The influence of elemental chemistry on the widths of otolith increments in the neon damselfish (*Pomacentrus coelestis*)

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Abstract—We examined the potential for water chemistry to affect the width of daily increments in reef fish otoliths using both mensurative and manipulative methods. We found significant differences in the widths of increments in otoliths of the neon damselfish (*Pomacentrus coelestis*) collected in different habitats at One Tree Island on the Great Barrier Reef. We then used manipulative experiments to determine if natural water masses (ocean water vs. lagoon plume) could produce different incremental widths in otoliths in the absence of potentially confounding factors. Fish exposed to ocean water had significantly wider otolith increments for two of the three experiments. Elemental analyses indicated that Ba/Ca ratios were significantly correlated with increment widths for two of the three experiments and Sr/Ca ratios did not correlate with increment width for any experimental period. Variation in crystal-lattice orientation did not explain differences in increment width between treatments. Differences in water chemistry can affect increment widths in otoliths of reef fishes, potentially confounding patterns previously attributed to growth rate or condition alone.

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Recent research has elucidated the importance of presettlement condition and growth rate of reef fish for survival and recruitment (Bergenius et al., 2002; Hoey and McCormick, 2004; Sponaugle and Pinkard, 2004). Studies on the condition of presettlement fishes have been based on a number of measures that include growth rate, biochemical development, and behavior (McCormick, 1998). More commonly, however, widths of increments in otoliths have been used as a means of examining growth on the assumption of a good relationship between somatic and otolith growth (Campana, 1999; Meekan et al., 2003). In addition, otolith increment widths may provide information about the condition of presettlement fishes; it has been demonstrated that fish with small lipid reserves generally deposit narrower increments than fish with adequate lipid reserves (Paperno et al., 1997; Molony and Sheaves, 1998). Indeed, periods of starvation are known to have a substantial influence on the increment widths of otoliths (Moksness, 1992; Molony, 1996), as can water temperature (Hüssy et al., 2003; Powell et al., 2004) and the onset of metamorphosis into a juvenile form (Wilson and McCormick, 1999). Thus, the increment widths of otoliths are an important, but not necessarily simple tool, by which larval condition and growth can be assessed.

Otoliths are composed of an aragonite lattice deposited on a protein matrix; this lattice incorporates trace elements, derived mainly from the surrounding water mass (Walther and Thorrold, 2006), as deposition occurs (Campana, 1999). Indeed, there is a close link between water and otolith chemistry for some elements (i.e., Ba; Bath et al., 2000; Walther and Thorrold, 2006); this relationship allows otolith chemistry to be used as a proxy for water chemistry. Although the process of elemental deposition is not entirely understood at this time, it is possible that the chemical composition of the water mass may influence increment widths in the otoliths by means of otolith chemistry and related crystallography, thus potentially confounding the interpretation of these increments.

Furthermore, some reef fish are capable of distinguishing and actively choosing particular water masses (Doherty et al., 1996; Atema et al., 2002; Gerlach et al., 2007), presumably as a means of choosing suitable habitat at settlement. This study demonstrates that otolith increment widths can vary between fish in dif-

ferent water masses that have a constant temperature and offer constant food conditions. Water masses, therefore, have the potential to influence increment width by affecting growth rates (Sponaugle and Pinkard, 2004) or influencing otolith chemistry; the challenge is to distinguish between these two mechanisms.

Materials and methods

This study was conducted on One Tree Island (OTI) in the southern Great Barrier Reef (GBR), Australia. This platform reef is closed off from the surrounding ocean for 3–5 hours during each low tide (Ludington, 1979). Thus, the geochemical properties of the lagoon water may be distinct from the surrounding ocean water (Atema et al., 2002); this feature may influence the increment widths of the otoliths of the fish residing in the lagoon. The otoliths of newly settled neon damselfish (*Pomacentrus coelestis*) collected from different water masses around OTI (i.e., reef slope (=oceanic water) vs. lagoon water) were examined and the increment widths were measured. A series of manipulative experiments were conducted with oceanic and lagoon water to examine the effects on increment widths of *P. coelestis* otoliths in the absence of potentially confounding factors, including food and temperature. The increment widths derived from these experiments were then compared with the results of another study (Patterson et al., 2004a) where otolith chemistry of the same experimental fish was examined as that used in this study to determine if otolith chemistry may affect increment width. In addition, scanning electron microscopy (SEM) was used to examine otolith microstructure because variation in elemental chemistry may potentially alter crystal orientation.

