STABILITY OF TRACE ELEMENTS IN OTOLITHS OF JUVENILE PACIFIC SARDINE *SARDINOPS SAGAX*

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ABSTRACT

We evaluated trace element analysis of otoliths of juvenile Pacific sardine *Sardinops sagax* by inductively coupled plasma mass spectrometry as a potential method to identify regional stocks. Otolith treatment experiments determined the stability of trace elements. Hydrogen peroxide treatment slightly affected Mn, Sr, and Ba, but it removed 35%–43% of Mg and 51% of P. Growth experiments identified ontogenetic and temperature factors that influenced trace element composition. Otolith weight was a major determinant of composition. Mn, Sr, and Ba each had unique ontogenetic and temperature responses. Mg/Ca and P/Ca ratios covaried and decreased with growth at 16°–19°C but increased with growth at 21°C. The decrease in Mg/Ca and P/Ca ratios with age was nonconservative, i.e., the total mass of initially deposited Mg and P decreased as the otoliths grew. The results contradict the assumption that all deposited trace elements are permanently incorporated in otoliths in living fish.

INTRODUCTION

Pacific sardine

Pacific sardine *Sardinops sagax* (Jenyns 1842) occur along approximately 5000 km of coastal waters of North America between Canada and the Baja California peninsula into the Gulf of California, Mexico. Integrated assessment of the fisheries in Canada, the United States (USA), and Mexico requires identification of regional stocks of mature and immature sardine along with knowledge of their spawning habits and migration patterns. It is believed that three North American stocks exist with synchronous, seasonal, north-south migration patterns (Félix-Uraga et al. 2004, 2005) although little genetic structure has been detected (Hedgecock et al. 1989; Grant and Bowen 1998; Pereyra et al. 2004; García-Rodríguez et al. 2011).

Methods that have had some success in identifying regional stocks and migrations include egg, larval, and adult surveys (Lo et al. 2005, 2010, 2011); vertebral counts and tags (Smith 2005); temperature at catch (Félix-Uraga et al. 2004, 2005); analysis of spawning habitats (Reiss et al. 2008; Demer et al. 2012); fish and otolith aging and morphometric analysis (Javor et al. 2011; Javor 2013; Vergara-Solana et al. 2013); and otolith stable isotope measurements (Valle and Herzka 2008; Dorval et al. 2011; Javor and Dorval 2014).

Differences in spawning and feeding regions and changes in seasonal temperature and salinity could offer further evidence of stock structure if they are reflected in trace element composition of otoliths. Sardine spawn at 12°–14°C off southern and central California in the spring with a peak around April (Lo et al. 2005; Reiss et al. 2008). However, the temperature range of sardine habitats is broad, from less than 10°C in the Pacific Northwest (Emmett et al. 2005) to over 25°C in their southern range in the Gulf of California (Mitchell et al. 2002; Félix-Uraga et al. 2004, 2005). Because mature sardine cross regions during migrations, trace elements incorporated into adult otoliths might not give clear signals of stock identity. Juveniles that are incapable of long-distance swimming are believed to remain within or near the water masses where they were spawned. They might provide relatively distinct regional chemical signatures in their otoliths.

Chemistry of biogenic calcium carbonate

Fish otoliths are biogenic calcium carbonate (aragonite) bodies that form in sacculus endolymph fluid secreted from blood plasma. Unlike biogenic calcium carbonate produced by bivalves and corals, otoliths remain internal within the growing fish. Endolymph is an extracellular fluid with gradients and diurnal fluctuations in dissolved ions, proteins, and pH that influence otolith growth (Edeyer et al. 2000; Payan et al. 2002, 2004; Borelli et al. 2003; Takagi et al. 2005; Guibbolini et al. 2006).

Some trace elements typically detected in otoliths may have important functions in animal metabolism (e.g., Mg and P roles in ATP and nucleic acid metabolism) while others have no known biological functions (e.g., Ba). Some trace elements associate with growing otoliths, clam shells, and corals without forming true

crystalline mineral structures in calcium carbonate. The associations may be strong (e.g., ionic bonds) or weak (e.g., adsorption), or chelation with organic ligands. For example, Mg is believed to be largely associated with the organic matrix in bivalves and corals (Watanabe et al. 2001; Takesue et al. 2008; Foster et al. 2008; Jacob et al. 2008; Schöne et al. 2010; Yoshimura et al. 2014; Poulain et al. 2015), while P (as phosphate) is associated either with small domains of hydroxylapatite or amorphous phosphate-carbonate microstructures in newly formed bivalve shells (Xu and Zhang 2014) and corals (Mason et al. 2011; Zhang et al. 2011). The stability of the associations between calcium carbonate and trace elements in biogenic aragonite depends on multiple factors including charge, ionic radii, steric criteria, competing ions, concentration, rate of formation, and the physical structure of the calcium carbonate crystals (Stipp 1998; Watson 2004; Gaetani and Cohen 2006; Gabitov et al. 2006, 2008).

The pioneering work of Campana (1983) showed ^{45}Ca incorporated into salmon otoliths did not resorb within two days after ^{45}Ca exposure. No studies have shown unequivocal calcium loss from otoliths, although an investigation using fluorescent staining techniques demonstrated Ca^{2+} can translocate in otoliths after initial deposition (Beier et al. 2004). It is generally accepted that "any elements or compounds accreted onto its growing surface are permanently retained" in an otolith and serve as permanent natural tags (Campana 1999). However until recently (Veinott et al. 2014), experimental investigations have not been conducted to demonstrate all trace elements are as stable as calcium during otolith growth.

Numerous studies have used otolith trace element chemistry to identify fish stocks. Investigations have addressed factors that influence elemental partitioning into otoliths such as temperature, salinity (Fowler et al. 1995; Hoff and Fuiman 1993; Elsdon and Gillanders 2002; Bath Martin and Wuenschel 2006), age, and ontogeny (Begg et al. 1998; Rooker et al. 2001; Brophy et al. 2003; Ruttenberg et al. 2005). Research has also focused on post-mortem processing and contamination that could alter trace element concentrations (Milton and Chenery 1998; Proctor and Thresher 1998; Rooker et al. 2001; Brophy et al. 2003; Swan et al. 2006). In general, instability of trace elements in otoliths has been treated as a problem to avoid rather than addressing it as a natural factor in vivo.

This investigation

In order to interpret otolith trace element composition in surveys to identify and differentiate regional populations of immature Pacific sardine, we conducted several experiments to determine factors that affected trace element incorporation. We captured juvenile sardine off San Diego, California, USA (32.7°N) to use to track otolith composition under controlled conditions: otolith treatment experiments to measure trace element stability using protocols similar to published methods for bivalves and corals (experiment 1); culture at ambient temperature to determine ontogenetic effects (experiments 2 and 3); and culture at different temperatures to evaluate thermal effects (experiment 4).

We postulated four hypotheses to explain possible outcomes of the growth experiments. The hypotheses compare the ratios of trace elements to calcium (TE/Ca) vs. otolith weight in whole otoliths at the beginning of the experiment (time-0, "core") with those at the end of the experiment (fig. 1, H1–H4). For the purpose of depicting the hypotheses, all time-0 otoliths are shown as lightly stippled figures whereas the color intensity at the end of the experiments represents higher (darker) or lower (no color) element ratios. H1 predicts TE/Ca ratios at the end of the experiment will be the same as the time-0 otoliths. H2 predicts TE/Ca ratios will increase with growth. H3 predicts TE/Ca ratios will decrease with growth with the most extreme case being no deposition of a trace element in the new aragonite. The H3 curve in Figure 1 represents the minimum TE/Ca ratios expected in the whole otoliths, i.e., a dilution curve of core trace elements. H4 is similar to H3 but it also predicts loss of trace elements from the core during the experimental growth period. The trajectory of the H4 curve will be lower than the dilution curve predicted by H3. In light of the results, we discuss the possibility that some elements that associate with noncrystalline matrices at the time of initial otolith formation might be subject to in vivo loss during subsequent growth.

