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Incorporation of strontium into otoliths of an estuarine fish

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Abstract

Patterns of Sr/Ca variability in fish otoliths have been widely applied as tracers of movement between freshwater and marine habitats, with the assumption that low salinity habitats correspond to lower otolith levels of Sr/Ca. On the other hand, fluvial estuaries can contain steep gradients in Sr/Ca, and in some estuaries, freshwater values of Sr/Ca can exceed marine values, which are relatively constant across marine habitats. Therefore, to interpret Sr/Ca variability in otoliths of fish that move through estuaries, information is needed about both the incorporation of strontium into otoliths and the nature of the gradient of Sr/Ca in the water. We conducted four experiments to evaluate the incorporation of strontium into fish otoliths under estuarine conditions, using white perch (*Morone americana*) as a model estuarine fish. One laboratory and the two field experiments tested the relationship between Sr/Ca in the otolith and that in the water. A fourth experiment investigated the effect of salinity, independently of the water chemistry (Sr was manipulated while maintaining a constant salinity and Ca level). All four experiments supported a direct relationship between Sr/Ca in the otolith and the water, across a range of estuarine salinities. Results also indicated that the incorporation of strontium into otoliths of estuarine fishes should be constant across broad gradients of Sr/Ca in estuarine waters. While the experiments supported past applications of tracing estuarine and diadromous movements with otolith Sr/Ca chronologies, we emphasize the need to understand the underlying nature of Sr/Ca gradients in estuaries, which may limit or confound reconstructions of estuarine habitat use.

Keywords: Otolith microchemistry; Strontium; Calcium; Estuarine dependency; White perch (*Morone americana*)

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1. Introduction

Information on otolith growth patterns and chemical composition potentially provide detailed, individual chronologies of habitat use that are of great value to ecological studies (Campana and Thorrold, 2001). In fishes that traverse estuarine salinity gradients during their life history, measurements of strontium across microstructural features in otoliths (otolith strontium chronologies) have been used to infer life history periods spent in fresh, brackish, and marine environments. The applications are broad, with recent examples including studies on estuarine dependency in eels (*Anguilla japonica*, Tsukamoto and Arai, 2001), shad (*Alosa sapidissima*, Limburg, 2001), sea trout (*Salmo trutta*, Limburg et al., 2001), and striped bass (*Morone saxatilis*, Secor et al., 2001). These studies and others have been based on the expectation that otolith strontium and salinity are positively correlated; an association that has received limited calibration (Secor and Rooker, 2000).

Strontium variability in otoliths is primarily a function of water chemistry and, to a lesser extent, temperature (Kalish, 1989, 1991; Campana, 1999; Bath et al., 2000; Wells et al., 2003). Dietary sources typically represent a small fraction of strontium uptake in fish otoliths (Berg, 1968; Farrell and Campana, 1996; Milton and Chenery, 2001; but, for an alternative view, see Kennedy et al., 2000). Physiological events such as metamorphosis (Otake et al., 1994) or vitellogenesis (Kalish, 1990) represent episodic signals in the strontium chronology of otoliths. In marine environments, where Sr/Ca is relatively constant (8.5–9 mmol mol^{-1}; Brown et al., 1989), past studies have stressed temperature and physiological effects on otolith strontium variability (e.g., Townsend et al., 1992). In contrast, Sr/Ca in estuarine environments may change significantly between freshwater and seawater, and, among species, much more variability in otolith strontium is attributable to location within the estuary (i.e., salinity) than is attributable to temperature or physiology (Secor and Rooker, 2000). Despite the importance of water chemistry, rigorous evaluation of the relationship between Sr/Ca in otoliths and water has only been conducted in freshwater (see Wells et al., 2003) or seawater conditions by spiking Sr and Ca levels in the water, resulting in ratios that are not often observed in nature (Bath et al., 2000; Milton and Chenery, 2001). Across an estuarine salinity gradient, published information on the incorporation of strontium from water into otoliths is lacking.