Wild fish

Newly settled *P. coelestis* were collected by divers using hand nets and clove oil from two sites on the reef slope (ocean 1 and 2) and two sites within the lagoon (lagoon 1 and 2) at OTI (23°30′S, 152°06′E; n=7 per site). The sagittae were removed and one was randomly chosen for analysis. Transverse sections were prepared by embedding the sagittae in Epofix resin (Struers, Milton, Australia) and polishing with 800-grit-size lapping paper on a grinding wheel and then fine-polishing with 3-μm lapping film until the primordium was visible and daily rings could be distinguished. The daily increments (validated by Flood, 2000) were counted and measured with a Leica image analysis system (Leica Microsystems, North Ryde, NSW, Australia). A drop of immersion oil was placed on each otolith to enhance its appearance, and an image of each otolith was captured and saved at a magnification of 400× with a Leica DC300 digital video camera. The distance between each increment on the distal side of each otolith was measured with the use of the software package Leica IM50.

Experimental fish

Experimental procedures were identical to those described in Patterson et al. (2004a), but are briefly repeated here. Presettlement (*sensu* Kingsford et al., 2002) *P. coelestis* were collected by using two to four moored light traps at several locations within 100 m of the reef slope (see Patterson et al., 2004a, Fig. 1) during three consecutive austral summer field seasons (January and February, 2000–02). Light traps were illuminated for three hours each night (21:00–22:00, 00:00–01:00, and 03:00–04:00) and were checked on the first high tide after sunrise. Samples from the light traps were sorted on the boat, and target species were taken to the laboratory to be counted and identified. Fish used in each experiment were collected over a period of two days. Additional individuals were immediately frozen to use as control fish (see *Crystallography* section).

The experimental design for each year differed only in the number of replicate fish placed in each tank at the beginning of the experiment (2000, n=6; 2001, n=10; 2002, n = 3; n varied because of among-year differences in the availability of fish). Experimental fish were allowed a minimum of 24 hours to acclimate to experimental conditions before the start of the experiment. It was not possible to measure the standard length (SL) of fish before the start of the experiment because this process stressed, and in some cases damaged the fish, which could have confounded the results. All fish were approximately the same size at the beginning of the experiment (~15 mm SL). Three replicates of each treatment (lagoon water vs. ocean water) were set up in 200-L tanks and fish were randomly placed in each tank. Aerated tanks were kept under shade cloth in a wet laboratory and were randomly positioned. Water (from lagoon ebb tide plumes and oceanic waters collected outside of plumes; see Patterson et al., 2004a, Fig. 1) was changed daily (~90%) and debris and potential food items were removed with a 30-μm net. The water had adequate time (~1 h minimum) to come to a standard experimental temperature before being placed in the tanks. Natural temperature variations were therefore controlled for. Fish were fed twice a day (am and pm) with cultured *Artemia* sp. and wild zooplankton collected with channel nets or with a 120-μm net during horizontal tows. Quantities of food were measured (500 mL per feeding time) and fish were fed in random order at each feeding time to eliminate any potential bias in food allocation. The water temperature was recorded at each feeding session. No differences were detected in water temperature during the experimental treatment (mean conditions for all years and treatments ±standard error [SE]: am feeding: 26.5°C ±0.09, pm feeding: 28.2°C ±0.09). For all years of the experiment, there were no significant differences in water temperature by tank nested in treatment (ANOVA: am: $F_{1,\ 4}$=0.09, P>0.05; pm: $F_{1,4}$=0.06, P>0.05) or by treatment (ANOVA: am: $F_{1,4}$=2.61, P>0.05; pm: $F_{1,4}$=5.13, P>0.05). Fish were maintained for a total of nine days in each experiment (i.e., nine days per year) after which three fish

from each tank were randomly selected and frozen (–20°C) for microstructural analysis of otoliths.