MATERIAL AND METHODS

Juvenile sardine for both the otolith treatment experiment (experiment 1) and growth experiments (experiments 2, 3, 4) were captured in waters off San Diego, California (USA) in the surf zone by beach seine or just offshore by a live bait seiner (Everingham Bros.). It was not possible to capture the same size fish for all the experiments.

Otolith treatment, experiment 1

Experiment 1 tested the effects of otolith treatment methods on weight change and trace element composition. Using both sagittal otoliths from each fish, one otolith was randomly selected and assigned to a specific treatment method, whereas the opposite otolith was used as the control (treated similarly in purified MilliQ™ water, MQ-H_2O, to account for aqueous leaching). The combined effects of time, chemical solution, and temperature were tested in five experimental treatments (table 1) including two time periods in unbuffered 30%

TABLE 1
Summary of factors and experimental treatments for otolith analysis in experiment 1. All samples were analyzed at SIO except treatment F (UCSB).

		Treatment					
Factor	Control	A	B	C	D	E	F
Time	16 h	30 sec	2 h	2 h	2 h	16 h	24 h
Solution	MQ-H_2O	HNO_3	SDS	Pro K	H_2O_2	H_2O_2	H_2O_2
Temperature	Ambient	Ambient	37°C	55°C	Ambient	Ambient	Ambient

TABLE 2
Experimental details of *S. sagax* culture experiments 2, 3, and 4. Growth parameters reported as averages ± S.D.

Experiment	Age at Time-0	Duration of expt	*n*, Time-0	*n*, End	SL (mm)	Fish wt (g)	Otolith wt (mg)
2	6 mo	8 mo					
Time-0			10		69.3 ± 4.2	2.8 ± 0.5	0.338 ± 0.027
End				32	105.2 ± 20.9	17.8 ± 10.9	0.832 ± 0.206
Range					71.0 – 138.0	3.9 – 39.2	0.496 – 1.197
3	~10 mo	5 mo					
Time-0			10		118.5 ± 14.5	13.2 ± 5.1	0.840 ± 0.155
Tank 1				10	147.2 ± 7.6	46.4 ± 9.0	1.037 ± 0.123
Tank 2				10	155.8 ± 10.6	61.8 ± 14.4	1.120 ± 0.115
4	~8 mo	11 mo					
Time-0			10		118.0 ± 8.7	14.9 ± 2.9	0.695 ± 0.055
13°C				10	158.9 ± 11.7	55.9 ± 10.7	1.135 ± 0.143
17°C				10	167.6 ± 10.9	64.7 ± 12.6	1.404 ± 0.130
21°C				12	156.0 ± 10.2	52.7 ± 8.7	1.348 ± 0.141

hydrogen peroxide (J.T. Baker Ultrex II Ultrapure). Watanabe et al. (2001), Krause-Nehring et al. (2011), and Holcomb et al. (2015) used unbuffered 30% H_2O_2 in their treatment protocols with bivalves and corals, and we followed suit. Watanabe et al. (2001) found little difference in the results between buffered and unbuffered H_2O_2 in their experiments. We tested 30 sec exposure to 0.1% nitric acid. We also tested two solutions to solubilize proteins: 2% sodium dodecyl sulfate (SDS; Gibco-BRL UltraPURE) dissolved in MQ-H_2O, and proteinase K (ProK, USB Corp.) dissolved in 100 mM Tris-HCl, pH 8.0, to a working concentration of 1 mg ml^{-1}. For weight change measurements after these treatments, an additional set of control otoliths was evaluated in which both left and right otoliths were soaked in MQ-H_2O for 2 h. For the control and treatments A–E, the average initial weight of the otoliths (± standard deviation, S.D.) was 0.720 ± 0.090 mg. The sardine were approximately one year old.

Except for the acid treatment that was performed on glass microscope slides, each otolith was treated in a small volume of the test solution in a 0.5 ml microfuge tube by periodically tumbling it gently with a pipette or by flicking the tube. After each treatment, the otoliths were washed with a pipette by gentle tumbling with at least four changes of MQ-H_2O. After drying they were reweighed. A follow-up assay was conducted to verify repeatability of the overnight treatment with H_2O_2. The experimental otoliths were treated for 24 h with unbuffered 30% H_2O_2 from a freshly opened bottle (treatment F, table 1). The juveniles in treatment F were captured in the same seine haul as those used for growth experiment 2 described below and in Table 2. Phosphorus was measured in treatment F, but not in treatments A through E.

Growth experiments 2, 3, and 4

Wild-caught juveniles were maintained in tanks at our facility on a 12 h light/12 h dark cycle. They were fed a commercial pellet diet, Bio-diet brood formulation (Bio-Oregon), *ad libitum* in temperature-regulated, circular (1.83 m diameter, 1600 L) or elliptical (1.5 x 3.0 m diameter, 3200 L) tanks with an approximate daily turnover of ten seawater volumes. Seawater was obtained from the Scripps Institution of Oceanography pier where little variation in salinity has been noted. Surface seawater salinity measured quarterly during 1990–2012 from three nearshore sampling stations within 27 km of the pier averaged 33.470 ± 0.168 (S.D., n = 279)

(www.calcofi.org, accessed 30 January 2014). Average monthly sea surface temperature at the site of collection ranges between 13.9° and 20.0°C, with an annual average of 16.6°C (www.nodc.noaa.gov/dsdt/cwtg/spac.html, accessed 12 February 2015). Fish that died during the experiments were removed but not enumerated. All the growth experiments were conducted between 2004 and 2006.

Table 2 lists experimental details for the growth experiments. The experiments were designed to evaluate whether any of the four hypotheses, H1–H4, described the outcomes of the trace element ratios in otoliths between the time-0 fish and the fish at the end of the experimental growth period. Ten fish were randomly selected at time-0. To determine ontogenetic effects on otolith trace elements, experiment 2 used juveniles from a single school that were raised in one tank for eight months at ambient temperature (16° to 19°C for most of the period). At time-0 the average age based on counts of daily otolith increments was six months (Takahashi and Checkley 2008). Experiment 3 further examined ontogenetic effects, this time with fish from a single seine haul maintained for five months in duplicate tanks. The age at time-0 was estimated to be about ten months based on a presumed April birthday.

In experiment 4, which assessed thermal effects on trace element deposition in otoliths, juveniles were cultured in duplicate tanks at 13°, 17°, or 21°C. Wild immature sardine near San Diego experience this temperature range during the course of a year (Javor and Dorval 2014). Experimental details are described in Dorval et al. (2011). Based on previous data, we converted fork length to standard length by subtracting 3 mm.

Otolith preparation

Growth experiments 2, 3, and 4 used primarily left otoliths for analysis. No difference in trace element composition was noted between left and right otoliths in preliminary measurements. After initially cleaning in MQ-H_2O, otoliths were dried, weighed on a Cahn C-33 microbalance (0.005 mg accuracy), and stored in plastic microfuge tubes.

All glass and plastic implements were cleaned in 10% nitric acid. Otoliths were further prepared in a Class 100 clean room. Sardine otoliths are fragile and often break during the sonication step of standard cleaning protocols. We developed a procedure that avoided breaking the otoliths while minimizing dissolution during cleaning: 30 min in 2% SDS, at least four MQ-H_2O washes, 3–5 min in unbuffered 30% H_2O_2, and four final MQ-H_2O washes. Each otolith was checked under a microscope after the washing steps, and further cleaned with glass probes if necessary. Most otoliths did not require additional cleaning after the washing protocols.