By considering the otolith in its environment as a calcium carbonate system with dissolved and solid phases, a proportional relationship is expected between otolith and water strontium (expressed as mmol Sr mol^{-1} Ca; Morse and Bender, 1990). The proportion, D_{Sr}, is called a partition coefficient,

$$D_{Sr} = \frac{(m_{Sr}/m_{Ca})_{otolith}}{(m_{Sr}/m_{Ca})_{water}}, \text{where}$$

m denotes molarity. The value of D_{Sr} depends, at least in part, on the uptake kinetics of both Sr and Ca. Calcium is highly regulated by fish and otoliths are almost pure calcium carbonate (~ 96% by mass; Campana et al., 1997). Further, in a model of whole body uptake in carp (*Cyprinus carpio*), uptake rates of calcium were saturated at low calcium levels in the water (Chowdhury and Blust, 2001).

Chowdhury and Blust (2001) observed that Ca and Sr act as competitive inhibitors: the two ions are similar in valence and radius and therefore compete for the same uptake pathway. In most aquatic environments, strontium is a trace element whereas calcium is a major ion, so strontium is unlikely to inhibit calcium uptake. Thus, it is the concentration of strontium relative to calcium in the water, and not the absolute concentration of strontium, that should determine uptake of strontium into the otolith. This point is best demonstrated by plotting a hypothetical scenario of Sr uptake across a range of aqueous Sr levels (Fig. 1) using the Michaelis–Menton model developed by Chowdhury and Blust (2001). Note that strontium uptake is insensitive to a wide range of strontium concentrations in the water (i.e., salinity levels), but there is a direct relationship between strontium uptake and Sr/Ca in the water (Fig. 1).

In fluvial estuaries, the concentrations of Sr and Ca change linearly (conservatively) with salinity (proxy for the mixing of freshwater and seawater), but the gradient of Sr/Ca is curvilinear with most of the variation occurring below salinity ~ 8 (e.g., Surge and Lohmann, 2002). Importantly, the mixing curve of Sr/Ca depends almost entirely on the freshwater value. A survey of coastal draining rivers and creeks in the U.S. (Fig. 2)

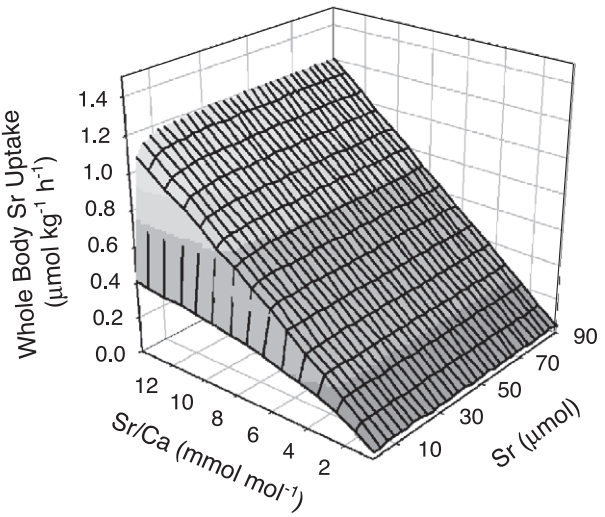

Fig. 1. Model of whole body strontium uptake across a range of strontium concentrations (range expected between freshwater and marine end members) and a range of possible aqueous Sr/Ca values. Uptake rate was calculated based on a carp model (*C. carpio*) developed by Chowdhury and Blust (2001). The model includes hydrogen ion concentration (H^+) as a variable; however, for this exercise we held it constant (neutral, pH = 7.0). The model takes a Michaelis–Menton form,

$$j_{Sr} = J_{maxSr}\frac{\beta_{Sr}(H^+) + K_{iH}}{(H^+) + K_{iH}}\frac{(Sr^{2+})}{(Sr^{2+}) + K_{mSr}[1 + (Ca^{2+})/K_{iCa}]},$$

where strontium and calcium act as competitive inhibitors, j_{Sr} is the uptake, and the model parameters used were those estimated by Chowdhury and Blust (2001): $J_{maxSr} = 293.0$, $\beta_{Sr} = 0.35$, $K_{iH} = 0.54$, $K_{mSr} = 96.3$, and $K_{iCa} = 28.5$. Note that Sr uptake rate is relatively insensitive to changes in strontium, but most sensitive to Sr as a relative concentration of calcium (expressed as Sr/Ca).

