

FEATURE ARTICLE



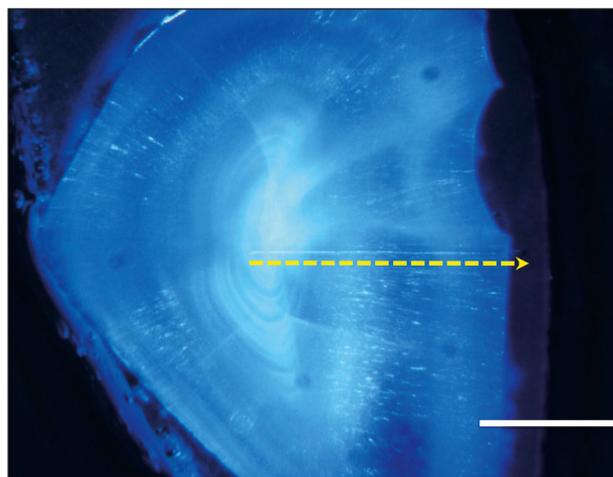
# Influence of constant and periodic experimental hypoxic stress on Atlantic croaker otolith chemistry

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**ABSTRACT:** The chemical composition of fish otoliths may provide information on the environmental exposure histories of fishes if 'vital effects' on element incorporation are minimal. In order to use redox-sensitive geochemical proxies, such as manganese, in otoliths to quantify sublethal exposure to hypoxia, the relative influence of endogenous and exogenous controls on otolith composition must first be validated. Controlled laboratory experiments were conducted on Atlantic croaker *Micropogonias undulatus* to examine the response of otolith Sr:Ca, Ba:Ca, Mg:Ca, Mn:Ca, and Na:Ca ratios to either constant or periodic hypoxia treatments for 4 and 10 wk, respectively. Although fish somatic growth and condition were affected by constant hypoxia, no difference in otolith chemistry relative to normoxic control treatments was detected. Similar to the 4 wk study, there was no difference in otolith chemistry between fish (males and females combined) exposed 10 wk to constant hypoxia and control normoxic fish. Periodic hypoxia significantly decreased otolith Ba:Ca and Mg:Ca in both males and females and reduced Sr:Ca in males, and there was a slight effect of sex on otolith Mn:Ca. Significant interactions between treatment and sex were detected for otolith Sr:Ca and Na:Ca, possibly related to combined stresses of gonadal development and periodic hypoxic stress. Although responses to treatments were observed for some elements, the magnitudes of responses were minimal compared to exogenous variation driven by water chemistry composition reported in previous laboratory and field investigations. The otolith chemistry of Atlantic croaker is therefore minimally influenced by endogenous factors in response to hypoxic stress, which has important implications for interpreting otolith chemical chronologies of wild fish collected within natural hypoxic regions.

**KEY WORDS:** Hypoxia · Otolith chemistry · *Micropogonias undulatus* · Manganese · Trace elements · Physiological stress



Sectioned otolith of Atlantic croaker *Micropogonias undulatus*, showing laser transect (faint line above the yellow dashed line) from core (early life) to edge (experimental period). Scale bar = 850  $\mu\text{m}$

Photo: J. Mohan

## INTRODUCTION

Hypoxia (dissolved oxygen  $<2 \text{ mg O}_2 \text{ l}^{-1}$ ) is increasing in frequency and severity in coastal ecosystems worldwide due to human activities (Diaz & Rosenberg 2008). Fish exposed to hypoxia have exhibited negative sublethal physiological effects including reduced growth (McNatt & Rice 2004), shifts in habitat (Craig 2012), and reproductive disorder (Thomas & Rahman 2012), yet less is known about natural exposure levels that elicit damaging organismal and ecological responses, especially for mobile species capable of sensing, tolerating, and avoiding hypoxia (Froeschke & Stunz 2011). A validated chronological geochemical proxy indicating hypoxia exposure in fishes is needed to assess the long-term sublethal effects of hypoxic exposure on aquatic communities.

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Fish respond to an oxygen challenge by first attempting to extract more oxygen from the water using strategies of increasing gill ventilation rates, red blood cell counts, and hemoglobin O<sub>2</sub>-binding capacity (Wu 2002). If the oxygen challenge persists, they will depress metabolism and 'turn down the pilot light' (Hochachka et al. 1996) and initiate energy conserving pathways by downregulating protein synthesis and modifying enzymes to decrease ATP use (Wu 2002, Bickler & Buck 2007). Many hypoxia-responsive pathways are turned on via the 'master switch', the hypoxia-inducible factor HIF- $\alpha$ , a heterodimeric transcription factor (Nikinmaa & Rees 2005, Rahman & Thomas 2007). HIF- $\alpha$  is expressed in a wide range of fish tissues and codes for genes related to red blood cell production, vascularization, apoptosis, and carbohydrate metabolism. Under normoxic conditions, the HIF protein is degraded, but the protein accumulates under hypoxic exposure. Short-term biomarkers, such as HIF- $\alpha$ , provide reliable molecular indicators of exposure to hypoxia in several fish (Thomas & Rahman 2009) and crustacean (Kodama et al. 2012) species on time scales of hours to days. However, HIF- $\alpha$  mRNA expression returns to basal levels within 24 h after recovery from hypoxia, offering no information on lifetime patterns of exposure and the potential for long-term sublethal effects (Rahman & Thomas 2007, Kodama et al. 2012).