The SL of the *P. coelestis* used in the experiment (mean=15.2 mm ±0.07 SE) did not differ between treatment groups for any year of the experiment (ANOVA: 2000: $F_{1, 16}$=3.31, P>0.05; 2001: $F_{1, 16}$=1.43, P>0.05; 2002: $F_{1, 16}$=2.4, P>0.05) or among years (ANOVA: $F_{2, 51}$=0.24, P>0.05). The otoliths (the left or right sagittae was chosen randomly), were rinsed three times in Milli-Q water (Millipore, Billerica, MA). and were allowed to dry. Otoliths were polished as described above (see *Wild fish* section. Following elemental analysis of the otoliths by means of laser ablation inductively coupled mass spectrometry (for a complete description of the elemental analysis see Patterson et al., 2004a), the daily increments were counted and measured in the same manner as described earlier. Note that all increments were counted and measured, not just those pertaining to the experiment. Settlement marks, based on the criteria defined by Wilson and McCormick (1999), were also noted.

Crystallography

The orientation of crystals was examined with SEM near the sulcus of sectioned sagittae. The surface of sectioned otoliths was lightly etched (EDTA 0.1 to 0.01M for 3 minutes) to observe the orientation of the crystal-lattice. Although etching altered the edge of crystals, orientation of the long-axis of the crystal-lattice was still easily seen (e.g., Linkowski et al., 1993). The crystal-lattice was observed for four fish from each treatment (ocean vs. lagoon) for the year 2000 experiment. The crystal-lattice of experimental fish was compared with that of 18 control fish. These fish were collected in the same light traps as fish used in the experiments, but were not subjected to any experimental conditions.

Statistical analysis

Data were tested for homogeneity of variances with a Cochran's C-test and were found to be homogeneous. Wild fish otolith increments were analyzed with a nested ANOVA design with the factors habitat (ocean vs. lagoon) and site nested within habitat. However, because site nested within habitat was found to be nonsignificant at the P = 0.25 level, sites were pooled with the residual to increase degrees of freedom and the ANOVA was undertaken again with only habitat as a factor (Underwood, 1997).

To analyze otolith increments corresponding to both the pre-experimental (nine days before the start of the experiment) and the experimental phase (nine days of the experiment), fully nested ANOVAs were used for each year of the experiment. This design used two factors (treatment and tank nested within treatment). Treatment was a fixed factor and tank was a random factor. One-factor ANOVAs and Tukey's HSD *post hoc* tests were used to examine differences among years within treatment groups. Paired t-tests were also used to examine the relationships of increment widths before and during the experiment for each experimental treatment. Based on the findings of Patterson et al. (2004a), correlation analyses were used to examine the relationships between incremental widths and elemental ratios (i.e., Ba/Ca, Sr/Ca).

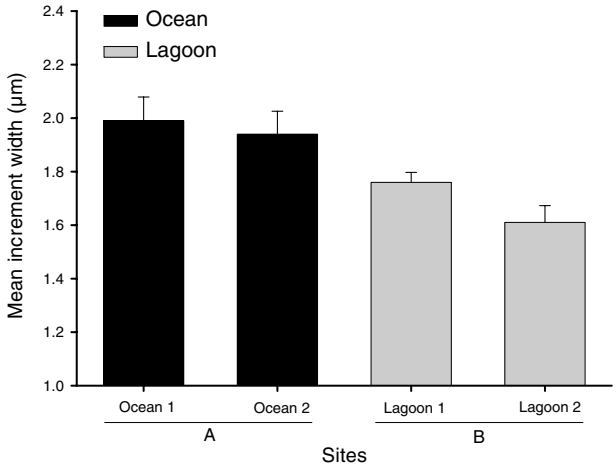

Figure 1

Mean (+1 standard error) increment width (µm) in otoliths of nonexperimental neon damselfish (*Pomacentrus coelestis*) (n=7 per site) collected from two sites on the reef slope in ocean water (Ocean 1 and 2) and within the lagoon (Lagoon 1 and 2) at One Tree Island, Great Barrier Reef, Australia. Tukey's HSD contrast groupings are indicated as letters (i.e., A, B) where overall differences were significant (P<0.05).