illustrates a wide range in potential freshwater end values. These data were obtained on-line from the U.S. Geological Survey, National Water Inventory Service (http://waterdata.usgs.gov/nwis), and are calculated from total dissolved Sr and Ca concentrations at a given site (the number of samples at a site varied). Only sites that might be encountered by a diadromous fish were selected. As expected, the distribution is skewed with most values much less than the marine end member (Fig. 2). Still, some freshwater values substantially exceeded Sr/Ca expected for seawater. Notably, these sites included tributaries of: the Raritan River, New Jersey; Choptank River, Maryland; small creeks near Sitka and Kenai, Alaska; and St. John's River, Peace River, Myakka River, Phillipi Creek, and Little Manatee River in Florida. While mean values of Sr/Ca >9 mmol mol^{-1} constituted 12% of all sites surveyed, the distribution in surveyed ratios may not be representative of all coastal draining rivers and creeks in the U.S. Nevertheless, if the aqueous concentration of Sr/Ca is the primary determinant of the concentration in the otolith, then we might expect that otolith Sr/Ca for fish reared in freshwater with Sr/Ca levels exceeding 9 mmol Sr mol^{-1} Ca could exceed otolith Sr/Ca for the same fish reared in seawater—a result never before reported in the literature.

The purpose of our study was to quantify experimentally the relationship between Sr/Ca in otoliths and the water, across an estuarine salinity gradient. Because most of the change in the mixing curve of Sr/Ca typically occurs at salinities < 8, we chose Patuxent River (Maryland, USA) white perch, *Morone americana* (Gmelin), as a model system.

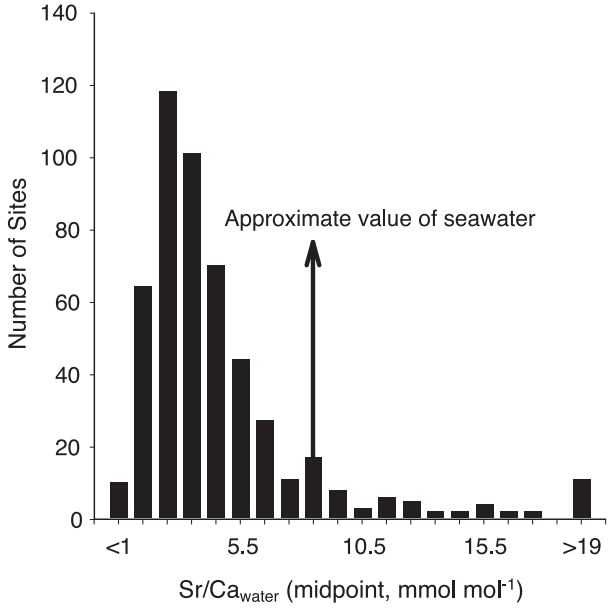

Fig. 2. Distribution of aqueous (surface water) Sr/Ca values for coastal draining rivers and creeks in U.S. coastal states. For reference, the value of Sr/Ca in seawater is indicated by the arrow. Note that the tail of the distribution has been compressed into a single category (>19 mmol mol^{-1}). Data were obtained from the U.S. Department of the Interior, U.S. Geological Survey (available at http://waterdata.usgs.gov/nwis), and constituted 7921 samples from 507 sites in 169 unique hydrologic units.

