval marine fishes are not fully understood (Brauner, 2009), nor are the cellular mechanisms involved in calcification

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**Table 1.** Average seawater chemistry parameters over the duration of the experiment for the nominated pH treatments. Salinity ( $S$ ), temperature ( $T$ ) total alkalinity ( $A_T$ ) and pH were measured directly. All other parameters were estimated in the program CO2SYS.

$pH_{NBS}$	$S$ ppt	$T$ °C	$A_T$ $\mu\text{mol kg}^{-1}$	DIC $\mu\text{mol kg}^{-1}$	$p\text{CO}_2$ $\mu\text{atm}$	$\text{CO}_2$ $\mu\text{mol kg}^{-1}$	$\text{HCO}_3^-$ $\mu\text{mol kg}^{-1}$	$\text{CO}_3^{2-}$ $\mu\text{mol kg}^{-1}$	$\Omega_{\text{Ar}}$
8.15	33	30	2043	1768	404.1	10.24	1567.22	190.45	3.14
7.8	33	30	2066	1946	1050.8	26.64	1820.68	98.83	1.63
7.6	33	30	2042	1990	1721.4	43.63	1881.85	65.01	1.06

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**Table 2.** Fish sample sizes and dimensions used in morphometric and Fourier analyses.

Treatment	Morphometrics			Fourier	
	n	Fish standard length (mm)			
		Mean ( $\pm$ SD)	Range		
pH 8.15	16	7.2 ( $\pm$ 1.75)	5.2–10.6	19	
pH 7.8	16	8.0 ( $\pm$ 1.22)	6.0–9.7	19	
pH 7.6	16	6.9 ( $\pm$ 1.22)	5.6–9.0	19	

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**Table 3.** Results of Kruskal-Wallis median test (area, maximum length) and ANCOVA (breadth, circularity, rectangularity) for left and right otoliths of larval clownfish reared at three pH treatments (8.15, 7.8, and 7.6).

(a) Left otoliths				
Variable	Source	d.f.	$\chi^2$ or $F$ value	$P$
Otolith area ( $\mu\text{m}^2$ )	pH treatment	2	$\chi^2 = 6.00$	0.049
Otolith maximum length ( $\mu\text{m}$ )	pH treatment	2	$\chi^2 = 9.50$	0.009
Otolith maximum breadth ( $\mu\text{m}$ )	pH treatment	2	0.583	0.562
	Fish standard length	1	3.029	0.089
Otolith circularity	pH treatment	2	1.280	0.288
	Fish standard length	1	59.70	0.000
Otolith rectangularity	pH treatment	2	0.893	0.416
	Fish standard length	1	4.739	0.030
(b) Right otoliths				
Otolith area ( $\mu\text{m}^2$ )	pH treatment	2	$\chi^2 = 0.50$	0.778
Otolith maximum length ( $\mu\text{m}$ )	pH treatment	2	$\chi^2 = 6.00$	0.049
Otolith maximum breadth ( $\mu\text{m}$ )	pH treatment	2	0.446	0.643
	Fish standard length	1	1.103	0.299
Otolith circularity	pH treatment	2	1.32	0.277
	Fish standard length	1	40.84	0.000
Otolith rectangularity	pH treatment	2	5.649	0.007
	Fish standard length	1	0.014	0.907