Fish otoliths are calcium carbonate structures that deposit permanent daily growth bands offering detailed information on age and growth. Otoliths are metabolically inert and not subject to resorption, even during periods of stress (Campana 1983) or starvation (Maillet & Checkley 1990). Although much otolith chemistry research has focused on chemical identifiers of migratory movements and stock discrimination (Walther & Limburg 2012), there is significant potential for geochemical indicators of hypoxia exposure to be recorded in otoliths. In particular, otolith manganese (Mn) is a viable geochemical hypoxia proxy (Limburg et al. 2011). Hypoxia alters redox conditions such that Mn oxides are reduced; the reduced forms (primarily Mn<sup>2+</sup> but also Mn<sup>3+</sup>) are soluble, and under suboxic/hypoxic conditions, dissolved Mn can be released into the water column (Thamdrup et al. 1994, Slomp et al. 1997) and remain dissolved for several days (Pakhomova et al. 2007). If elevated dissolved Mn leads to increased incorporation of Mn in otoliths, a direct link between residence in hypoxic waters and otolith composition could be employed. However, in order to effectively interpret potential hypoxia proxies such as Mn/Ca, it is important to validate whether hypoxic stress alone, in the absence of ambient chemical differences, could alter otolith chemistry.

During otolith accretion, aragonite is crystallized onto an organic protein matrix, and dissolved elements from the ambient environment that have passed through several biological barriers (i.e. gill, intestine, blood, and endolymph interfaces) are incorporated into the crystal structure (Campana 1999). While the concentration of some elements may directly reflect water concentrations, there is significant opportunity for physiological regulation of uptake and transport of certain ions at each barrier. Sturrock et al. (2012) employed the hard and soft acid and base theory to describe the behavior of certain metals in seawater and in the blood and endolymph of marine fish. Hard acid cations, such as Mg, Sr, Ca, and Ba, exist as free hydrated ions in seawater and in the blood of the fish, so they are more likely to passively diffuse across blood–endolymph membranes, and are more readily accepted into the crystal lattice by substituting for Ca during otolith precipitation. Soft acid cations such as Cu, Pb, and Zn are strongly bound to organic ligands in seawater and strongly bind proteins in the blood and thus require more active transport across biological membranes and are susceptible to being rerouted to the liver or actively excreted. The soft acid cations are more likely incorporated with the organic protein matrix of the otolith, or in interstitial spaces of crystal defects. The behavior of intermediate cations, such as Mn, may depend on the environmental or physiological context (Sturrock et al. 2012). The potential influences on blood–endolymph chemistry variation include both environmental (salinity, temperature, dissolved oxygen) and physiological (ontogeny, sex, growth rate) factors (Walther et al. 2010). Understanding whether endogenous (physiological) or exogenous (environmental) processes dominate otolith elemental incorporation is essential for effective interpretations of biogenic proxies.

Here, we examined the elemental chemistry of Atlantic croaker *Micropogonias undulatus* otoliths in response to hypoxia exposure using both short-term (4 wk) and long-term (10 wk) hypoxia exposure laboratory experiments. This species was chosen because of its extensive use as a model for identifying individual (Thomas & Rahman 2009, 2012) and population-level effects (Craig & Crowder 2005, Creekmore 2011) of hypoxia exposure. We hypothesized that hypoxic stress will affect element incorporation into the otolith, potentially driven by physiological alteration of blood chemistry through changes in protein and ion content. Experiments were performed in seawater with unmanipulated chemical composition to test the degree of endogenous alteration of otolith chemistry after exposure to hypoxic stress. In the first

experiment, fish were exposed for 4 wk to constant hypoxia or normoxia (controls), and otoliths were analyzed along the sulcal axis by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). For the second experiment, fish were exposed for a longer duration of 10 wk to normoxia, constant hypoxia, or periodic alternating hypoxia/normoxia, and otoliths were analyzed along the ventral axis. Investigating the influence of internal physiological effects on otolith chemistry is essential to discern exogenous environmental signals from endogenous chemical signals contained in otoliths.

## MATERIALS AND METHODS

### Laboratory hypoxia experiments

#### Experimental fish

Atlantic croaker (100–110 mm total length, TL; 25–35 g wet mass, M), were caught by shrimp trawl in the Texas (USA) gulf coast by local fisherman and transported to fish holding facilities at the University of Texas Marine Science Institute. Fish were treated with Paracide-F at 170 ppm in seawater for 1 h to minimize parasite infections and transferred to large indoor recirculating seawater tanks (4727 l) under ambient temperature (22–23°C) and photoperiod (13 h dark:11 h light) conditions for at least 1 mo prior to experimentation. Fish were fed chopped shrimp once a day (3% M d<sup>-1</sup>). At the end of the experiments, fish were sacrificed following guidelines approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

#### 4 wk constant hypoxia experiment

Details of the experimental setup were described by Rahman & Thomas (2012). Briefly, in the hypoxia exposure tanks, the flow of air was reduced through the air flow meter gradually from 100–80% to 60, 40, and 20%, and finally adjusted until the dissolved oxygen (DO) level reached 1.7 mg l<sup>-1</sup>, which was achieved within 2 d. A

YSI multiprobe was used to monitor DO, pH, temperature and salinity 3 times daily (Table 1, and see Fig. S1 in the Supplement at [www.int-res.com/articles/suppl/b020p001\\_supp.pdf](http://www.int-res.com/articles/suppl/b020p001_supp.pdf)). Sodium bicarbonate buffer was added as needed to adjust and maintain pH between 7.7 and 7.9 during the experiment. At the end of the study, fish TL in mm and M in g were measured, and the heads of the experiment subjects containing the otoliths were separated by treatment and frozen until analysis. TL and M data were used to calculate Fulton's condition index,  $K = (M/TL^3) \times 100$ .

#### 10 wk constant and periodic hypoxia experiment

Identical experimental methods as described above were used for the 10 wk hypoxia study; however, an additional treatment of periodic hypoxia exposure was produced by adjusting air flow to the tanks at weekly intervals to expose fish to hypoxia and normoxic conditions every other week. Salinity, temperature, DO concentration, oxygen saturation, and pH were recorded daily for each of 3 replicate tanks for each treatment (Fig. S1). At the end of the experiment, fish were measured for TL. Fish sex was determined by examining the gonads, and sagittal otoliths were immediately removed, cleaned with ultrapure water, and stored in 1.5 ml polyethylene vials to air dry.