Results

Neon damselfish in natural habitats displayed significant variation in increment widths; increments were narrower in otoliths of fish collected in the lagoon (ANOVA: $F_{1, 26}$=14.76, P<0.001; Fig. 1). For experimental fish, which all originated in ocean water, no difference in incremental spacing was found before the start of the experiment by treatment or by tank nested within treatment for any year of the experiment (ANOVA: 2000: $F_{1, 4}$=1.34, P>0.05; 2001: $F_{1, 4}$=0.17, P>0.05; 2002: $F_{1, 4}$= 0.04, P>0.05; Fig. 2). In addition, no difference in Ba/Ca ratios was found before the experiment (Fig. 3; Patterson et al., 2004a). Fish in ocean water in 2000 and 2002 had wider increments than those in the lagoon treatment groups (ANOVA: 2000: $F_{1, 4}$=28.53, P <0.01; 2001: $F_{1, 4}$= 1.62, P>0.05; 2002: $F_{1, 4}$=26.42, P<0.01), as well as a significant difference in Ba/Ca ratios (Figs. 2 and 3; Patterson et al., 2004a). In addition, there was a significant difference in increment widths among tanks within treatments for 2002. A Tukey's HSD test (P<0.05) indicated this was due to a tank of lagoon water where fish

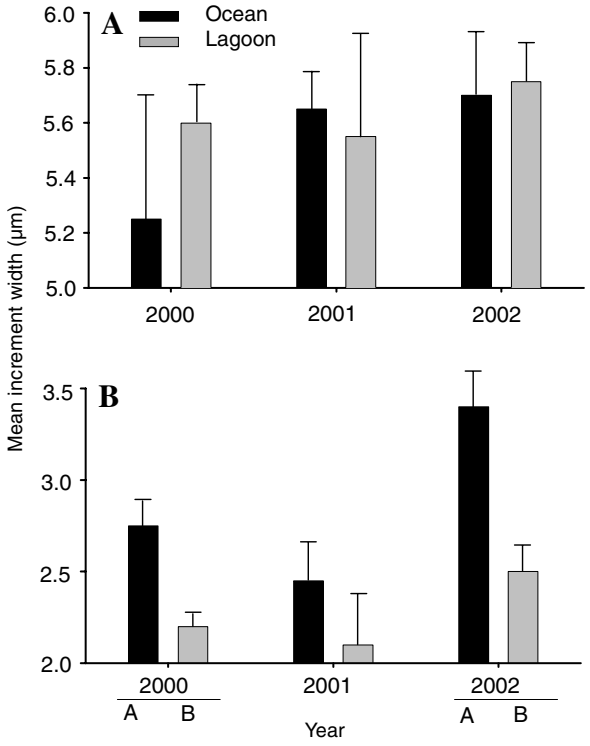

Figure 2

Mean (+1 standard error) increment width (μm) in otoliths of neon damselfish (*Pomacentrus coelestis*) (n=9 per treatment per year) for all years from ocean and lagoon treatments (**A**) before the start of the experiment and (**B**) during the experiment for each year of the experiment. Tukey's HSD contrast groupings are indicated as letters (i.e., A, B) where overall differences were significant (P<0.05). Note the difference of the y-axis scale in A and B.