Inductively coupled plasma mass spectrometry (ICPMS)

For treatment experiment 1 and growth experiments 2 and 3, otoliths were dissolved in 2% HNO_3 (Fisher Optima) with 2 ppb In for trace element analysis by solution-based ICPMS on a Finnegan MAT Element 2 instrument. Due to unavoidable circumstances, different spectrometers were used during the course of the study. The instruments were at Scripps Institution of Oceanography (SIO, La Jolla, California), University of California Santa Barbara (UCSB), Old Dominion University (ODU, Norfolk, Virginia), or Woods Hole Oceanographic Institute (WHOI; Woods Hole, Massachusetts). Given the documented variations in trace element measurements determined in different laboratories and with different instruments (Campana et al. 1997), we tried to minimize any biases that the different spectrometers might have introduced (described below).

Analysis of whole otoliths by solution-based ICPMS allowed us to calculate mass balances and relative gain or loss of trace elements in treatment experiment 1 and at the beginning and end of growth experiments 2 and 3. In experiment 1, otolith samples from treatments A through E plus the control set were analyzed in a single run at SIO, whereas treatment F was analyzed at UCSB. Because we were interested in measuring percent gain or loss in each treatment, bias in elemental concentration measurement due to laboratory-specific spectrometer procedures would not have had any significant effects on our results. For experiment 2, analysis was conducted in a single run at ODU so the results were internally consistent. For experiment 3, analysis at SIO was conducted in a single run.

Solution samples were randomized in sets of eight samples in blocks between a blank (to determine minimum detection limits and baseline) and a repeating standard (to determine drift). The detection limits were similar for the different spectrometers. Average detection limits were: ^{24}Mg, 0.01 ppb; ^{31}P, 0.22 ppb; ^{48}Ca, 0.01 ppm; ^{55}Mn, <0.01 ppb; ^{88}Sr, 0.01 ppb; and ^{137}Ba, <0.01 ppb (determined at ODU). Standards for solution-based ICPMS were prepared by diluting a stock mixture in 2% HNO_3 (Fisher Optima) with similar element ratios as determined in preliminary analyses of sardine otoliths: 200 ppm Ca, 30 ppb Mg, 300 ppb P, 2 ppb Mn, 300 ppb Sr, 8 ppb Ba, and 2 ppb In as internal standard. Conversion of gravimetric units to molar ratios of the standards yielded the following ratios: Mg/Ca, 0.247 mmol mol^{-1}; P/Ca, 1.935 mmol mol^{-1}; Sr/Ca, 0.685 mmol mol^{-1}; Mn/Ca, 7.280 μmol mol^{-1}; and Ba/Ca, 11.660 μmol mol^{-1}. All elements except P were prepared from certified trace element standards obtained from High Purity Standards, CPI International, or Spex CertiPrep. The phosphorus stock

solution was prepared from reagent grade sodium phosphate (Fisher Scientific).

For experiment 4 in which temperature was the tested variable, laser ablation ICPMS (LA-ICPMS) of the distal edges of whole otoliths was conducted at WHOI using a UP 213 (New Wave Research) laser ablation sampler to monitor changes between time-0 fish and experimental fish. We did not compare measured trace element concentrations by the two analytical methods (solution vs. laser ablation) because we did not intercalibrate the two methods. Rather, we evaluated the results between the beginning and the end of each temperature treatment to assess trends in trace element ratios. LA-ICPMS employed two solid standards: Canadian National Research Council FEBS-1, a certified otolith reference material from the red snapper *Lutjanus campechanus*; and a second otolith material prepared from the red emperor *Lutjanus sebae* (Yoshinaga et al. 2000). Neither standard quantified phosphorus, which is reported as the ratio of counts per second (cps) of P/Ca after subtracting the blank values. For intact otoliths sampled by laser ablation, mean RSD(%) were: Mg, 6.16%; Ca, 5.27%; Sr, 2.85%; and Ba, 3.68%. RSD values for P and Mn were not provided by WHOI.

Inductively coupled plasma-optical emission spectrometry

The elemental composition of seawater is generally conservative, and surface seawater near SIO pier showed little variation in salinity over 23 years (noted above). To corroborate those findings for our experiments, a PerkinElmer 3700 optical emission plasma spectrometer at SIO was used to measure Ca, Mg, and Sr in seawater in the sardine-rearing tanks. Seawater was first diluted with MQ-H_2O. Analysis of each of the tanks on 11 monthly dates during growth experiment 4 revealed no significant variations in the concentrations and ratios of the three elements (data not shown). No further seawater analyses were conducted for the other experiments.

Data analysis

Relationships between otolith weight and trace element ratios were primarily determined by correlation analysis and linear regressions either as individual fish or as the averages of pooled fish as described in Results. For growth experiments 2 and 3, theoretical dilution curves of trace element ratios were calculated based on the molar concentrations of the trace elements and Ca in otoliths of known weight in time-0 samples, assuming the increase in otolith weight with growth was due to the accretion of $CaCO_3$ only (H3, fig. 1). We compared empirical data with the H3 curves for each element ratio using nonlinear models. Our criteria for testing the H4 hypothesis to explain the results after the growth

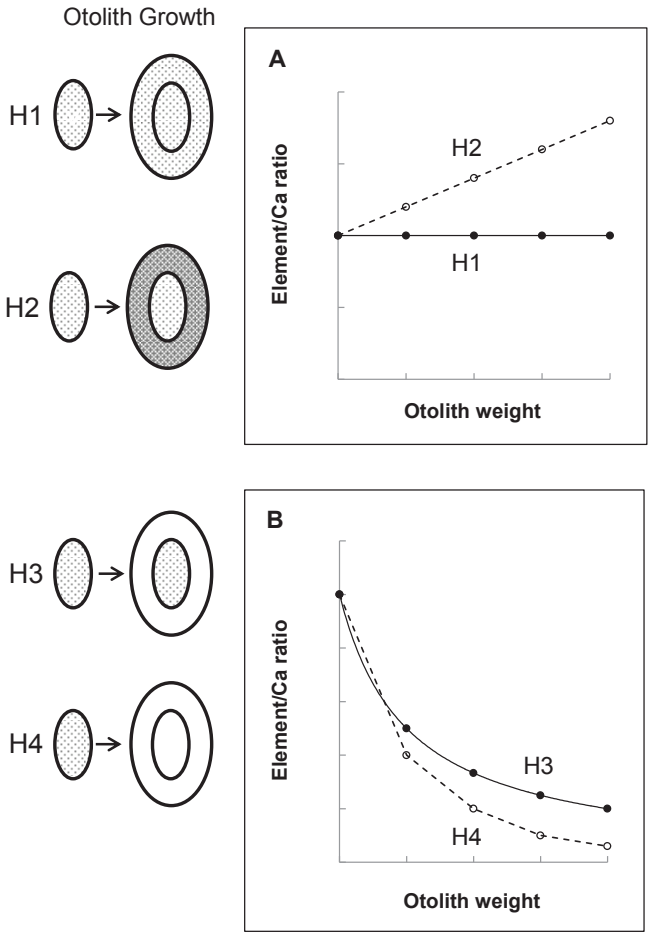

Figure 1. Four hypotheses (H1-H4) explaining possible outcomes of trace element ratios vs. otolith weight in the growth experiments. See explanation in the text.

period were based on determining whether the theoretical dilution curve described by H3 fell within the 95% confidence intervals (CI) of the predicted TE/Ca curve derived from experimental data.