Table 1. Ambient physicochemical parameters of the experimental tanks from the 4 wk experiment (mean ± SD). Dissolved elemental chemistry data and ambient physicochemical parameters for both control and hypoxia tanks sampled at 2 and 4 wk in the 10 wk experiment. Time series for experimental tank conditions monitored daily can be found in Fig. S1 in the Supplement. DO: dissolved oxygen

Experiment	Physicochemical parameter	Experimental tank/sampling time			
		Control		Hypoxia	
4 wk	Salinity (ppt)	32.50 ± 0.04		32.55 ± 0.07	
	Temperature (°C)	21.87 ± 0.12		21.85 ± 0.07	
	DO (%)	99.84 ± 1.17		26.56 ± 0.81	
	DO (mg l <sup>-1</sup> )	7.27 ± 0.12		1.80 ± 0.06	
	pH	7.69 ± 0.02		7.55 ± 0.01	
10 wk	Sr:Ca (mmol mol <sup>-1</sup> )	2 wk Control		4 wk Control	
		Hypoxia		Hypoxia	
	Ba:Ca (μmol mol <sup>-1</sup> )	8.21	8.21	8.04	8.07
	Mn:Ca (μmol mol <sup>-1</sup> )	12.52	13.16	27.9	28.89
	Mg:Ca (mol mol <sup>-1</sup> )	6.8	13.4	7.02	9.53
	Na:Ca (mmol mol <sup>-1</sup> )	5.12	5.14	5	5.06
	Salinity (ppt)	45.07	45.19	43.88	44.63
	Temperature (°C)	39.04	39.08	34.49	34.79
	DO (%)	22.3	22.3	22.1	22
	DO (mg l <sup>-1</sup> )	76	23.3	82.4	26.4
	pH	5.34	1.64	5.97	1.85
		7.35	7.44	7.53	7.45

## Otolith preparation and chemical analysis

### 4 wk exposure study

Otoliths were weighed to the nearest 0.001 g, and no difference in mass was detected between left and right otoliths (paired *t*-test, *df* = 57, *t* = 0.097, *p* = 0.9225). Therefore, either the left or right otolith was randomly chosen for analysis. Otoliths (*N* = 46) were embedded in Epoxi-Cure resin, sectioned in the transverse plane using a low-speed diamond blade saw, mounted on petrographic slides with Crystal Bond thermoplastic cement, and polished with lapping film (30 and 3  $\mu\text{m}$ ) to expose the core. Polished sections were then transferred to new petrographic slides (6 to 7 sections per slide) and sonicated in ultrapure water (18.2 m $\Omega$ ) 3 times for 5 min and dried in a class 100 laminar flow hood before analysis.

### 10 wk exposure study

Indium chloride (30 ppm) was added to the Epoxi-Cure resin and Crystal Bond embedding/mounting media to serve as an elemental indicator during the laser analysis, thus allowing precise determination of the otolith edge/resin interface in the data set. Left otoliths (*N* = 72) were weighed, embedded, sectioned, polished, cleaned, and air-dried as described above.

LA-ICP-MS analyses were conducted at the University of Texas Jackson School of Geosciences, Austin, Texas. An Aligent 7500ce ICP-Q-MS coupled to a New Wave UP 193-FX laser with a 26  $\mu\text{m}$  laser spot diameter, 30% power (mean  $\pm$  SD: 4.37  $\pm$  0.16 J  $\text{cm}^{-2}$ ), and 5  $\mu\text{m s}^{-1}$  scan speed was used to quantify  $^{43}\text{Ca}$ ,  $^{88}\text{Sr}$ ,  $^{55}\text{Mn}$ ,  $^{24}\text{Mg}$ ,  $^{23}\text{Na}$ , and  $^{137}\text{Ba}$  from transects ablated across otoliths following an initial pre-ablation (35  $\mu\text{m}$  spot diameter, 10% power, 25  $\mu\text{m s}^{-1}$  scan speed) to remove potential surface contamination. MACS-3 calcium carbonate and NIST 612 glass certified standards were run at the beginning and after every 60 min of analysis to convert raw intensity counts to molar concentrations (using MACS-3), correct for instrument drift, and assess analytical precision (with NIST 612). Elemental intensity count data were converted to concentrations using the Trace Elements IS Data Reduction Scheme in the software Iolite that uses calcium (as 37.69 wt% in aragonite) as an internal standard (Paton et al. 2011) and then converted to molar ratios. Relative standard deviations (RSDs) based on repeated measurements of the NIST 612 standard for the 4 wk data collected October

2010 over 2 d were: Sr = 3.0%, Ba = 5.4%, Mn = 7.3%, Mg = 7.5%, and Na = 12.9%; while the RSDs for the 10 wk experimental fish analyzed in December 2012 over 4 d were: Sr = 10.2%, Ba = 11%, Mn = 19.6%, Mg = 10.7%, and Na = 15.7%.

For the 4 wk study otoliths, laser transects were initiated at the otolith core and run parallel to the sulcal groove to the otolith edge, obtaining a lifetime elemental profile for each otolith. For the 10 wk study, the laser was run from the core to the edge along the ventral otolith axis, which displays wider growth increments than the sulcal axis (Fig. S2a in the Supplement). Elemental concentrations were only compared within a given experiment using similar laser transect paths (treatments versus controls), given the potential for different concentrations obtained from different ablation axes. To estimate otolith precipitation rates, a subset of fish (*N* = 4) were bathed 24 h in calcium-staining alizarin red solution (400 mg  $\text{l}^{-1}$ ) and held for 4 or 10 wk in the same size tank and fed the same food as experimental fish in normoxic conditions. At the end of the holding period, the fish were sacrificed and otoliths were embedded, sectioned, and polished as described above. The stained otolith sections were then viewed with fluorescent light to capture digital images to measure the distance between stained growth increment and otolith edge using ImageJ software to estimate otolith precipitation rates ( $\mu\text{m d}^{-1}$ ) along the sulcal and ventral growth axes (Fig. S2b,c, Table S1 in the Supplement). Mean otolith precipitation rates for the fish held for 4 wk and analyzed along the sulcal groove was 1.17  $\mu\text{m d}^{-1}$ , while those otoliths from the 10 wk study lasered along the ventral axis displayed mean precipitation rates of 1.35  $\mu\text{m d}^{-1}$  (Fig. S2d). All fish tagged in the alizarin study lost between 6 and 14% body weight due to poor feeding, and thus our estimates of otolith precipitation are conservative estimates to ensure that no pre-experimental aragonite was included in treatment comparisons (Table S1). The otolith chemistry data that were precipitated during the experiment were extracted from each fish otolith time series and averaged together for analysis.