White perch have a unique anadromous migratory behavior in that they remain within the estuary for the duration of their lives. Spring spawning migrations to tidal freshwater (where eggs and larvae develop) are followed by a return to brackish habitats, and adults are rarely found at salinities greater than 20 (Mansueti, 1961). As young-of-the-year, juveniles disperse throughout the estuary from freshwater to salinities no greater than 15 (unpublished seine survey data, Maryland Department of Natural Resources, Annapolis), and during this life-stage (May through November), the Patuxent River estuary has a relatively stable salinity gradient with a mean salinity between 10 and 16 at the entrance (Ritchie and Genys, 1975). Thus, juvenile white perch in the Patuxent River represent a closed population, living across a range of salinities in which aqueous Sr/Ca is expected to show large variation.

The objectives of our study were to: (1) quantify the relationship between Sr/Ca in otoliths and water between freshwater and mesohaline salinities in the laboratory, (2) conduct analogous field experiments by placing caged fish at different locations within the Patuxent River estuary, and (3) determine whether salinity has an effect on Sr/Ca in otoliths independent of aqueous Sr/Ca. For the first two objectives, we designed experiments that used salinity as a measure of the amount of mixing between freshwater and seawater end members, and then quantified otolith Sr/Ca and the aqueous Sr/Ca mixing curve for comparison. Our third objective was driven by the hypothesis that salinity might have an independent effect from aqueous Sr/Ca on Sr uptake rates due to osmoregulatory activity or the effects of other seawater constituents. In the laboratory experiment related to this hypothesis, salinity levels were varied under constant conditions of aqueous Sr/Ca.

2. Methods

During June 2001, juvenile perch (<65 mm TL) to be used in the laboratory and caging experiments that year were captured with a beach seine at river km 38 (river km is distance from river mouth) in the Patuxent River estuary, and transported to a holding tank at Chesapeake Biological Laboratory (CBL), near the entrance of the estuary. Flow-through holding tank conditions were maintained at salinity 6 and 23 °C (similar to the conditions at capture) by mixing groundwater with filtered (ca. 0.1 μm) water from the laboratory pier (salinity ca. 13–14). Chironomid larvae (from San Francisco Bay Brand, Inc., California, USA) were supplied *ad libitum*. In the 2002 caging experiment, juvenile perch were captured in July at river km 70, and the experiment was initiated without a holding period (see following sections).

At the beginning of each experiment, fish were batch marked with alizarin complexone (20 mg l^{-1}, by immersion for 24 h), then anesthetized with MS-222© (by immersion at 90 mg l^{-1}), and total length and weight were recorded. Individuals were then randomly assigned to experimental tanks or cages. At the end of each experiment, fish were sacrificed and total length and weight were again recorded. Immersion in alizarin complexone produced a red band, observed through epifluorescent microscopy, which demarcated the beginning of the experiment within the otolith's microstructure. Otolith growth (i.e., precipitation rate) was proxied by the distance from the alizarin mark to the

edge of the otolith along the ventral side of the sulcus. The measurement axis was oriented perpendicular to the growth rings.

2.1. Experimental design

Four experiments were conducted, and are hereafter referred to as the dilution experiment, the 2001 and 2002 caging experiments, and the salinity experiment. The dilution experiment was designed to quantify otolith Sr/Ca at four salinities (1, 3, 8, and 13), representing the natural range across which juvenile fish occur. Four blocks of four tanks each were used (blocks represented the positional arrangement of tanks within the laboratory), and within each block, the salinity treatments were randomly assigned to each tank (70 l), such that salinity was replicated four times. Within each tank, salinity treatments were achieved by mixing groundwater (salinity = 0) with filtered estuarine water (salinity ca. 13–14) from the laboratory pier. To maintain a constant temperature (ca. 27 °C) across all tanks, groundwater was heated to match the pier water temperature before it was mixed within the tanks. It should be noted that despite our efforts to maintain a constant temperature across all tanks, there was a significant correlation between mean observed salinity and mean observed temperature among all tanks (Pearson: $r = 0.99$, $p < 0.001$). This temperature differential was small, only 1.5 °C, with a coefficient of variation (CV) of 2%. Within each salinity treatment level there was also a correlation between mean observed salinity and mean observed temperature, and the magnitude of this correlation was equivalent to that observed among all tanks. However, at these temperatures and with our otolith assay (see *Chemical Analyses* below), previous work indicated that we would be unable to detect a response in otolith chemistry to such small changes in temperature (Toole and Nielsen, 1992; Campana, 1999; Bath et al., 2000). Flow-through circulation was maintained for the duration of the experiment (28 days, from 16 July to 12 August 2001). Each tank held 10 perch, which were fed two to three times daily. Salinity, temperature and consumption (as wet weight of chironomid larvae) were recorded daily.