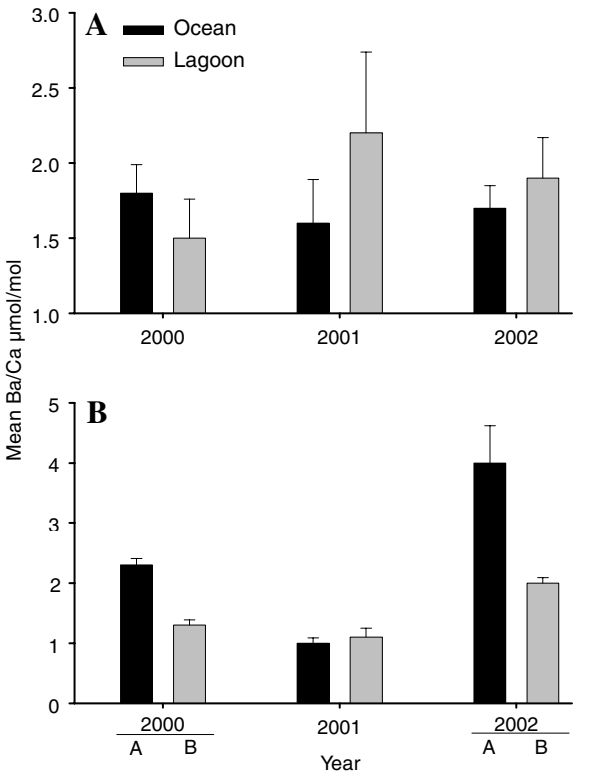

Figure 3

Ba/Ca ratios from otoliths of neon damselfish (*Pomacentrus coelestis)* (n=9 per treatment per year) for all years from ocean and lagoon treatments (**A**) before the start of the experiment and (**B**) during the experiment. Tukey's HSD contrast groupings are indicated as letters (i.e., A, B) where overall differences were significant (P<0.05). Note the difference of the y-axis scale in A and B.

had a higher mean increment width than the fish in the other two tanks (2.8 μm vs. 2.4 μm and 2.4 μm), but this did not obscure major differences between treatments. No significant difference in increment widths between treatment groups was detected for 2001. Finally, a significant difference in increment widths was detected among years for the ocean treatment group, but not for the lagoon treatment group (ANOVA: ocean: $F_{2, 24}$=16.56, P<0.05; lagoon: $F_{2, 24}$=2.28, P>0.05). Fish in the 2002 ocean treatment group had the widest increments of any fish in the experiments. Individual paired t-tests indicated that for both ocean and lagoon treatment groups there was a significant difference in increment width between the pre-experimental and the experimental increments (paired t-test: ocean: T_{242}=90.45, P <0.05; lagoon: T_{242}=322.05, P<0.05).

There were significant relationships between mean increment widths and Ba/Ca ratios for two of the three experimental years (2000: Pearson correlation coefficient, r=0.644, P<0.01; 2001: r=0.052, P>0.05; 2002: r=0.671, P<0.01; Fig. 4). There was no significant correlation between increment widths and Sr/Ca ratios for any of the experimental years (2000: Pearson coefficient, r=–0.417, P>0.05; 2001: r=0.033, P> 0.05; 2002: r=0.266, P>0.05; Fig. 4).

SEM indicated that the aragonite crystal-lattice within experimental otoliths was orientated perpendicularly to daily increments (Fig. 5). This pattern was consistent for ocean and lagoon treatments (angle from daily increments: ocean: 90° ±0°; lagoon: 90° ±0°) and control fish (90° ±0°). Variation in increment width could only be explained by variation in the length of the intra-incremental lattice rather than by a change in orientation or crystal packing.