## Collection and analysis of water samples

During the 10 wk experiment, water samples were collected from experimental tanks at 2 and 4 wk in order to assess variation in water chemistry. Samples were pumped from the bottom of a control and hypoxic tank using acid-washed Teflon tubing and a peristaltic pump (Masterflex). The sample water was

filtered with 0.45 and 0.2  $\mu\text{m}$  polytetrafluoroethylene filters in sequence with acid-washed 30 ml syringes to remove any particulate matter and retain dissolved fractions and immediately acidified to  $\text{pH} < 2$  with trace metal grade nitric acid into acid-washed 30 ml low-density polyethylene Nalgene bottles to be stored at  $4^\circ\text{C}$  until analysis. Water samples were analyzed for trace elements using solution-based ICP-MS at the University of Texas Jackson School of Geosciences. Samples were diluted 100 $\times$  with 2% trace metal grade  $\text{HNO}_3$ , and a subset of samples was spiked with matrix-matched solutions to assess recoveries. Replicated blanks and certified reference standards were used to convert isotope counts to concentrations and then converted to element:calcium ratios. Mean spike recoveries ( $N = 6$  spiked samples) were, means  $\pm$  SD: Sr =  $95.9 \pm 0.4\%$ , Ba =  $95.4 \pm 0.4$ , Ca =  $97.0 \pm 1.7$  Mn =  $93.8 \pm 0.3$ , Mg =  $87.9 \pm 8.7$ , Na =  $67.9 \pm 23.7$ .

### Data analysis

Student's  $t$ -tests were used to compare condition factor ( $K$ ) and otolith mass between control and constant hypoxia-exposed fish from the 4 and 10 wk experiments. Elemental otolith data were examined for normality and homoscedasticity, and a natural log transformation was performed for those elements that did not meet assumptions (Ba:Ca, Mg:Ca, and Mn:Ca). We found a significant difference in otolith mass between control and hypoxia-exposed fish in the 4 wk exposure study ( $t$ -test,  $t = 2.596$ ,  $\text{df} = 56$ ,  $p = 0.012$ ); therefore, a 1-way ANCOVA was used to compare elemental ratios between control and hypoxia-exposed fish, with otolith mass as a covariate.

Similarly, for the 10 wk exposure study, mean experimental otolith elemental ratios (Ba:Ca, Mn:Ca) were log-transformed to meet assumptions of normality and equal variance. Initially, a generalized linear model was performed to test the effect of Treatment, Sex, Treatment  $\times$  Sex interaction, with Tank nested within Treatment  $\times$  Sex. There was no significant effect of tank on otolith elemental ratios, so the 3 replicate tanks per treatment were pooled together to run a 2-way ANOVA to examine the effect of Treatment, Sex, and Treatment  $\times$  Sex on otolith elemental ratios and otolith mass. When a significant factor effect was detected with the 2-way ANOVA, Holm-Sidak multiple comparison methods were used to determine which treatment or sex was different by pooling data within a treatment (for

Ba:Ca and Mg:Ca) or sex (for Mn:Ca). All statistical analyses were performed using SYSTAT 12.

## RESULTS

### 4 wk exposure

The condition factor ( $K$ ) and otolith mass of hypoxia-exposed fish were significantly reduced compared to the control ( $K$ :  $t = 4.207$ ,  $\text{df} = 61$ ,  $p < 0.001$ ; otolith mass:  $t = 2.596$ ,  $\text{df} = 56$ ,  $p = 0.012$ ; Fig. 1). However, no significant difference in otolith Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca, or Na:Ca ratios were detected between control and hypoxia-exposed fish when accounting for otolith mass variation using ANCOVA (Fig. 1, Table 2).

### 10 wk exposure

The dissolved elemental chemistry of the experimental tank water displayed limited variability for all elements except Ba:Ca and Mn:Ca ratios. Ba:Ca molar ratios increased almost 2-fold from Week 2 to Week 4 in both control and hypoxia treatment tanks, which was accompanied by a decrease in salinity of approximately 5 ppt (Table 1). The Mn:Ca ratio was elevated in the hypoxic tanks, which had 50% less dissolved  $\text{O}_2$  than the control tanks by  $6.6 \mu\text{mol mol}^{-1}$  at 2 wk and  $2.5 \mu\text{mol mol}^{-1}$  at 4 wk (Table 1). In contrast to the 4 wk study, otolith mass was not different between control or hypoxia-exposed fish (Table 3).  $K$  of fish exposed to constant hypoxia was significantly decreased compared to controls ( $t = 3.234$ ,  $\text{df} = 45$ ,  $p = 0.0023$ ; Fig. 1). Similar to the results of the 4 wk exposure study, there were no significant differences in otolith Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca, or Na:Ca ratios between the control and fish exposed to constant hypoxia for 10 wk (Fig. 1). Only minor treatment and sex differences in the molar ratios of elements were observed in the periodic hypoxia treatment, varying less than 20%, whereas molar ratios of Sr:Ca, Ba:Ca, Mg:Ca, and Zn:Ca in Atlantic croaker otoliths have been found to vary 60 to 90% in environmental samples collected from Chesapeake Bay and Pamlico Sound, North Carolina, USA (Thorrold et al. 1997, Thorrold & Shuttleworth 2000). Interestingly, a significant treatment effect was detected for otolith Ba:Ca and Mg:Ca (Table 4), with post hoc tests revealing that Ba:Ca and Mg:Ca ratios were significantly reduced in the periodic treatment when males and females were pooled (Fig. 2). A

