

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 pH_{NBS} and $p\text{CO}_2$ levels (control: pH 8.15 & 404 $\mu\text{atm CO}_2$; intermediate: pH 7.8 & 1050 $\mu\text{atm CO}_2$; extreme: pH 7.6 & 1721 $\mu\text{atm CO}_2$) to test the possible effects of ocean acidification on otolith development. There was no effect of the intermediate treatment (pH 7.8 & 1050 $\mu\text{atm CO}_2$) 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 & 1721 $\mu\text{atm CO}_2$) otolith area and maximum length were larger than controls, although no other traits were significantly 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 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 (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 (CaCO_3) shells and skeletons (Feely et al., 2004; Orr et al., 2005). As

the saturation states of aragonite and calcite decline, the calcification rate of corals, molluscs, echinoderms and other invertebrates that secrete these forms of CaCO_3 is predicted to decrease (Gattuso et al., 1998; Riebesell et al., 2000; Gazeau et al., 2007; Kleypas and Yates, 2009). If the current trajectory of global CO_2 emissions is maintained, atmospheric CO_2 concentrations could reach between 730-1020ppm by the end of the century (Meehl et al., 2007; Raupach et al., 2007). This would cause ocean pH to decline 0.3-0.4 units compared to current-day levels (Caldeira and Wickett 2005) and reduce the concentration of carbonate ions in the shallow ocean by nearly 50% compared to the pre-industrial era. The consequences of such a reduction in carbonate ion concentration is likely to be serious for many calcifying species (Hoegh-Guldberg et al., 2007; Fabry et al., 2008; Doney et al., 2009; Hofmann et al., 2010). Possible impacts of elevated CO_2 and ocean acidification on non-calcifying species, such as fishes, is still poorly known, but could potentially include effects on a range of physiological (Pörtner and Farrell, 2008), developmental (Pankhurst and Munday, 2011) and behavioural processes (Munday et al., 2009, 2010). Early life history stages are likely to be most vulnerable because physiological homeostasis might not be fully developed and their small body size makes them more sensitive to environmental variation (Ishimatsu et al., 2008; Melzner et al., 2009).

In general, marine fish appear to be relatively tolerant to mild increases in ambient CO_2 , presumably because well-developed mechanisms for acid-base regulation allow them to compensate for cellular acidosis caused by exposure to elevated $p\text{CO}_2$ (Pörtner et al., 2005; Ishimatsu et al., 2008; Melzner et al., 2009). A concern, however, is that fish otoliths (earbones) are composed of aragonite, and therefore, could be susceptible to the declining carbonate ion concentrations associated with ocean acidification. Just as calcification rates in corals decrease as the aragonite saturation state falls, it may become more difficult for fish to precipitate aragonite in their otoliths as the availability of carbonate ions in seawater declines.

Alternatively, otolith growth may be affected by regulatory mechanisms used by fish to maintain their internal pH as ambient $p\text{CO}_2$ increases. Changes in extracellular concentrations of carbonate and bicarbonate caused by acid-base regulation in a high CO_2 environment could affect the precipitation of CaCO_3 in the otolith (Payan et al., 1997). Indeed, one recent study reported that otoliths were larger in larval fishes exposed to elevated 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 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; [Popper and Lu 2000](#)). 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 increased in larval seabass exposed to ~1000 $\mu\text{atm CO}_2$ for eight days. In contrast, Munday et al. (2011) detected no effects of ~850 $\mu\text{atm CO}_2$ 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 & 1050 $\mu\text{atm CO}_2$) and 2200-2300 (pH 7.6, 1721 $\mu\text{atm CO}_2$) based on a business-as-usual trajectory of 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. Four Aadult breeding pairs were kept in separate 70 l aquariums supplied with a continuous flow of filtered seawater at 18 l 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 eggs, the parental aquarium was assigned to one of three pH_{NBS} levels (8.15 (control), 7.8, 7.6) and pH adjusted as described below. The breeding pairs produced new egg clutches on a 2-4 week cycle. The pH treatment assigned to each clutch was alternated so that each of the four parents were assigned to each pH treatment during the experiment. On the ~~evening~~night of hatching (6-8 days after laying) the ~~pot~~ egg clutch -was removed from the parental aquarium and transferred to a 100 l larval rearing aquarium set to the same pH as the parental aquarium. Eggs hatched within several hours of darkness. Larvae were reared in a semi-closed system, where each aerated aquarium had no water flow during the 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⁻¹ each morning for the first three days. *Artemia naupli* were added at 1 individual ml⁻¹ each morning from day three. The ratio of *Artemia naupli* to rotifers was increased each day until larvae were only fed 5 *Artemia naupli* m⁻¹ 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.