Nonlinear models. Nonlinear models were developed for relating trace element ratios to otolith weight in growth experiment 2. A 3-parameter, negative exponential model was fitted to the data for Mg/Ca, P/Ca, Sr/Ca, and Ba/Ca ratios (Eq. 1), whereas a 2-parameter exponential model was used for Mn/Ca ratios (Eq. 2). The Marquardt optimization method was used to estimate parameters for all models using SAS software (version 9.2; Cary, North Carolina). The 95% confidence intervals were calculated for the expected value of element ratio-at-weight predicted from each model. These confidence intervals allowed comparison between the trajectory of element ratio-at-weight from the theoretical dilution curve to the expected value (H3) and associated uncertainties predicted from the empirically-derived models. The percentage of the variability explained from the data by each model is

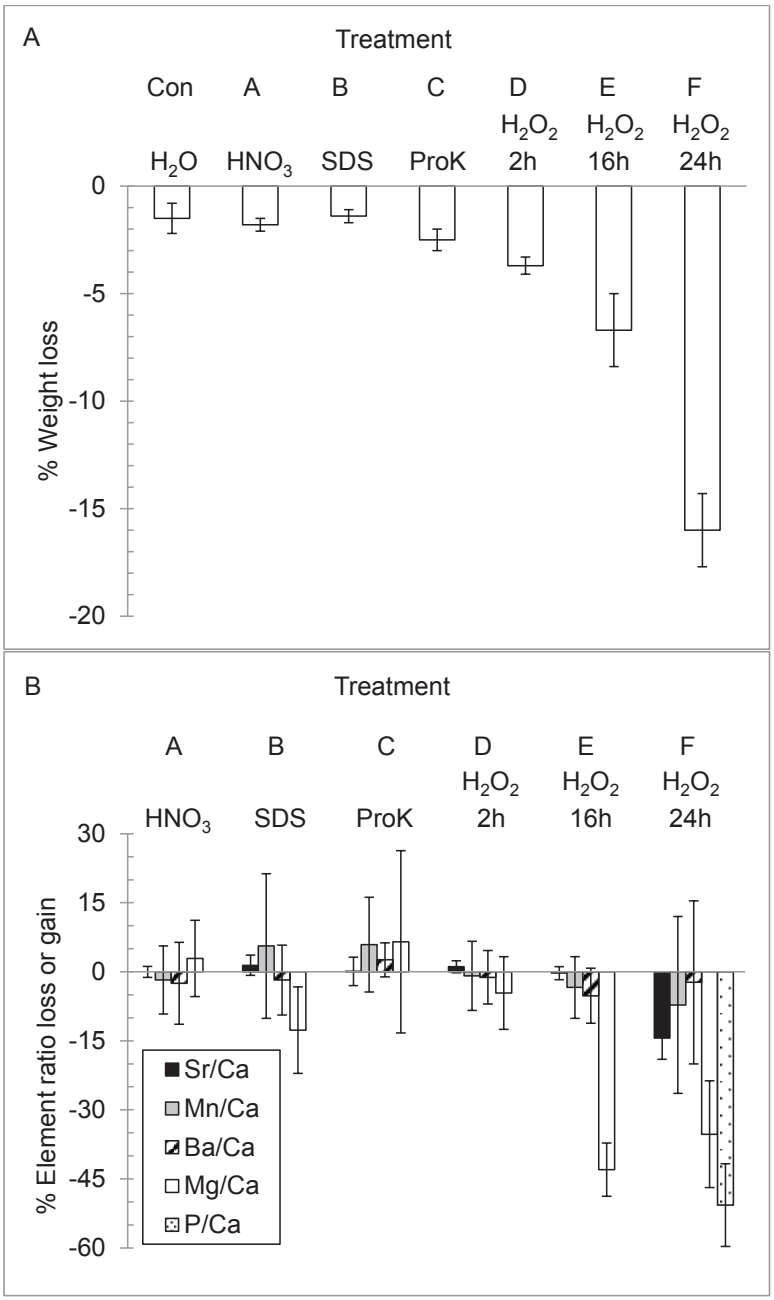

Figure 2. Experiment 1: Effects of treatments (Table 1) on juvenile *S. sagax* otoliths. The loss of otolith weight and changes in trace element composition are compared to otoliths treated with MQ-H_2O. A: Weight loss ± S.D., n = 6-8. B: Element loss (based on molar ratio to Ca) ± S.D., n = 5-8. Details are in the text.

reported using the R^2 value after adjustment for the degree of freedom:

$$(1) \quad y = y_0 + a \times e^{(-b \times w)}$$

$$(2) \quad y = a \times e^{(-b \times w)}$$

where y is the element ratio measured for each otolith, y_0 is the intercept of the model, a and b are the regression coefficients, and w is the otolith weight (mg) from each individual fish.

RESULTS

Otolith treatment, experiment 1

Weight losses of <3% after brief exposure to weak acid and protocols to dissolve proteins (treatments A–C) were similar to the control treatment in experiment 1 (fig. 2A). Overnight immersion in H_2O_2 caused more substantial weight loss (7%–16%) and a change of the sheen of the otoliths from shiny to dull (treatments E and F). The percent change of each element (calculated from molar ratios