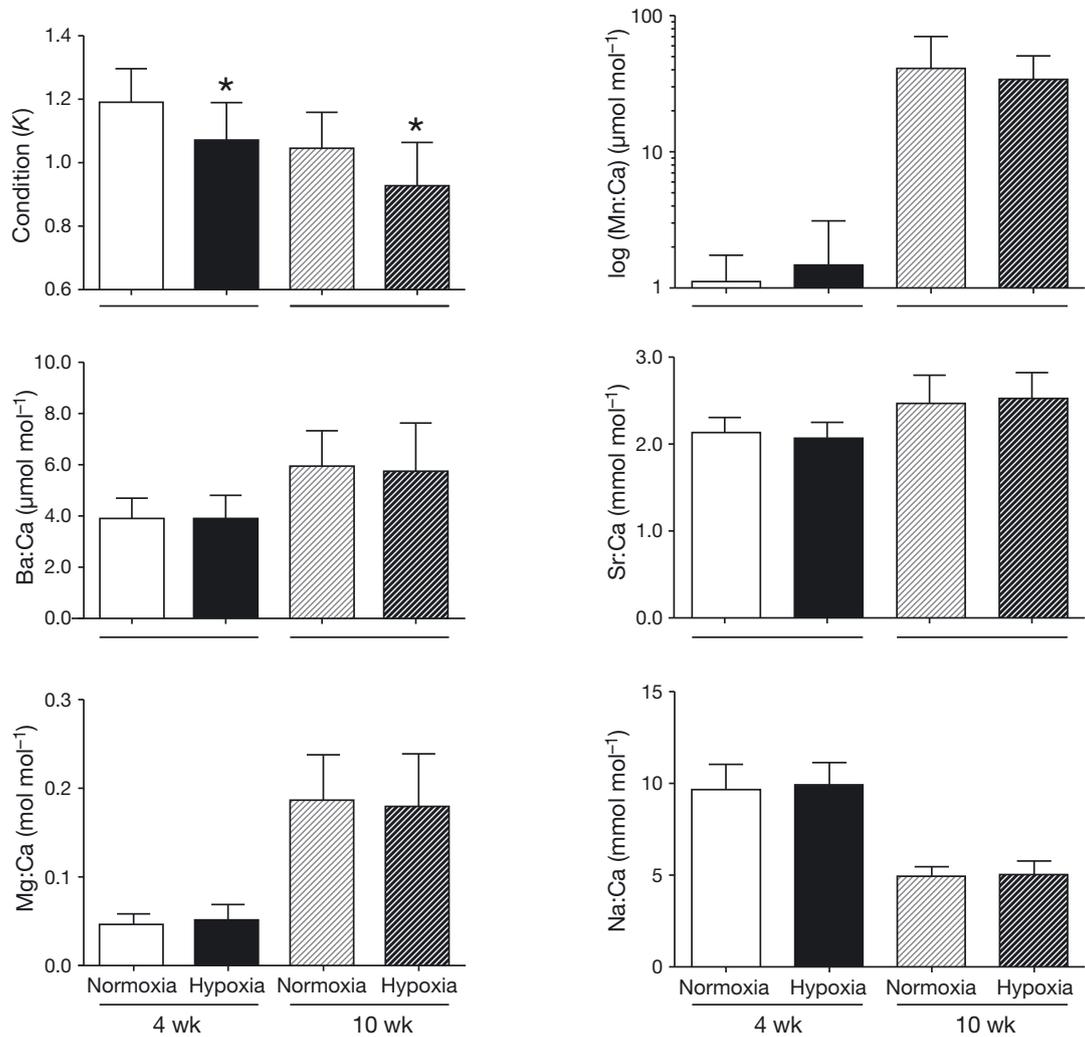


Fig. 1. *Micropogonias undulatus*. Fulton's condition index ( $K$ ; see 'Materials and methods' for calculation) and otolith element:Ca ratios of Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca, and Na:Ca for Atlantic croaker exposed for either 4 or 10 wk to normoxia (control) or constant hypoxia. Error bars indicate standard deviation and \* indicates significant difference ( $p < 0.05$ ) by Student's  $t$ -test. Note that differences in elemental concentrations between the 4 and 10 wk experiments were due to ablations on different otolith growth axes

significant interaction between treatment and sex was detected for both otolith Sr:Ca and Na:Ca ratios (Table 4). For otolith Sr:Ca, females had decreased ratios in the control treatment, and males had decreased ratios in the periodic hypoxia treatment, but we found no sex differences in the constant hypoxia treatment (Fig. 2). Otolith Na:Ca was significantly lower in males in the periodic hypoxia treatment, but there were no sex differences in the control or constant hypoxia treatments (Fig. 2). Sex had a significant effect on otolith Mn:Ca when all treatments were pooled (Table 4), with females having higher ratios than males (Fig. 2).