Settlement stage larvae (11-d post hatching) were sacrificed with a lethal dose of clove oil anaesthetic (Munday and Wilson 2007). Larvae from one breeding pair in the control group, and a different breeding pair in the pH 7.8 group, did not survive, therefore

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each treatment contained larvae from at least three of the four parental genotypes. A sample of 10-15 larvae from each clutch were stored in 75% ethanol for otolith analysis. The remaining larvae were stored in formaldehyde for other analyses.

2.2 Seawater manipulation

Seawater pH_{NBS} in the parental aquariums and larval rearing tanks that had been allocated to the 7.8 or 7.6 treatments was adjusted by CO_2 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_2 . The solenoid injected a slow stream of CO_2 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_2 into the diffuser. Using this method it was possible to constantly maintain pH within ± 0.05 units of the desired level and there was no detectable gradient in seawater pH within the aquarium. A diffuser without CO_2 injection was placed in each aquarium and rearing tank assigned to control conditions. The pH_{NBS} of each aquarium was independently checked each day using a WP80 pH meter (TPS, Australia) calibrated with fresh pH buffers (Merk, Germany). CO_2 was only injected into aquariums 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 $30^\circ\text{C} \pm 0.6$ (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 & 7.6 pH treatments was estimated to be 404, 1050 & 1721 μatm , 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 Larvae~~ were removed from the preservative, blotted dry, weighed (nearest mg) and photographed in a lateral position under a stereomicroscope. Standard length (SL) to nearest 0.01mm was estimated for each fish from the digital photograph using image analysis (Optimas 6.5, Media Cybernetics). 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, μm^2 ; maximum length, μm ; maximum breadth, μm ; 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 ($a + bi$) 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.

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 three different parental genotypes. Samples at each treatment level contained 3-10 individuals from at least 3 different breeding pairs.

2.4 Otolith chemistry

Elemental chemistry of otoliths was quantified using laser ablation inductively coupled plasma mass spectrometry. Otolith chemistry was conducted on 5-7 randomly selected individuals from each treatment. One otolith chosen at random from each fish was mounted on microscope slides with a cyanoacrylic glue, ground to the midplane using $3\mu\text{m}$ Al_2O_3 lapping film, sonified in ultrapure water for 2 minutes 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 μm spot centered approximately 50 μm outside of the nucleus using a laser repetition rate of 5 Hz at 70% power and a dwell time of 60 sec. 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% HNO_3) supplied by a self-aspirating (20 μmin^{-1}) PFA nebulizer in the concentric region of the quartz dual inlet spray chamber. Initial testing found that isotopes from six elements (^7Li , ^{25}Mg , ^{43}Ca , ^{55}Mn , ^{88}Sr and ^{138}Ba) had count rates significantly higher than blank values and were free of isobaric interferences. Instrument blanks (2% HNO_3) and a otolith certified reference material (Sturgeon et al. 2005), dissolved in 2% HNO_3 and diluted to a final Ca concentration of 8 $\mu\text{g}\cdot\text{g}^{-1}$, were run every eight samples and used to correct for blank values and instrument mass bias, respectively, following FitzGerald et al. (2004). Detection limits were calculated as 3 standard deviations of the blanks that were run periodically throughout the analyses (n=3). These limits were 5% of the average sample intensity for ^7Li , 3% of ^{25}Mg , 0.01% for ^{43}Ca , 8% for ^{55}Mn , 0.004% for ^{88}Sr , and 7% for ^{138}Ba . We estimated external precision (relative standard deviation, RSD) of the analyses by running a powdered otolith lab standard, dissolved in 2% HNO_3 and diluted to a Ca concentration of 8 $\mu\text{g}\cdot\text{g}^{-1}$. Estimates of RSD (n = 3) were 2.2% for Li:Ca, 1.7% for Mg:Ca, 14% for Mn:Ca, 0.5% for Sr:Ca and 0.6% for Ba:Ca.