Discussion

The pattern of wider increments observed for experimental *P. coelestis* held in ocean waters was consistent with

the pattern observed for wild fish. Additionally, these experiments demonstrated that increment widths could be altered in the absence of food and temperature differences. There are a number of factors that can potentially influence the increment widths of otoliths, such as feeding regime (McCormick and Molony, 1992), lipid reserves (Paperno et al., 1997; Molony and Sheaves, 1998), the onset of metamorphosis (Wilson and McCormick, 1999), water temperature (Powell et al., 2004), and metabolic rate (Mosegaard et al., 1988; Wright et al., 2001). Temperature and food, the most well-studied factors, were not relevant here because both were held constant during the experiments. Additionally, metabolic rates were expected to be consistent among individuals, given the similar size, age, and the constant temperature and feeding regimes of these individuals. It is also unlikely that metamorphosis played a role; experimental fish immediately "settled" in the tanks. Settlement marks were noted to form with narrower post-settlement increments, consistent with what is known for this species (Wilson and McCormick, 1999). However, these narrower increments were unlikely to have affected the results as they occurred in all fish in the experimental treatments over all years.

It is possible that the differences noted in increment widths were related to initial size or the condition of the fish during the presettlement phase. Although the standard length of the fish was measured after the study and no differences were found, it was not possible to measure fish before the study (see Methods) and more explicit measures of condition (e.g., Fulton's *K*; Hoey and McCormick, 2004) were not applied before the experiment. Therefore, pre-existing differences in size or condition may have influenced the results to some degree (e.g., some fish may have started the experiment with higher lipid reserves which could have influenced their growth rate and increment widths irrespective of experimental conditions). The randomized allocation of fish between treatments on three separate occasions, however, makes this scenario highly unlikely. In addition, any size or conditional differences were more likely to have introduced variation within the treatment groups rather than the between treatment differences observed in this study.

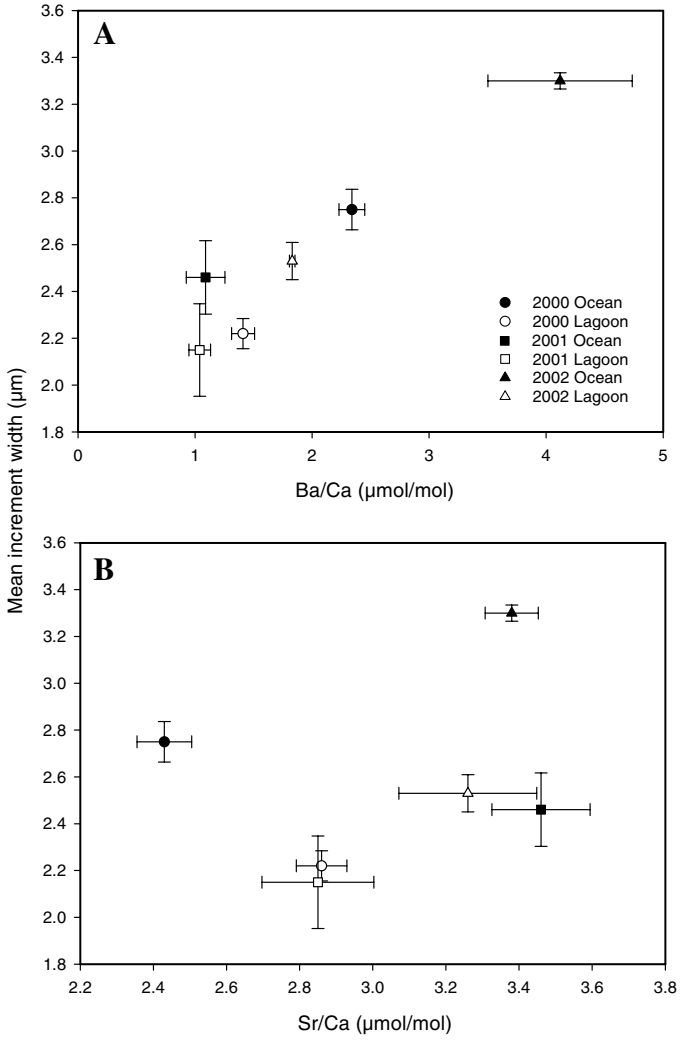

Figure 4

Relationship (mean +1 standard error) between increment width (μm) in otoliths of neon damselfish (*Pomacentrus coelestis*) and elemental ratios for all experimental years for each treatment group (n=9 per treatment per year) for (**A**) Ba/Ca and (**B**) Sr/Ca.