Table 2. One-way ANCOVA results with otolith mass as covariate for Atlantic croaker exposed 4 wk to control or hypoxic conditions

Variable	Effect	Type III SS	df	MS	$F$	$p$
Sr:Ca	Treatment	0.054	1	0.054	1.659	0.205
	Otolith mass	0.006	1	0.006	0.176	0.677
ln(Ba:Ca)	Treatment	0.000	1	0.000	0.049	0.827
	Otolith mass	0.003	1	0.003	0.299	0.588
ln(Mn:Ca)	Treatment	0.020	1	0.020	0.345	0.560
	Otolith mass	0.073	1	0.073	1.256	0.269
ln(Mg:Ca)	Treatment	0.012	1	0.012	0.794	0.378
	Otolith mass	0.000	1	0.000	0.011	0.918
Na:Ca	Treatment	0.901	1	0.901	0.534	0.469
	Otolith mass	0.160	1	0.160	0.095	0.759

Table 3. *Micropogonias undulatus*. Sample size (N), total length (TL), and otolith mass of Atlantic croakers exposed 4 or 10 wk to control, constant hypoxia, or periodic hypoxia experimental treatments (mean  $\pm$  SD). \*Significant difference ( $p < 0.05$ ) by Student's *t*-test. –: not applicable

Treatment	4 wk			10 wk		
	N	TL (cm)	Otolith mass (mg)	N	TL (cm)	Otolith mass (mg)
Control	28	15.18 $\pm$ 0.58	115.89 $\pm$ 9.25	24	15.69 $\pm$ 0.54	125.92 $\pm$ 10.45
Constant hypoxia	30	15.00 $\pm$ 0.56	109.88 $\pm$ 8.38*	23	15.50 $\pm$ 0.60	124.26 $\pm$ 10.26
Periodic hypoxia	–	–	–	24	15.796 $\pm$ 0.59	126.42 $\pm$ 9.151

Table 4. Two-way ANOVA investigating effects on Atlantic croaker otolith mass and elemental ratios, of Treatment (control, constant hypoxia, periodic hypoxia), Sex (male, female), and the interaction of Treatment  $\times$  Sex in the 10 wk hypoxia study. Significant effects ( $p < 0.05$ ) are shown in **bold**

Variable	Effect	df	MS	F	p
Otolith mass	Treatment	2	32.871	0.323	0.725
	Sex	1	125.343	1.232	0.271
	Treatment $\times$ Sex	2	6.123	0.0602	0.942
ln(Mn:Ca)	Treatment	2	0.0369	0.628	0.537
	Sex	1	0.242	4.117	<b>0.047</b>
	Treatment $\times$ Sex	2	0.0247	0.42	0.659
Mg:Ca	Treatment	2	0.0131	4.334	<b>0.017</b>
	Sex	1	0.00311	1.032	0.313
	Treatment $\times$ Sex	2	0.00104	0.346	0.709
Sr:Ca	Treatment	2	0.0564	0.729	0.487
	Sex	1	0.00167	0.0215	0.884
	Treatment $\times$ Sex	2	0.451	5.822	<b>0.005</b>
ln(Ba:Ca)	Treatment	2	0.0823	5.717	<b>0.005</b>
	Sex	1	0.00655	0.455	0.503
	Treatment $\times$ Sex	2	0.0101	0.702	0.499
Na:Ca	Treatment	2	0.0357	0.105	0.9
	Sex	1	0.312	0.919	0.341
	Treatment $\times$ Sex	2	1.228	3.621	<b>0.032</b>

## DISCUSSION

We have demonstrated with 2 independent controlled laboratory experiments that physiological stress associated with short- and long-term constant sublethal hypoxic exposure has minimal influence on the otolith chemistry of the hypoxia-tolerant teleost Atlantic croaker. In the first experiment, after 4 wk of constant hypoxia exposure, fish condition was significantly reduced, indicating that somatic growth was impaired; however, elemental ratios did not differ between control and hypoxia-exposed fish. Similarly, after 10 wk of constant hypoxia exposure, the otolith chemistries of both male and female croakers were not different from controls, although condition factors of hypoxic fish

were decreased, which lends further support to our initial finding. The variation in otolith Sr:Ca, Ba:Ca, Mg:Ca, and Na:Ca ratios that was detected in response to the periodic hypoxia treatment was small compared to variation seen in field studies. In our study, the variation in otolith element ratios between control and periodic hypoxia treatments was only 2  $\mu\text{mol mol}^{-1}$  for Ba:Ca, 0.25  $\text{mmol mol}^{-1}$  for Sr:Ca, 20  $\mu\text{mol mol}^{-1}$  for Mn:Ca, and 0.5  $\text{mmol mol}^{-1}$  for Na:Ca. In contrast, Thorrold & Shuttleworth (2000) reported 2 to 10 times the variation found in our study of 4  $\mu\text{mol mol}^{-1}$  for Ba:Ca, 2.5  $\text{mmol mol}^{-1}$  for Sr:Ca, and 40  $\mu\text{mol mol}^{-1}$  for Mn:Ca in otoliths of Atlantic croaker collected from northeastern estuaries. These data suggest that environmental factors dominate element incorporation with minimal influence from physiological stress.

### Redox-sensitive otolith proxies

The most promising geochemical proxy for hypoxia, Mn:Ca ratio, did not show statistically significant variation by treatment. The experimental tank water from the constant hypoxic tanks did contain higher dissolved Mn:Ca, perhaps due to lower oxygen saturation allowing  $\text{Mn}^{2+}$  to remain dissolved, but these differences were relatively small compared to field observations of dissolved Mn:Ca, which can vary over orders of magnitude in both space and time (Slowey & Hood 1971, Statham et al. 2005). Only 2 controlled laboratory studies have directly examined otolith composition following addition of dissolved Mn to water, and neither reported positive relationships (Elsdon & Gillanders 2003, Miller 2009). In contrast, several field studies have reported positive correlations between ambient dissolved Mn:Ca and otolith Mn:Ca (Forrester 2005, Dorval et al. 2007, Mohan et al. 2012). Additional