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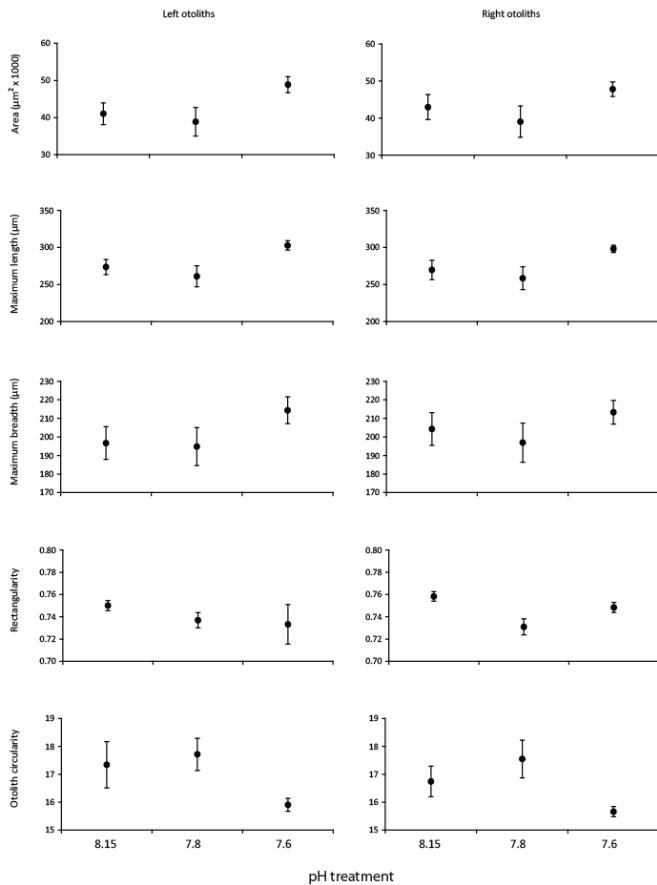
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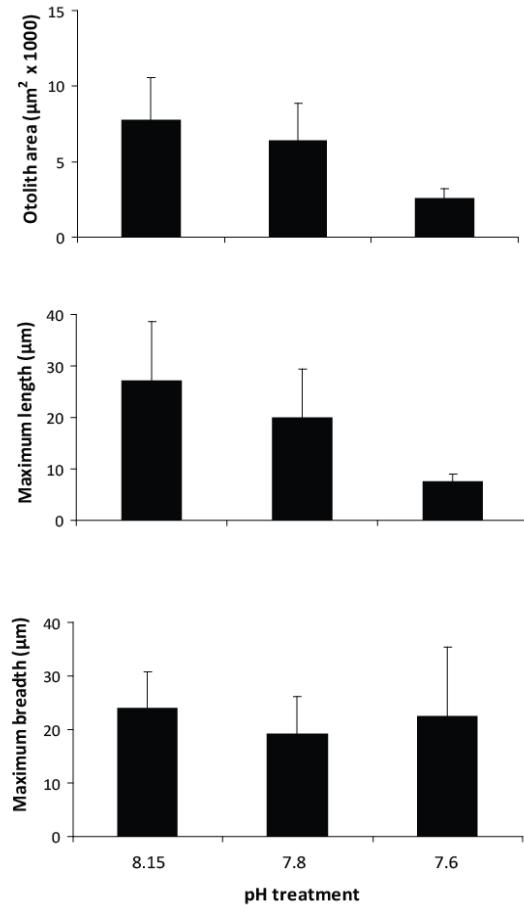
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**Fig. 1.** Mean ( $\pm\text{SE}$ ) otolith area, maximum length, maximum breadth, rectangularity and circularity for left and right otoliths of larval clownfish reared at  $\text{pH}_{\text{NBS}}$  8.15, 7.8, and 7.6.

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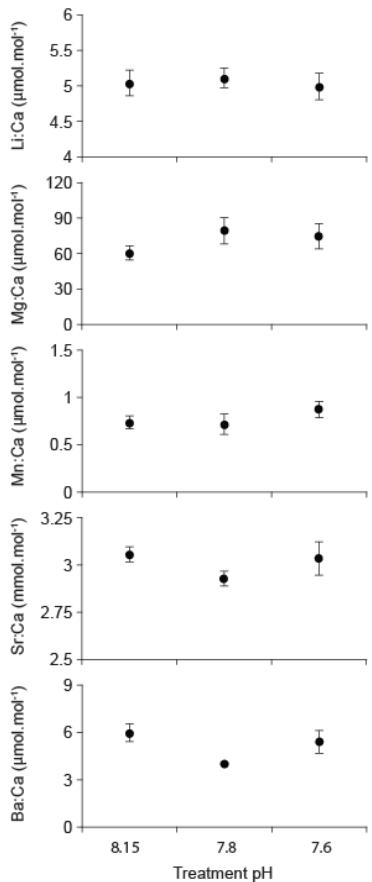
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**Fig. 2.** Mean ( $\pm\text{SE}$ ) unsigned differences in area, maximum length and maximum breadth between left and right otoliths of larval clownfish reared at  $\text{pH}_{\text{NBS}}$  8.15, 7.8, and 7.6.

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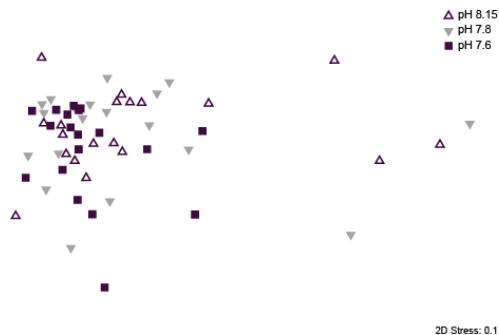
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**Fig. 3.** Mean ( $\pm$ SE) Li:Ca, Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca ratios in otoliths of larval clownfish reared at  $\text{pH}_{\text{NBS}}$  8.15 ( $n = 7$ ), 7.8 ( $n = 5$ ), and 7.6 ( $n = 6$ ).

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**Fig. 4.** MDS plot of Fourier descriptors of otolith shape for each individual sampled, grouped by pH treatment.

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