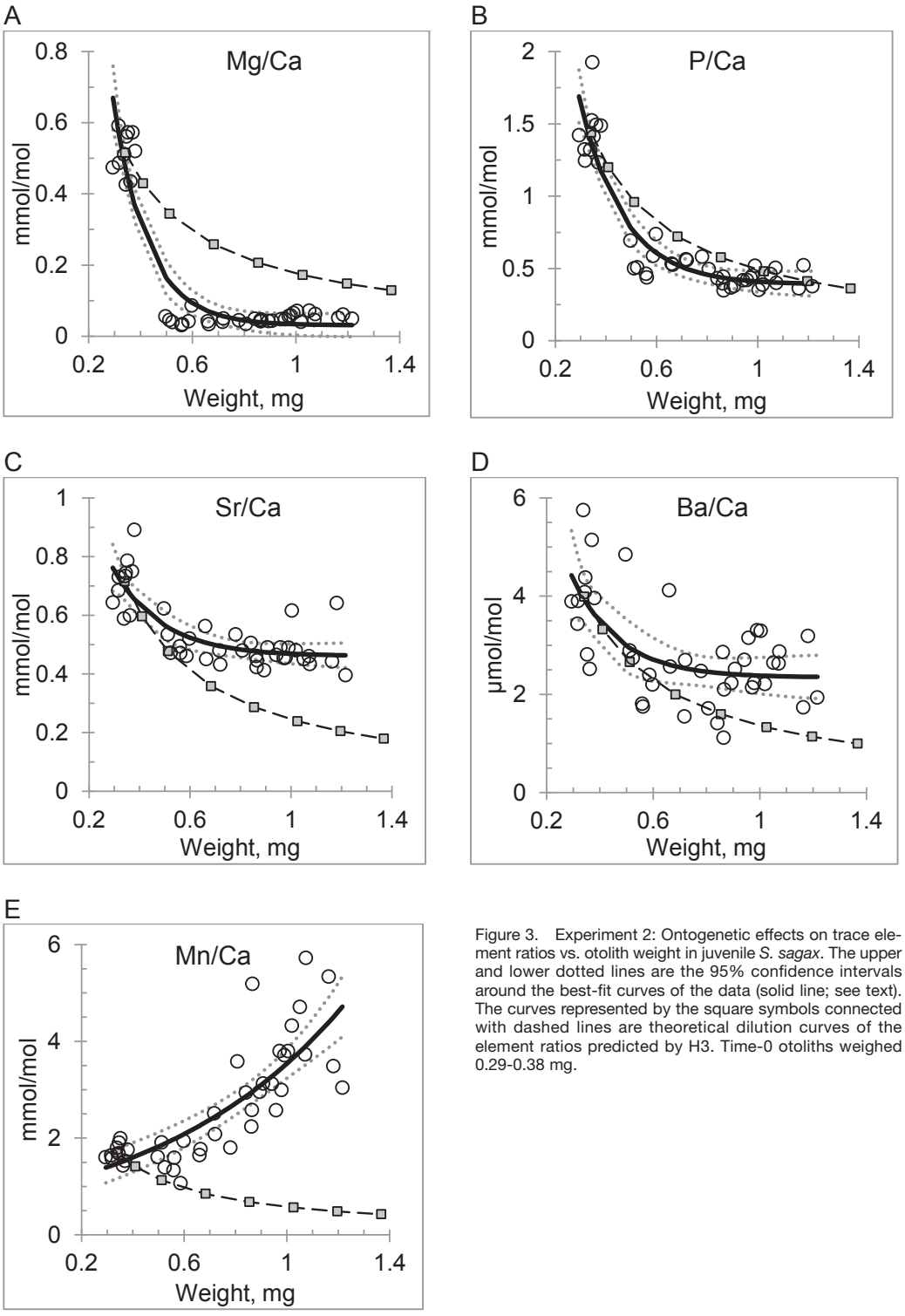

Figure 3. Experiment 2: Ontogenetic effects on trace element ratios vs. otolith weight in juvenile *S. sagax*. The upper and lower dotted lines are the 95% confidence intervals around the best-fit curves of the data (solid line; see text). The curves represented by the square symbols connected with dashed lines are theoretical dilution curves of the element ratios predicted by H3. Time-0 otoliths weighed 0.29-0.38 mg.

to Ca) in the treated otolith relative to the control otolith from the same fish indicated Sr/Ca, Mn/Ca, and Ba/Ca ratios were generally unaffected by the treatments, gaining or losing an average of ≤6% of the untreated value (fig. 2B). Mg/Ca ratios decreased by 13% after SDS treatment, and by 35%–43% after overnight treatment in 30% H_2O_2. Overnight immersion in 30% H_2O_2 from a freshly opened bottle also resulted in the loss of 51% of the P/Ca ratios in the otoliths in treatment F. Although the average weights of the otoliths were different in treatments A–E (0.720 mg) and F (0.339 mg), overnight immersion in H_2O_2 resulted in similar percent losses of Mg.

TABLE 3
Trace element ratios in otoliths of *S. sagax* in growth experiment 3. Average molar ratios (± S.E.) for Time-0, after growth in duplicate tanks (Time-End), and theoretical ratios if no further trace elements were incorporated after Time-0 based on H3 predictions. Mg/Ca, P/Ca, and Sr/Ca are mmol mol^{-1}; Mn/Ca and Ba/Ca are µmol mol^{-1}.

Sample	Mg/Ca	P/Ca	Mn/Ca	Sr/Ca	Ba/Ca
Time-0	0.267 ± 0.029	1.239 ± 0.132	2.563 ± 0.431	0.522 ± 0.024	3.108 ± 0.527
Time-End	0.063 ± 0.007	0.681 ± 0.078	3.276 ± 0.279	0.436 ± 0.018	1.937 ± 0.480
H3 Prediction	0.198	0.920	1.903	0.388	2.308

Growth experiments 2 and 3 to test ontogenetic effects

In experiment 2 in which juveniles from a single school grew in one tank, growth after eight months was very heterogeneous (table 2). Otolith weight correlated with standard length (correlation coefficient = 0.965). Trace element ratios plotted against otolith weights revealed different trends (fig. 3). We evaluated whether decreases in trace element ratios with growth indicated conservation or loss of each trace element as postulated by hypotheses H3 and H4. Each element's H3 curve was constructed based on the average trace element mass and average weight of the otoliths at time-0, e.g., Mg/Ca at time-0 was 0.515 mmol mol^{-1} in 0.342 mg otoliths (n = 10). The best-fit curves for measured element ratios and their predicted 95% CIs varied for each element. The equations describing the best-fit curves (± standard error, S.E.) for element ratios calculated against otolith weight and adjusted R^2 values were:

(3) $Mg/Ca\ (mmol\ mol^{-1}) = 0.031\ (\pm 0.02) + 5.95\ (\pm 2.46) \times e^{(-7.59\ (\pm 1.28) \times w)}$, $R^2 = 0.87$.

(4) $P/Ca\ (mmol\ mol^{-1}) = 0.39\ (\pm 0.05) + 7.29\ (\pm 2.56) \times e^{(-7.59\ (\pm\ 1.11) \times w)}$, $R^2 = 0.86$.

(5) $Sr/Ca\ (mmol\ mol^{-1}) = 0.46\ (\pm 0.02) + 1.36\ (\pm 0.83) \times e^{(-5.13\ (\pm 1.95) \times w)}$, $R^2 = 0.60$.

(6) $Ba/Ca\ (\mu mol\ mol^{-1}) = 2.35\ (\pm 0.24) + 11.09\ (\pm 11.88) \times e^{(-5.69\ (\pm 3.39) \times w)}$, $R^2 = 0.37$.

(7) $Mn/Ca\ (\mu mol\ mol^{-1}) = 0.94\ (\pm 0.14) \times e^{(-1.33\ (\pm 0.17) \times w)}$, $R^2 = 0.64$.

The calculated total mass of Mg and P in the otoliths decreased with growth, i.e., the measured Mg/Ca and P/Ca ratios (±95% CI) were below the H3 dilution curves and thus resembled H4 curves (Eq. 3 and 4, and figs. 3A and 3B, respectively). Best-fit curves in Eq. 3 and 4 had the greatest adjusted R^2 values, indicating these models can explain most of the variability in Mg/Ca and P/Ca ratios in response to growth.

Sr/Ca ratios also decreased with growth, following or exceeding the H3 dilution curve (fig. 3C) which indicated the time-0 mass of Sr was conserved in the otoliths. The values of Ba/Ca ratios generally decreased with growth, but the results were scattered with some values below the H3 dilution curve (fig. 3D). Correspondingly, Eq. 6 explained only 37% of the variability of the Ba/Ca data. Mn/Ca ratios increased with growth, and the predicted values from Eq. 7 were clearly greater than minimum expected values computed from the H3 curve and more closely resembled the H2 response (fig. 3E).

We repeated the protocol in experiment 3 using juveniles maintained in duplicate tanks. Although the time-0 juveniles were approximately four months older and larger than those used in experiment 2 (table 2), and experiment 3 ran for five months rather than eight, the overall trends in trace element composition were comparable (table 3). The results of the duplicate tanks in experiment 3 showed little variability, as indicated by the low standard errors when the data were combined. Mg/Ca and P/Ca ratios decreased with growth, and Mn/Ca ratios increased in a manner predicted by H2. Mg/Ca and P/Ca ratios calculated from H3 trace element dilutions were significantly greater than the ratios (±S.E.) measured at the end of the experiment. The results indicate Mg and P were not conserved, and their behavior resembled the ratios predicted by H4. Measured values for Sr/Ca exceeded the H3 dilution values, indicating conservation. The Ba/Ca ratios from the two tanks were inconsistent, with some measured values exceeding the H3 dilution prediction and others below it.

Comparison of growth experiments 2 and 3 showed that Mg/Ca and P/Ca ratios were highly correlated in both trials, 0.95 and 0.98, respectively. Further, the experimental data (tables 4A and 4B) demonstrated strong correlation between Mn/Ca and sardine growth assessed as otolith weight in the two experiments, 0.75 and 0.79, respectively.