Water chemistry may have influenced increment width in an indirect manner. Late-stage reef fish have well-developed olfactory systems and are capable of detecting chemical differences of ocean and lagoon waters found around OTI (Atema et al., 2002). Some taxa prefer lagoon water (e.g., apogonids), whereas it has been shown that *P. coelestis* actively avoid settling in the lagoon and prefer the coral rubble habitat found on reef slopes (Doherty et al., 1996). Indeed, *P. coelestis* were relatively rare in the lagoon at OTI, and this finding confirms the previous report of Doherty et al. (1996). Although not well-studied in reef fish (but see Arvedlund et al., 1999 and Gerlach et al., 2007), natal imprinting and homing has been documented in fish species, most notably in the olfactory-driven homing of salmonids (Dittman et al., 1996). Because *P. coelestis* are likely spawned and undergo their presettlement life outside of lagoon habitats, they may have imprinted on nonlagoon waters. Lagoon water, therefore, may be an indication of substandard habitat that *P. coelestis* actively avoid. Holding fish in lagoon water may have triggered a physiological response (e.g., stress), resulting in reduced growth and narrower otolith increment widths for fish held in nonlagoon waters (e.g., Marchand et al., 2003). However, this seems unlikely to have been

the primary mechanism driving our results. Although it was not possible to measure fish before each experiment, there was no difference in standard length between treatment groups for any year, indicating that fish in both groups had similar growth rates.

Alternatively, it is possible that lagoon water lacks some physiologically important trace element which limits growth. Again, this seems unlikely to fully account for our results because *P. coelestis* do inhabit and spawn in the lagoon, albeit not in great numbers, as do other pomacentrid species. This would not be expected if lagoonal waters were lacking an element of such physiological importance. Furthermore, Ba is not this limiting element because it has been clearly shown that Ba concentrations in otoliths are indicative of Ba concentrations in water (Bath et al., 2000; Walther and Thorrold, 2006) and that Ba is not influenced by the physiology of the fish (Campana, 1999). Because there was a significant relationship between increment widths and Ba/Ca, it seems likely that another mechanism was involved in producing the results observed.

A related study, in which the otolith chemistry of the same otoliths used here was examined, indicated significant differences in the otolith chemistry of the two treatment groups for 2000 and 2002, primarily driven by differences in Ba/Ca and Sr/Ca (Patterson et al., 2004a). Similar to the results of the present study, no difference in otolith chemistry between the treatment groups was detected for 2001. The differences in otolith chemistry between the treatment groups probably reflected differences in the composition of the water masses (Bath et al., 2000). Oceanographic features that may affect water chemistry (i.e., upwelling, phytoplankton blooms; Patterson et al., 2004a, 2004b) are not temporally stable. This may explain why significant differences in otolith chemistry and microstructure were found only for fish from the 2000 and 2002 experiments. In addition, there was a significant relationship between Ba/Ca ratios and increment widths, and annual variation in increment widths reflected differences for Ba. No significant correlation was found between Sr/Ca ratios and incremental widths, which concurs with previous studies (Kalish, 1989; Gallahar and Kingsford, 1992).

The mechanisms by which trace elements are deposited into otoliths are not entirely understood at this time. Therefore, the exact mechanisms by which otolith chemistry may directly influence increment widths remain unknown. Otoliths are composed primarily of calcium carbonate in the form of aragonite. Divalent metal ions of similar size to Ca, such as those of Ba and Sr, can substitute directly for Ca in the crystal-lattice (Campana, 1999). However, other elements such as Zn and Mn may occupy different positions in the otolith matrix (i.e., in the interstitial spaces or in association with the proteinaceous matrix; Campana, 1999). Although it is known that otolith crystal orientation is protein-mediated (Lowenstam, 1981), it is not known how physiological stress can affect protein deposition, or how trace element inclusion may influence increment width. The orientation of the crystal-lattice did not change in fish treated with ocean and lagoon waters and crystal orientation was similar to other fishes (e.g., Linkowski et al., 1993). Elemental availability in the location of the trace metal inclusions may be capable of creating differences in increment width by increasing the length, or number, of