For the 2001 caging experiment, perch were placed in cages at different locations in the Patuxent River estuary, corresponding to similar salinity regimes as in the dilution experiment. Two cages were placed at different locations at river km 72 in freshwater, and three other cages were placed at different brackish water sites, river km 53, 38, and 4 (Fig. 3). Brackish and freshwater habitats formed the basis for a statistical comparison of otolith Sr/Ca (see *Statistical Analyses section*). Temperature and salinity measurements for each site were obtained in various ways. They were recorded (daily) on weekdays at river km 4 (CBL pier), and at a fossil fuel power generating facility near river km 38. The cage at river km 53 was equipped with a data storage tag to record temperature and salinity (15-min intervals), and routine (15-min intervals) monitoring of water quality at Jug Bay National Estuarine Research Reserve (data available at http://cdmo.baruch.sc.edu/cbm.html; collected under NOAA/NERRS System-Wide Monitoring Program, Julie Bortz—principal investigator) was conducted adjacent to the river km 72 site.

The cages were constructed of a steel frame (102 × 102 × 51 cm deep) suspending a polyester pen (79 × 79 × 43 cm deep), with approximately 1 cm diameter mesh. Preliminary caging trials indicated that biofouling of the cages could serve as a natural food

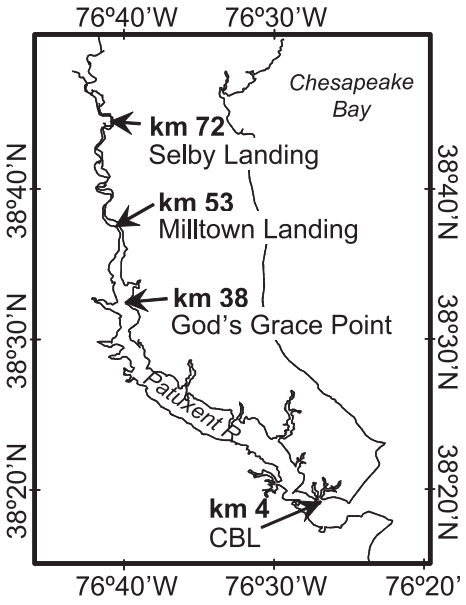

Fig. 3. Locations used in the white perch caging experiment, Patuxent River estuary, Chesapeake Bay, Maryland, USA. River km is distance from the mouth of the river, and CBL is Chesapeake Biological Laboratory.

source for caged perch; therefore, cages were placed off the CBL pier for 2 weeks prior to the experiment to initiate fouling. Ten perch were placed in each cage, and the experiment lasted 30 days (17 July to 15 August 2001). The cages were checked on day 17 to enumerate perch and inspect and clean the data storage tag.

The 2002 caging experiment was designed to test whether movements between freshwater and brackish habitats would be recorded in the Sr/Ca otolith chronologies, and in this experiment juveniles held in cages were manipulated by moving some of the cages to either freshwater or brackish sites. The experiment was a modification of Ebbutt's (1984) three-period crossover design with two treatments where each animal receives all treatments. The two treatments were freshwater (FW) and brackish (BR) habitats, and three sequences were used (Table 1). The sequences were chosen to represent early (FW–BR–BR) and late (FW–FW–BR) movements from the natal (freshwater) habitat and a return to the natal habitat (FW–BR–FW). Each period was approximately 1 month, but it should be noted that the first period occurred before initial capture (Table 1). Because individuals used in this experiment were captured in freshwater, we made the assumption that otolith material that was deposited immediately prior to the first alizarin complexone mark could be used as a freshwater treatment, constituting the first period (salinity was assumed to be 0.5 during this period). There were three replicates of each sequence, such that there were paired cages at each of three brackish sites and a single cage at each of three freshwater sites (Table 1). Thus, each group of fish in each of the freshwater cages would be transferred to one of the brackish cages between periods II and III, and one group from each pair at each of the brackish water sites would be transferred to a cage in