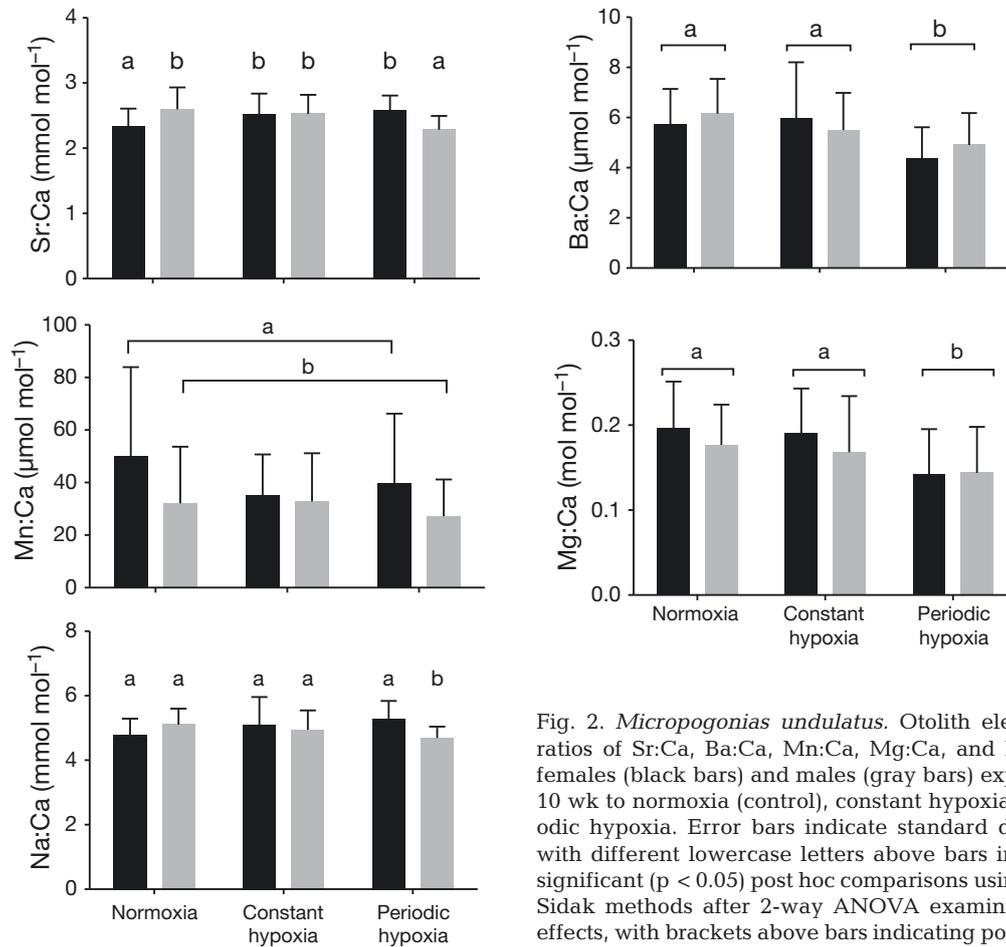


Fig. 2. *Micropogonias undulatus*. Otolith element:Ca ratios of Sr:Ca, Ba:Ca, Mn:Ca, Mg:Ca, and Na:Ca of females (black bars) and males (gray bars) exposed for 10 wk to normoxia (control), constant hypoxia, or periodic hypoxia. Error bars indicate standard deviation, with different lowercase letters above bars indicating significant ( $p < 0.05$ ) post hoc comparisons using Holm-Sidak methods after 2-way ANOVA examining main effects, with brackets above bars indicating pooled data

field studies have attributed elevated otolith Mn to hypoxic redox conditions in Pamlico Sound (Thorrold & Shuttleworth 2000), Chesapeake Bay (Dorval et al. 2007), and the Baltic Sea (Limburg et al. 2011). The mechanism of Mn incorporation into otoliths is unclear, but substitution with  $\text{Ca}^{2+}$  ions in the aragonite crystal lattice has been suggested, as Mn has not been detected bound to proteins in cod otoliths (Miller et al. 2006). As discussed by Sturrock et al. (2012), the chemical behavior of  $\text{Mn}^{2+}$  is intermediate between hard acids/bases and soft acids/bases, meaning it can exist in the plasma and endolymph in both free and bound forms. We did detect a slight statistically significant effect of sex ( $p = 0.047$ ) on otolith Mn:Ca, with higher ratios in females, but both males and females in the constant hypoxia treatment were similar. Unlike the other elements we investigated that tend to vary with salinity, dissolved Mn:Ca will flux out of sediments in dissolved form during low-oxygen events, thus the non-effect of hypoxia on otolith Mn:Ca observed in this experiment is perhaps due to lack of sedimentary supply of  $\text{Mn}^{2+}$ . The small variation in dissolved Mn:Ca between control and

hypoxic experimental tank water was not enough to induce changes in otolith chemistry, as neither was the physiological effect of hypoxic stress. These results, along with other field studies that have measured ambient dissolved Mn (Dorval et al. 2007, Limburg et al. 2011, Mohan et al. 2012), support the hypothesis that variation in otolith Mn:Ca is strongly driven by exogenous, environmental dissolved ambient  $\text{Mn}^{2+}$  variability that may be linked to sediment redox biogeochemistry.

#### Effects of sex and stress on otolith chemistry

Many studies have documented intrinsic vital effects on otolith chemistry, but few have examined differences between male and female fish. Kalish (1991) examined seasonal variation in the chemistry of the blood plasma, endolymph, and otoliths of male and female bearded rock cod, and also investigated changes in the protein content of the blood. Seasonal variation in the trace element chemistry of the endolymph was related to changes in the protein content

of the blood plasma in response to gonad development. It was hypothesized that as fish invest energy into reproduction, more calcium-binding proteins, such as the egg-yolk precursor vitellogenin, will be present in the blood and endolymph, which reduces the amount of free Ca available for otolith accretion resulting in increases in other trace metal impurities such as Sr (Kalish 1991). Earlier studies related otolith Sr:Ca to changes in temperature (Radtke 1989) or somatic growth rate (Sadovy & Severin 1992) without considering the effect of gonad development that is initiated by temperature changes and results in reduced somatic growth (Kalish 1991). The significant interaction between sex and treatment that we detected for Sr:Ca and Na:Ca ratios may have been the product of differential gonad investment in addition to the stress of the periodic hypoxia treatment.