Temperature effects on trace element composition (experiment 4)

In experiment 4, growth was similar at 13° and 21°C, and greatest at 17°C (table 2). Using LA-ICPMS to sample the posterior edge of the otoliths, element ratios of the time-0 otoliths were compared with ratios in new aragonite in the otoliths at the end of the experiment.

Differentiating the effects of growth and temperature on trace element incorporation was complex as was

TABLE 4
Correlation coefficients between trace element ratios and otolith weight (mg) in *S. sagax* juveniles in growth experiments 2 and 3. Ratios are mmol mol^{-1} for Mg/Ca, P/Ca, and Sr/Ca; and µmol mol^{-1} for Mn/Ca and Ba/Ca. ICPMS analyses were conducted in a single run for experiment 2 at ODU, and in a single run for experiment 3 at SIO.

A. Growth experiment 2. Data are shown in Figure 3.

	Mg/Ca	P/Ca	Mn/Ca	Sr/Ca	Ba/Ca
P/Ca	0.949				
Mn/Ca	–0.417	–0.514			
Sr/Ca	0.845	0.840	–0.442		
Ba/Ca	0.635	0.624	–0.354	0.671	
Weight	–0.744	–0.801	0.792	–0.683	–0.526

B. Growth experiment 3. Data are summarized in Table 3.

	Mg/Ca	P/Ca	Mn/Ca	Sr/Ca	Ba/Ca
P/Ca	0.983				
Mn/Ca	–0.157	–0.098			
Sr/Ca	0.648	0.688	–0.308		
Ba/Ca	0.177	0.187	–0.050	0.629	
Weight	–0.751	–0.707	0.745	–0.602	–0.021

evaluating the outcomes according to the four hypotheses. Temperature rather than growth rate or otolith size influenced the incorporation of trace elements in new otolith aragonite. Ratios of Mg/Ca and P/Ca were the highest and most variable (over 10-fold range in values) in sardine maintained at 21°C (figs. 4A and 4B). In fish cultured at 13° and 17°C, ratios of Mg/Ca and P/Ca were similar for each trace element at the two temperatures and time-0, indicating growth rate and temperature were not coupled to the composition of new otolith growth across all temperatures. Because the behavior of Mg and P had similarities, we compared them to each other. P/Mg ratios showed a linear negative correlation with temperature (fig. 4C).

Mn/Ca and Sr/Ca ratios corresponded positively and linearly with respect to temperature, but each temperature resulted in a different trend when compared to the time-0 otoliths (figs. 4D and 4E). In addition to responding positively to temperature, all Mn/Ca ratios increased with growth from time-0 values similarly to the results of growth experiments 2 and 3. Although Sr/Ca ratios showed a positive temperature effect, the ratio of Sr/Ca relative to the time-0 otoliths confounded interpretation of thermal influence. At 13°C growth temperature, otoliths accreted less Sr than that measured in the time-0 otoliths. At 17°C they accreted approximately the same amount of Sr as the time-0 otoliths, and at 21°C they incorporated greater concentrations of Sr.

Ba/Ca ratios responded differently to temperature than the other trace elements (fig. 4F). Ba/Ca ratios had a broader range of values at 13°C (over 40-fold range) than at the other temperatures, with a similar decrease in ratios at 17° and 21°C from time-0 otoliths. No trace element besides Ba demonstrated such variability at 13°C or a lack of thermal effects between 17° and 21°C.

DISCUSSION

Trace elements in treatment experiment 1

Mg and P demonstrated instability with H_2O_2 treatment which likely oxidized matrix organic matter and solubilized weakly bound trace elements in the otoliths. While weight loss indicated dissolution of aragonite under the moderately acidic conditions of unbuffered 30% H_2O_2, the selective removal of these two elements suggests their association was largely outside the carbonate crystal lattice. The percent decreases in Mg/Ca ratios after 16 and 24 h were similar although the two treatments were analyzed on different ICPMS instruments. These results validated the analyses in different instruments.

Trace elements in growth experiments 2 and 3

Growth rate heterogeneity of fish is well known in aquaculture (Kelly and Heikes 2013). Such variability occurred in experiment 2 which included sardine that hardly grew and others that doubled in size. These results allowed us to evaluate both size and age effects on trace element incorporation. The results for Mg and P indicate age was a factor in their composition because all the fish, regardless of size, had similar low concentrations at the end of the experiment. The results for Mn and Sr suggest that overall growth was a significant factor in their incorporation because there were gradients in composition that corresponded to otolith weight. The scatter in the values of Ba/Ca ratios made it difficult to interpret the relative roles of size and age in the incorporation of Ba.

The decrease or increase in trace element/Ca ratios with growth and the magnitude of change varied for each element. Among the trace elements assayed, there was at least one example for each of the outcomes predicted by the four hypotheses, H1–H4. The greatest decreases as postulated by H4 were in Mg/Ca and P/Ca ratios which also highly correlated with each other. Increases as postulated by H2 were noted in Mn/Ca ratios. The trends in thermal responses of Sr/Ca ratios in experiment 4 followed the outcomes postulated by H1, H2, and H3.

The analyses of otolith samples for experiment 2 were conducted on a different ICPMS instrument than experiment 3, yet the correlations of trace element ratios were consistent for the two experiments for Mg/Ca vs. P/Ca, Sr/Ca vs. Ba/Ca, and for all trace element ratios vs. otolith weight except Ba/Ca. Like the treatment assays in experiment 1, the results of experiments