Table 1
Caging experiment results from 2002, where juvenile white perch in cages were moved between estuarine locations (either freshwater, FW, or brackish, BR) in a sequence of 3-month-long periods (I, II, and III)

Cage	Sequence	*N*	S		Otolith Sr/Ca		
			Period-II	Period-III	Period-I	Perio-II	Period-III
1	–	0	–	–	–	–	–
2	FW–BR–FW	4	7.1	2.1	1.28	2.49	2.12
3	FW–BR–FW	9	8.5	1.1	1.34	2.74	2.51
4	FW–BR–BR	9	6.4	7.8	1.35	2.47	2.77
5	–	0	–	–	–	–	–
6	–	0	–	–	–	–	–
7	FW–BR–BR	4	8.5	9.4	1.54	2.78	3.11
8	FW–FW–BR	2	2.1	9.4	1.40	2.11	2.56
9	–	0	–	–	–	–	–

Approximate salinity (*S*) values are shown, and the least-squares mean otolith values of Sr/Ca (in mmol mol^{-1}) are based upon individuals that survived the experiment (*N*). Four of the cages were lost due to various complications; therefore, zero sample size is indicated along with no data for other columns. Period-I salinity was assumed to be 0.5 across all treatments (see text).

freshwater. Though the cages were not physically moved, for convenience we refer to the groups of juveniles as 'cages' (Table 1). At the beginning of the experiment, there were nine cages each with 10 juvenile white perch.

In the 2002 caging experiment, juveniles were held for 2 months (periods II and III), and between the first and second month (27 August), juveniles from the cages were returned to the lab for a second marking with alizarin complexone (weight, and length were again recorded during this event). The three freshwater sites were situated at river km 72 and approximately 2 km upstream and downstream. The three brackish sites were situated at river km 53 and approximately 3 km upstream and downstream. Approximate salinities were calculated as the mean of measurements at the beginning and end of each month-long period. Mortality between cages was highly variable and some cages experienced complete mortality (Table 1). Therefore, for the 2002 caging experiment, we did not make inferences about growth (somatic or otolith) or mortality with respect to site location or treatment. In addition, to minimize the potential for carry-over effects (see Ebbutt, 1984 or discussion in Kuehl, 2000), we limited our analysis to microprobe results that corresponded to the last half of each experimental increment.

The salinity experiment was a completely randomized factorial design with three salinity levels (0, 7, and 14) and two strontium levels (1 and 4 mg l^{-1}) for six treatments total. There were two replicate tanks (40 l) per treatment, and calcium was maintained at a constant level (90 mg l^{-1}) in all 12 tanks. The strontium and salinity treatments represented a range of typical estuarine values, and the calcium level represented an intermediate value that was not limiting (based on Chowdhury and Blust, 2001) and an intermediate estuarine value. Mean calcium and strontium concentrations in the water were measured (from two samples per treatment) and found to be close to target levels (Ca = 92 mg l^{-1}, S.E. 0.7; Sr_{Low} = 0.9 mg l^{-1}, S.E. = 0.03; Sr_{High} = 4.6 mg l^{-1}, S.E. = 0.07), indicating that treatments were formulated properly. Tanks were supplied with aeration, and water conditions were static with complete water changes on alternate days. Five

perch were placed in each tank and fed ad libitum. Temperature and salinity were recorded daily, for the duration of the experiment (28 October to 21 November, 2001). Batches of water that were used to conduct water changes were mixed in advance using groundwater and appropriate amounts of ultrapure calcium chloride (dihydrate) and ACS reagent-grade strontium chloride (hexahydrate). Amounts were determined based on the amount of sodium chloride used to generate the correct salinity. The use of reagent grade sodium chloride was determined to be prohibitively costly for this experiment; therefore, screening of alternative sources of sodium chloride was conducted. It was found that a commercial grade of non-iodized table salt contained levels of calcium (<1.0 mg g^{-1} dry salt) and strontium (below detection limits) low enough to permit target experimental treatment levels to be achieved.