2.5 Statistical analyses

2.5.1 Otolith size and shape

Fish size could potentially influence otolith size and shape, therefore, we first examined the relationship between SL and each of the otolith morphometric traits. [Otolith circularity was](#)

~~the only trait that exhibited a significant relationship with SL. A relationship between SL and maximum breadth, otolith rectangularity and circularity was detected for either right or left otoliths, therefore and~~ ANCOVA was used to determine if ~~this~~ these morphometric traits differed among pH treatments, using fish standard length as the covariate. There was no relationship between SL and ~~the other otolith traits. ANOVA was used to compare otolith breadth and rectangularity differed among pH -otolith area or maximum length. Furthermore, the treatments. The~~ variance distributions ~~for otolith area and maximum length of these two traits~~ were not homogeneous (Levene's test: $P < 0.05$) and were not improved by transformations. Therefore, non-parametric Kruskal-Wallis median tests were used to determine if these morphometric traits differed among pH treatments.

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), 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 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

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 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 7.6 treatment, there were no apparent differences in average size or shape of otoliths in the 7.8 treatment compared with controls (Fig. 1).

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).

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.

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 linear relationship between otolith circularity and fish standard length (left otoliths: $y = 1.457x + 6.507$; $r^2 = 0.517$; right otoliths: $y = 1.060x + 8.880$; $r^2 = 0.522$); Circularity is defined as otolith perimeter²/otolith area. The minimum value of 4pi (12.75) is achieved only for a circular boundary, with values of ~16 for a square boundary, and ~ 20 for a triangular boundary. Circularity increased from ~ 15 in the smallest fish (5-6 mm SL) to >20 in the largest fish (9-11 mm SL) indicating a shift from a square-shaped boundary ~~in smaller fish~~ towards a more triangular shape with increasing fish length.

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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.

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.

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).

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 CO₂ emissions (pH 7.8 & 1050 μatm CO₂). However, in a more extreme treatment (pH 7.6 & 1721 μatm CO₂) 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 CO₂ during the egg stage and up to 8 days post-hatching. Otoliths area was 7-9% and 15-17% larger than controls for fish reared at 993 and 2558 μatm CO₂, respectively. In contrast, Munday et al. (2011) did not detect any effect of elevated CO₂ on otolith size of juvenile spiny damselfish, *Acanthochromis polyacanthus*, reared for 3 weeks in treatments up to 841 μatm CO₂. The different results of these two studies could have been due to: 1) the higher pCO₂ 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 CO₂, or 3) different durations of the two experiments. Our results presented here suggest that otolith size can indeed be affected by reduced pH and elevated pCO₂, as proposed by Checkley et al. (2009), however, sensitivity varies among species. Clownfish otoliths were not affected at ~ 1000 μatm CO₂, whereas seabass otoliths were significantly larger at this concentration, but otoliths of both species were larger at higher pCO₂ concentrations. Thus, elevated pCO₂ appears to affect otolith development in both species, but larval clownfishes are more tolerant of elevated CO₂ than larval seabass. There is increasing evidence that ocean acidification affects different 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 [extensive](#) carbonate skeletons.

The variable responses observed among the three species studied to date may be related to their life histories. The spiny damselfish, *Acanthochromis polyacanthus*, 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 CO₂ levels are likely to become elevated due to respiration. Consequently, newly hatched *A. polyacanthus* may be adapted to periods of high ambient CO₂. The clownfish, *Amphiprion percula*, has pelagic larvae, but is a demersal spawner. Benthic eggs are likely to experience significant fluxes in ambient CO₂ due to consumption of CO₂ by photosynthesis during the day and release of CO₂ by respiration of reef organisms at night (Munday et al., 2008). Hatching from benthic eggs may precondition larval clownfishes to moderate increases in ambient CO₂. In contrast, the seabass *Atractoscion nobilis* is a pelagic spawner, so both eggs and larvae are likely adapted to the relatively low and stable CO₂ environment [characteristic of epipelagic ocean waters](#), and may be more susceptible to elevated CO₂. Further studies on a range of benthic and pelagic spawners are required to test this hypothesis.