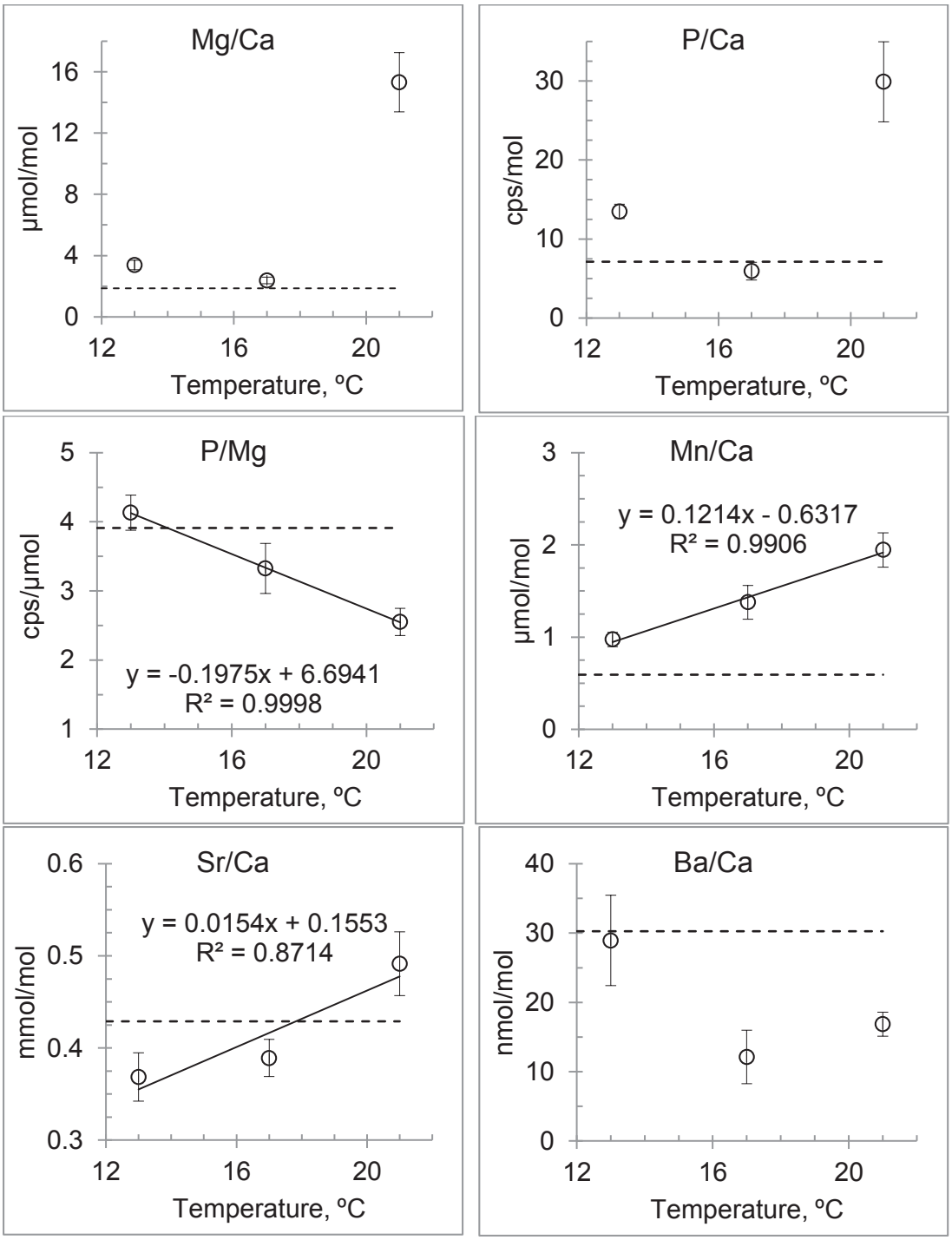

Figure 4. Experiment 4: Temperature effects on element ratios in the distal edges of *S. sagax* otoliths, averages ± S.E. Due to lack of a P standard for the LA-ICPMS analyses, P/Ca concentrations are presented as ratios of counts per second (cps) after subtraction of blank values. The average time-0 ratios are shown as a dashed line for comparison. Growth data and sample sizes are noted in Table 2. Linear regressions were drawn through the mean values of P/Mg, Mn/Ca, and Sr/Ca ratios.

2 and 3 validated the analyses conducted by different ICPMS instruments.

The elemental concentrations in our study were similar to those reported in otoliths of a number of other fish (Campana 1999), including *Sardinops sagax* from Australia (Edmonds et al. 1995). Edmonds et al. found P exceeded Sr concentrations, which agrees with our data. Some of their otoliths might have been from late age-0 sardine (0.8–1.0 mg), but most of their otoliths were larger and probably were from age-1 and older fish. Their Mg and

P concentrations overlapped with the lowest concentrations measured in our study of juvenile *S. sagax*.

Temperature effects on trace element composition (experiment 4)

Temperature positively correlated with Sr/Ca and Mn/Ca ratios, but the results for Mg, P, and Ba were mixed. Taken in conjunction with demonstrated ontogenetic influence on trace element ratios, it may be difficult to differentiate growth and thermal effects in otoliths of wild sardine of differing sizes when assigning fish to stocks and natal regions. Among the published investigations that have addressed the effects of temperature on element ratios in fish otoliths, there is little agreement across species (Campana 1999). We postulate some of the disparities may have been due to the confounding effects of growth and age.

For example, juvenile Atlantic croaker *Micropogonias undulatus* otoliths showed an inverse relationship between temperature and Mg concentration (Fowler et al. 1995). However, the authors did not account for the disparate sizes of the otoliths between the treatments. A reevaluation of their data shows clear relationships for Mg and P with respect to otolith weight when the results are compared by weight without regard to salinity and temperature. These relationships are similar to the results in our investigation in which size (and therefore growth or ontogeny) was a factor for Mg and P concentrations in juvenile sardine otoliths.

In another example, a study measuring Mg in juvenile black bream *Acanthopagrus butcheri* otoliths reported highly variable concentrations between individual fish and replicate tanks, and higher Mg/Ca ratios after growth at 24°C than at 16°C and 20°C (Elsdon and Gillanders 2002). The variability could have reflected the inherent instability of Mg in otoliths. At the end of their experiments, otoliths from fish maintained at 24°C were twice as heavy as otoliths from fish maintained at 16°C, indicating growth rather than temperature may have influenced Mg incorporation. The behavior of Mg was opposite of that noted in Pacific sardine (our study) and Atlantic croaker (Fowler et al. 1995) otoliths, i.e., Mg/Ca ratios increased with growth.

The results of our unpublished survey of otolith composition in juvenile Pacific sardine captured between the USA and Mexico concur with the findings of the present study. Investigations of other fish species have also noted an inverse relationship between Mg concentrations and otolith size (Begg et al. 1998; Rooker et al. 2001; Brophy et al. 2003; Ruttenberg et al. 2005). Begg et al. (1998) also demonstrated an inverse relationship between Mg and P concentrations in two species of mackerel (*Scomberomorus cavalla* and *S. commerson*). This trend was opposite the relationship between the two elements in sardine otoliths, suggesting the causative factors driving the disparate trace element partitions in different species are complex.

The behavior of Mg and P in juvenile sardine otoliths in our experiments was not predicted based on the generally accepted premise that trace elements are permanent chemical tags in otoliths (Campana 1999). Based on mass calculations, the results indicate Mg and P underwent in viv*o* losses that can likely be attributed to physicochemical factors of otolith structure and trace element stability.

Otolith structure and stability

Otoliths grow in sacculus endolymph fluid that exhibits diurnal changes and gradients in dissolved ions, proteins, pH, and pCO_2 (Edeyer et al. 2000; Payan et al. 2002, 2004; Borelli et al. 2001, 2003; Takagi et al. 2005; Guibbolini et al. 2006). Due to their porous structure and permeability (Gauldie et al. 1998; Gauldie 1999), otoliths are sensitive to contamination as a result of extraction, storing, and cleaning methods (Proctor and Thresher 1998; Milton and Chenery 1998; Rooker et al. 2001; Brophy et al. 2003; Swan et al. 2006). Instability noted as possible in vivo loss of Mg from otoliths in *Salmo trutta* juveniles has been described (Veinott et al. 2014). Interpretations of our findings on element stability in sardine otoliths are supported by research on non-biogenic aragonite and biogenic aragonite in other phyla.

The calcium carbonate "host" in non-biogenic and biogenic aragonite

Aragonite is an orthorhombic calcium carbonate mineral that can host small amounts of foreign ions within or between layers of the crystal lattice (e.g., at dislocations), or associated with the proteinaceous organic components in otoliths (Campana 1999; Miller et al. 2006). Foreign ions can associate with aragonite with ionic bonds, chelation, or weak attraction (e.g., adsorption). The strength of the attractions and stability of the associations between the host crystal, organic matrix, and foreign ions are dictated by charge, ionic radii, steric criteria, competing ions, concentration, temperature, crystal imperfections, and rate of crystal growth.

The incorporation of foreign ions into aragonite may not be in equilibrium with the surrounding fluid. Rapid crystal growth promotes the entrapment of ions in disequilibrium with bulk fluid concentrations, and the degree of entrapment depends on the competition between growth rate and crystal lattice diffusivity (Watson 2004). Mg, Sr, and Ba ions were enriched in non-biogenic aragonite grains relative to the concentrations predicted from equilibrium concentrations, indicating kinetic processes and entrapment controlled their distribution (Gaetani and Cohen 2006; Gabitov et al. 2006,

2008). Entrapment and growth rate effects could explain the 100-fold differences in Mg concentrations detected within individual otoliths (Milton and Chenery 1998), and some of our results.