Few other studies have examined the effects of stress on otolith chemistry (Kalish 1992) or blood and endolymph chemistry (Payan et al. 2004a). Thermal and osmotic stress resulted in low condition factors, a decrease in otolith Na:Ca, and an increase in otolith Sr:Ca in Australian salmon (Kalish 1992). Rainbow trout that were stressed with Cl<sub>2</sub> gas exhibited decreased Na<sup>+</sup> and Cl<sup>-</sup> in the blood plasma and endolymph and increased proteins in the proximal endolymph, but no changes in total Ca (Payan et al. 2004a). The stress-induced changes in endolymph chemistry also produced a 'check' mark and caused a reduction in otolith growth rate. Growth rate effects on otolith chemistry have been regarded as kinetic or physiological (Walther et al. 2010). Kinetic effects are controlled by the calcification rate of the otolith and can either result in increased element:Ca ratios if more trace elements get entrapped within the crystal during rapid calcification due to increased crystal defects (Gaetani & Cohen 2006), or decreased element:Ca if higher supplies of Ca<sup>2+</sup> in the endolymph dilutes the concentration of other trace elements in the calcifying fluid (Sinclair 2005). Physiological effects are mediated by metal-binding protein levels in the endolymph, which is coupled to somatic growth and affects the availability of free ions to be incorporated into the otolith. Otolith Mg:Ca was correlated with somatic growth and otolith precipitation rates in spot (Bath Martin & Thorrold 2005) and in Chinook salmon (Miller 2011), and Mg is subject to strong physiological regulation in fish (Woodcock et al. 2012). The stressful periodic hypoxia decreased otolith Mg:Ca as would be expected if Mg is under endogenous control, but otolith Ba:Ca also decreased. In many euryhaline species, otolith Ba:Ca typically displays positive relationships with ambient

dissolved Ba, as observed in both controlled lab experiments (Bath et al. 2000, Elsdon & Gillanders 2002, 2003, 2004, 2005, Bath Martin & Thorrold 2005, Walther & Thorrold 2006, Miller 2009), and field studies (Dorval et al. 2007, Walther & Thorrold 2008, Mohan et al. 2012). Some studies have found an effect of somatic/otolith growth on otolith Ba:Ca (Walther et al. 2010, Miller 2011), or temperature effects which can be related to growth (Elsdon & Gillanders 2002, 2004), while others have detected no growth/temperature effects (Bath et al. 2000, Bath Martin & Thorrold 2005, Bath Martin & Wuenschel 2006). More work is needed to assess the potential effects of stress and growth on otolith Mg:Ca and Ba:Ca ratios.

Interestingly, the periodic hypoxia treatment did result in significant Sex × Treatment interactions for otolith Sr:Ca and Na:Ca ratios and also decreased both otolith Ba:Ca and Mg:Ca ratios, perhaps because alternating between hypoxic and normoxic conditions is more physiologically stressful than remaining in constant hypoxic conditions. Hypoxia-tolerant species exhibit 3 specialized adaptations when they encounter low-oxygen conditions: (1) metabolic suppression, (2) tolerance of pH and ionic disturbances, and (3) ability to avoid free-radical injury during reoxygenation (Bickler & Buck 2007). Fish exposed to constant hypoxia would only need to enact the first 2 defense mechanisms, but those fish exposed to alternating weeks of hypoxia and normoxia would need to utilize all 3 mechanisms and at weekly intervals. The periodic hypoxia treatment did not significantly inhibit somatic growth, since condition factors did not differ from control fish, but otolith Ba:Ca and Mg:Ca were decreased. Dissolved Mg:Ca, Sr:Ca, Ba:Ca, and Na:Ca ratios were similar between control and constant hypoxia tanks throughout the experiment. Only dissolved Mn:Ca was different between control and hypoxic tanks, but a difference of 7 μmol mol<sup>-1</sup> is very minor compared to field observations of dissolved Mn:Ca in the northern Gulf of Mexico, which can vary over 100 μmol mol<sup>-1</sup> at low O<sub>2</sub> levels (J. Mohan unpublished data), and Mn can be elevated several orders of magnitude in coastal regions compared to offshore Gulf waters (Slowey & Hood 1971).

### Chemistry differences in otolith growth axis

We found differences in elemental chemistry between the sulcal and ventral axes of the croaker otoliths, as expected. Compared to the sulcal axis, the longer ventral axis exhibited approximately 1.5×, 4×,

and 40× higher values for Ba:Ca, Mg:Ca, Mn:Ca ratios, respectively. Only Na:Ca was increased 2-fold on the sulcal axis, while Sr:Ca was similar between both axes. These patterns may have arisen simply due to differences in precipitation rates and therefore incorporation dynamics of elemental impurities (Strasser et al. 2008, Walther et al. 2010). In addition, other studies have quantified the elemental composition of the endolymph fluid from which the otolith is mineralized and found heterogeneity between dissolved elements and proteins in the proximal and distal regions of the endolymph (Payan et al. 1999, 2004b). The endolymph fluid in trout was found to contain 20% higher Na<sup>+</sup> concentration on the proximal side, which is the same side on which the sulcus is formed (Payan et al. 1999). Consistent with our results, Sr:Ca ratios displayed a uniform distribution along the proximo-distal axis in turbot otoliths that was related to homogeneous dissolved Sr<sup>2+</sup> concentration in the endolymph (Payan et al. 1999).

## CONCLUSIONS

This is the first controlled laboratory study to examine the effects of hypoxic stress on otolith chemistry and one of the few studies to investigate the influence of sex on otolith elements. Constant hypoxia exposure over 4 or 10 wk did not affect element incorporation in the otoliths of Atlantic croaker. Periodic hypoxia did influence element incorporation in complex and interactive ways, and the exact mechanism of the hypoxic stress and sex influence is unclear. Otolith Mn:Ca was the only element ratio not affected by endogenous hypoxic stress, and thus may serve as a promising environmental indicator of hypoxic redox conditions.

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