If reduced seawater carbonate ion (CO₃²⁻) 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 (HCO₃⁻) 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 pCO₂. Changes to extracellular HCO₃⁻ and CO₃²⁻ concentrations caused by acid-base regulation at high pCO₂ could potentially influence the precipitation of CaCO₃ in structures such as otoliths (Payan et al., 1997). Checkley et al. (2009) proposed that larval fish controlled the concentration of H⁺ and Ca²⁺ ions in the blood plasma and endolymph (the fluid surrounding an otolith), but not the neutral molecule CO₂. Consequently, maintenance of a constant pH in the endolymph

despite elevated extracellular $p\text{CO}_2$ would lead to increased CO_3^{2-} concentrations in the endolymph due to diffusion of CO_2 from blood plasma across the endolymphatic membrane. Increased CO_3^{2-} concentrations in the endolymphatic fluid may, in turn, increase the rate of aragonite precipitation at the otolith surface. However, CO_2 concentrations are generally higher in endolymphic fluid than in blood plasma (Takagi, 2002), perhaps related to the removal of H^+ ions produced during disassociation of HCO_3^- to CO_3^{2-} at the time of aragonite precipitation (Payan et al. 1999). Under this scenario there would be net diffusion of CO_2 out of, not into, the endolymph. Alternatively, HCO_3^- accumulation as a result of acid-base compensation, either in the plasma or endolymph, may promote otolith accretion if HCO_3^- is the preferred dissolved inorganic carbon species for calcification (Allemand et al. 2007; Herfort et al., 2008). 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 of many marine species (Hofmann et al., 2010). Nonetheless, acid-base regulation and biological control of endolymph pH are likely to be central to the changes in otolith growth observed in these two species of fish when exposed to high CO_2 (Romanek and Gauldie, 1996; Payan et al., 1997; 1998; Allemand et al. 2007).

~~Although we detected an increase in otolith area and maximum length in the more extreme treatment (pH 7.6, 1721 $\mu\text{atm CO}_2$), we did not observe any effects on other otolith characteristics, such as otolith shape or symmetry. Otoliths have an important role in fish hearing and body orientation. However, if the changes in otolith area and maximum length that we observed in the highest CO_2 treatment (pH 7.6, 1721 $\mu\text{atm CO}_2$) would be sufficient to affect these important functions is unknown. Otolith size can vary considerably among individual fish of the same somatic size, and otoliths of some species continue to grow even once somatic growth has ceased (Campana 1990; Thorrold and Hare 2002; Munday et al. 2004). Furthermore, we observed considerable variation in otolith morphometric traits among~~

individuals in the control and less extreme CO₂ groups. Therefore, some variation in otolith size is commonplace and it is not clear that the relatively small change in otolith size observed here in the highest CO₂ treatment would be sufficient to cause substantive effects on larval fish.

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Although a number of studies have shown that physiological stress can increase otolith asymmetry (Gagliano and McCormick 2004; et al., 2008; Payan et al. 2004); however, we found no evidence that ocean acidification is likely to decrease affect the magnitude of symmetry between left and right otoliths, even at relatively high CO₂ concentrations. In fact, there was a trend for smaller differences in otolith area and length between left and right otoliths in the highest CO₂ treatment, and for there to be less variation among individuals (i.e. lower sample variance). Increased calcification in the higher CO₂ treatment might have tended to ameliorate differences in otolith shape and size, and thus been responsible for the apparent increase in symmetry between otoliths, both within and among individuals. Variation in the size and shape of left and right otoliths may affect the ability of individuals to detect and localize sound (Popper and Lu 2000; Gagliano et al. 2008) and decreased otolith symmetry has been associated with higher mortality rates in larval reef fishes (Gagliano et al. 2008). Consequently, any trend toward increased symmetry, such as that observed here, is unlikely to have a negative impact on individual fitness.