Once trace elements are entrapped in calcium carbonate, they may retain a degree of mobility at ambient temperatures, moving in or out of crystals by solid-state diffusion along planar crystal defects (Stipp 1998; Stipp et al. 1998). Using fluorescent labeling, Beier et al. (2004) showed calcium can translocate in vivo in fish otoliths. Because otoliths have a relatively porous architecture, these lines of evidence suggest they may contain high-diffusivity pathways that could promote open-system behavior (Bruce Watson, pers. comm.).

Stable and metastable trace elements in biogenic aragonite

Many studies of trace elements in bivalves, corals, and foraminifera have been conducted because of their potential utility to reconstruct paleotemperatures of the oceans based on element ratios in skeletal carbonates. It is important to differentiate stable associations (e.g., lattice-bound within $CaCO_3$ crystals) from metastable associations (e.g., non-lattice-bound by absorption to extra-crystalline matrices) to calculate element partition coefficients in carbonates based on temperature and salinity. Various treatment protocols have been tested and compared to analyze the stability of trace elements in bivalves and corals (Watanabe et al. 2001; Schöne et al. 2010; Krause-Nehring et al. 2011; and Holcomb et al. 2015). No single method is ideal to resolve the composition of stable and metastable trace elements. In general, most investigators agree Sr and Ba are lattice-bound, and other trace elements are partly or largely non-lattice-bound. Sr has been shown to substitute well for Ca in aragonite (Plummer and Busenberg 1987; Finch and Allison 2007).

As biogenic aragonite structures, otoliths should share basic physicochemical features with bivalve shells and corals, i.e., crystalline microstructures interlayered with organic matrices, and inclusion of stable and metastable trace elements. One potentially significant difference is that otoliths remain sealed from direct contact with seawater and microorganisms in the fish sacculus while mollusk shells and corals may be subject to relatively unregulated interactions with environmental agents after initial formation.

Strontium and barium. Sr/Ca ratios are generally unaffected by treatments in bivalves (Takesue et al. 2008) and corals (Holcomb et al. 2015). Sr/Ca ratios have been shown to be relatively immune to various methods of otolith storage and cleaning (Milton and Chenery 1998; Proctor and Thresher 1998; Rooker et al. 2001; this study). We showed the effects of ontogeny and growth temperature on Sr/Ca ratios in juvenile sardine otoliths were predictable as well.

Ba/Ca ratios are also generally unaffected by treatments in bivalves (Takesue et al. 2008), corals (Holcomb et al. 2015), and otoliths (our study). Ba has been shown to be both relatively stable (Rooker et al. 2001) and relatively unstable (Swan et al. 2006) in studies addressing post-mortem artifacts in otolith preparation. The variability of Ba/Ca ratios in our experiments with sardine otoliths suggests Ba binding is controlled by a complex of factors.

Manganese. Hydrogen peroxide treatment greatly reduces Mn/Ca ratios in bivalves (Takesue et al. 2008; Krause-Nehring et al. 2011), indicating Mn is metastable and not bound to the aragonite lattice. In our otolith study, overnight exposure to 30% H_2O_2 did not affect Mn/Ca ratios. Protein binding of transition metals may largely explain the presence of these ions in otoliths (Asano and Mugiya 1993; Miller et al. 2006). As in some bivalves (Schöne et al. 2010), it is possible Mn in sardine otoliths may associate with organic matter that would not completely dissolve except under extremely acidic conditions that we did not test.

Mn concentration in otoliths has been shown to reflect ontogeny. Mn was enriched by up to a hundred-fold or more in otolith cores of diverse fish, while Mg and Ba were enriched up to about two- or three-fold in cores, and Sr was not enriched at all (Ruttenberg et al. 2005). Those results indicate larval and juvenile otolith composition changes with age in a variety of fish. In Pacific sardine, the increase in Mn/Ca ratios with both growth and temperature could make it difficult to differentiate ontogeny from environmental parameters affecting Mn incorporation.

Magnesium. Treatment experiments with bivalve and coral aragonite (Watanabe et al. 2001; Takesue et al. 2008; Schöne et al. 2010; Krause-Nehring et al. 2011; and Holcomb et al. 2015), molecular dynamics simulations (Ruiz-Hernandez et al. 2012), and X-ray analytical methods (Yoshimura et al. 2014) have confirmed Mg is largely non-lattice-bound and is associated with organic matter or extra-crystalline matrix in nanodomains of unknown nature (Finch and Allison 2007; Foster et al. 2008). In bivalves, significant amounts of Mg were enriched in the insoluble organic material that did not dissolve in dilute nitric acid commonly used to solubilize aragonite for ICPMS (e.g., 2%), but rather required stronger acidic conditions (Schöne et al. 2010).

Mg appears to be metastable in corals, bivalves, and fish otoliths (Milton and Chenery 1998; Rooker et al. 2001; this study). In H_2O_2 treatment experiments, about 40% of the Mg in the coral skeleton was removed (Watanabe et al. 2001), and about 33% of the Mg in bivalve shells was removed (Takesue et al. 2008). In our otolith

treatment experiments, overnight immersion in H_2O_2 resulted in similar losses of Mg. Instability of Mg was also detected in otoliths of living sardine. A rearing experiment with juvenile sardine using multiple sampling times would better detail the decreases of Mg with age or growth. Tracking Mg in otoliths of wild juvenile sardine to detect regional differences might lead to incorrect conclusions if element loss and otolith size and age are not considered.

Phosphorus. Evidence indicates phosphate is metastable in biogenic carbonates. Various analytical and imaging technologies have detected conformations of phosphate in bivalves and corals (Zhang et al. 2011; Mason et al. 2011). In bivalves, newly formed shell consists of nanospheres of amorphous carbonated Ca-Mg phosphate with high molar ratios of Mg/Ca (0.625 mol mol^{-1}) and P/Ca (0.714 mol mol^{-1}) (Xu and Zhang 2014). Ontogenetic effects recorded as variability in P/Ca ratios in new growth relative to bulk shell composition have been shown at the growing edge of bivalves (Takesue et al. 2008) and early in life close to the umbo (Strasser et al. 2008). If phosphate is largely adsorbed in biogenic aragonite in amorphous forms, extra-crystalline domains of hydroxylapatite, or phosphoproteins, concentrations could change due to temperature or growth. Adsorption and desorption of phosphate on non-biogenic calcium carbonate are temperature-dependent, multistep processes in seawater (Millero et al. 2001). It is likely that phosphate interactions with biogenic aragonite are similarly complex.

The covariance of Mg and P in juvenile sardine otoliths did not reflect a stoichiometry associated with magnesium phosphate minerals. Correlation between Mg and P should be analyzed in invertebrate biogenic aragonites as well to establish whether Mg-P associations are common. Like Mg, the metastability of P may result in uncertainty when evaluating regional attributes of juvenile sardine otoliths unless ontogenetic effects and possible in vivo loss are factored.

Conclusions

A major determinant of trace element composition in juvenile Pacific sardine otoliths was weight or age. Except for Mg and P, growth temperature affected each element differently. H_2O_2 treatment showed Mg and P were largely metastable while growth experiments demonstrated the apparent in vivo loss of Mg and P from juvenile sardine otoliths. The results of this study challenge the assumption that all trace elements in otoliths are permanently bound in living fish. Some trace elements may be inherently metastable due to otolith architecture, the physicochemical properties of aragonite and the associated organic matrix, and the effects of growth that might modify composition. Further studies with time-course sampling of cultured juvenile fish and mollusks might clarify some of the early processes during biogenic aragonite formation.

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