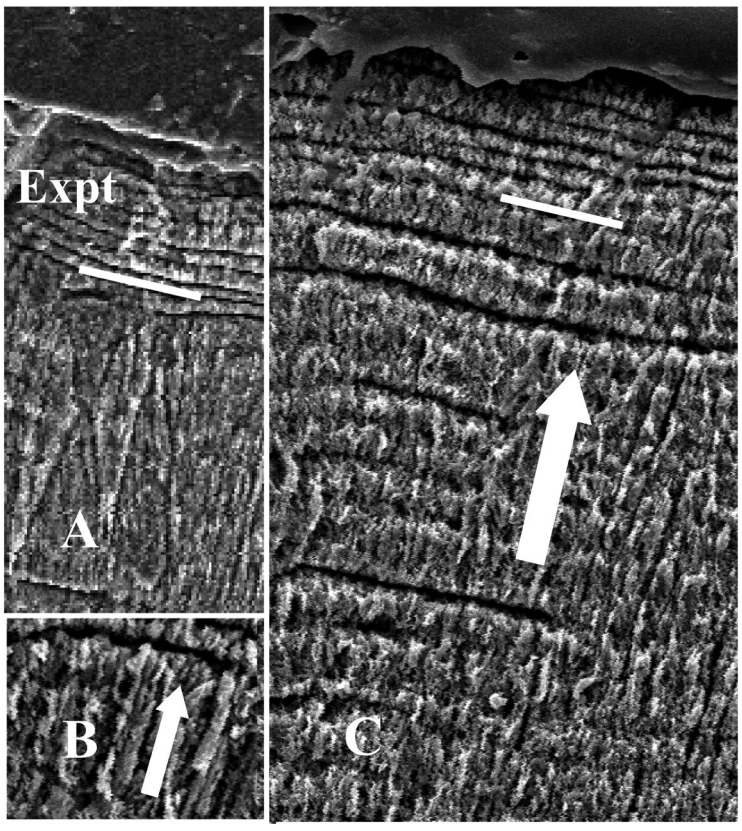

Figure 5

Scanning electron microscope images of the crystal lattice in otoliths of neon damselfish (*Pomacentrus coelestis*). (**A**) Image of an otolith of an experimental (Expt) fish. "Expt" indicates the area (above the white line) where increments were laid down in the otolith of a fish exposed to ocean waters. (**B**) Detail of crystal-lattice from a control fish; (**C**) Detail of the crytalline lattice of a postsettlement control fish; the white line indicates the settlement mark. Large white arrows indicate the orientation of the crystal-lattice. Scale: increments near the edge of the otolith are separated by approximately 1.5 μm.

crystals that are perpendicular to incremental zones, or by altering the way in which crystals are bundled (tightly packed vs. chaotic bundles). The SEM used in this study did not have the resolution to distinguish between these alternatives.

In summary, this study has provided convincing evidence that water chemistry can affect the increment widths of reef fish otoliths by either direct or indirect mechanisms. It is also possible that multiple mechanisms have interacted to produce the results observed. Although the results presented here are preliminary, they indicate that interpreting increment widths may be more complex than has previously been noted and, at least under some circumstances, differences in water chemistry may confound patterns previously attributed only to growth and condition. Further research on this topic is therefore warranted and should include controlled experiments with temporally comparable measurements of water chemistry, otolith chemistry, and increment widths to tease apart the complex mechanisms at work. Lagoons are also found on other reefs (Atema et al., 2002), and further studies using other water masses are needed to determine if the results presented here are prevalent in coral reef fish or are specific to the system at One Tree Island. Finally, continuing research on natal imprinting may elucidate to what extent reef fish imprint on specific water masses, and the physiological consequences of that imprinting.

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