Otolith circularity was the only trait that varied significantly with fish length. There was a linear relationship between SL and circularity, indicating a shift from a square-shaped boundary in smaller fish towards a more triangular shape in larger fish. Otolith deposition does not occur evenly around the otolith, instead, growth often occurs more rapidly on one axis than the other, leading to an otolith that is increasingly more elongate and triangular in shape (Gagliano and McCormick 2004; Green et al. 2009). Otolith growth is often correlated

with somatic growth and therefore larger, faster growing fish within treatments may be expected to have a more triangular otolith profile, as observed.

This is the first study to examine the potential effects of ocean acidification on otolith chemistry and we found no effects of altered seawater carbonate chemistry on the elemental chemistry of the otoliths. Elemental ratios of all five elements were within the range typically reported for aragonitic marine fish otoliths (Campana and Thorrold, 2001).

Although sample sizes were relatively small (5-7 individuals per treatment) the low variance and consistency of results suggests that a larger sample sizes would not have changed our interpretation in any meaningful way. We also found no visual evidence of vaterite or calcite in any of the treatment otoliths, which if present would have resulted in relatively low Sr:Ca values (Veizer, 1983; Tomas and Geffen, 2003). Taken together, our data suggest that the larval clownfish were capable of regulating endolymphic fluid chemistry even in waters with pH values significantly lower than open ocean values.

Our results demonstrate a number of important points. First, the tendency for otolith size to increase and become less variable in the higher CO₂ treatment is consistent with an effect on otolith development caused by the physiological process of pH regulation in fishes. Further studies are required to pinpoint the exact processes involved. Second, our results indicate that otolith development in larval clownfishes is robust to the levels of ocean chemistry change that may occur over the next 50-100 years, even if higher levels are likely to influence otolith size. Finally, we emphasize that there is likely to be considerable variation among species in their sensitivity to elevated CO₂ and reduced pH. Determining the traits that make some species more susceptible than others will aid in making predictions about the longer-term and ecosystem level effects of ocean acidification.

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Table 1. Average (\pm SD where estimated) seawater chemistry parameters over the duration of the experiment for the nominated pH treatments.

Salinity (S), temperature (T) and total alkalinity (A_T) were measured directly. All other parameters were estimated in the program CO2SYS.

pH_{NBS}	S	T	A_T	DIC	PCO₂	CO₂	HCO₃⁻	CO₃²⁻	Ω
	ppt	°C	μmol kg ⁻¹	μmol kg ⁻¹	μatm	μmol kg ⁻¹	μmol kg ⁻¹	μmol kg ⁻¹	Ar
8.15	33	30 (0.6)	2043 (131)	1768	404.1	10.24	1567.22	190.45	3.14
7.8	33	30 (0.6)	2066 (116)	1946	1050.8	26.64	1820.68	98.83	1.63
7.6	33	30 (0.6)	2042 (132)	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)		n
		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

Table 3 Results of Kruskal-Wallis median test (area, maximum length), ANOVA (breadth, rectangularity) 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). Fish standard length (SL) is the covariate for ANCOVA.

a) Left otoliths

Variable	Source	d.f.	χ^2 or <i>F</i> value	<i>P</i>
Otolith area	pH treatment	2	$\chi^2=6.00$	0.049
Otolith maximum length	pH treatment	2	$\chi^2=9.50$	0.009
Otolith maximum breadth	pH treatment	2	1.488	0.237
Otolith rectangularity	pH treatment	2	0.62	0.54
Otolith circularity	pH treatment	2	1.280	0.288
	Fish SL	1	59.70	0.000

b) Right otoliths

Variable	Source	d.f.	χ^2 or <i>F</i> value	<i>P</i>
Otolith area	pH treatment	2	$\chi^2=0.50$	0.778
Otolith maximum length	pH treatment	2	$\chi^2=6.00$	0.049
Otolith maximum breadth	pH treatment	2	0.878	0.422
Otolith rectangularity	pH treatment	2	6.31	0.004
Otolith circularity	pH treatment	2	1.32	0.277
	Fish SL	1	40.84	0.000

Figure legends

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

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

Fig. 3. MDS plot of Fourier descriptors of otolith shape for each individual sampled, grouped by pH treatment.

Fig. 4. Mean (\pm SE) Li:Ca, Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca ratios in otoliths of larval clownfish reared at pH_{NBS} 8.15 (n = 7), 7.8 (n = 5), and 7.6 (n = 6).