2.2. Chemical analyses

In the dilution experiment, five perch were subsampled from each tank for otolith chemistry analysis, whereas otoliths from all perch were analyzed in the caging and salinity experiments. Extracted otoliths were dried and embedded in epoxy resin. A low-speed saw with a diamond-coated blade was used to cut a transverse thin-section from each otolith. The sections were mounted on glass slides with thermoplastic glue, and were ground and polished by hand with aluminum oxide lapping paper and alumina powder until the core of the otolith was exposed. Preparation of otolith sections for microprobe analyses was conducted such that an ideal section was flat, approximately 0.25–0.5 mm thick, and smooth in appearance at 200× magnification.

Electron microprobe wavelength dispersive X-ray spectroscopy (WDS) has been used frequently for quantifying calcium and strontium in otoliths and was chosen for use in this study. Electron microprobe WDS analysis has been shown to provide reliable measurements of Sr and Ca that compare favorably to more sensitive methods (Campana et al., 1997; Secor et al., 2001). Our approach was to probe five spots, each approximately 100 μm^2 that were arranged along the margin of the otolith between the sulcus and the ventral tip (Fig. 4). In the 2002 caging experiment, five spots were probed in each otolith increment that corresponded to each experimental period, for a total of 15 spots per sample. The probed areas of the otolith sections represented the last 1–2 weeks of the experimental periods, which were identified through the demarcation of the experimental start with alizarin complexone and daily increment counts. The mean Sr/Ca from the five probed spots was used as the response value from each fish. Analyses were conducted at the University of Maryland Center for Microanalysis with a JEOL JXA-8900 electron probe microanalyzer. Operating conditions were 25 kV and 20 nA, under high vacuum, and samples were carbon coated in an evaporator. Calcite (Smithsonian Institute-National Museum of Natural History USMN136321) and strontianite (USMN R10065) standards were used, and during probe sessions, detection limits averaged 68 (S.E. = 0.04) and 93 mg g^{-1} (S.E. = 0.08) for calcium oxide and strontium oxide, respectively (see Secor, 1992 for further details on the microprobe precision and beam–sample interactions).

Water chemistry was analyzed to characterize Sr/Ca mixing curves and to confirm that treatments in the salinity experiment were correctly formulated. Samples were collected (unfiltered) with acid-washed Teflon vials and acidified with nitric acid (to 1%)

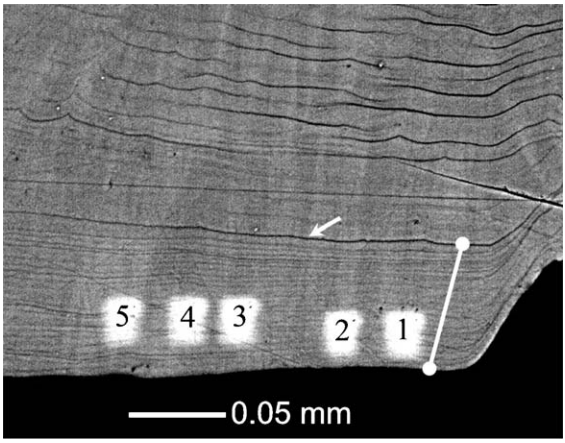

Fig. 4. Electron backscatter image of representative juvenile white perch otolith section from the dilution experiment. Electron microprobe sampling spots, which are numbered, are evident as 'burn' marks in the carbon coating. There is also a check mark within the otolith chronology (indicated by the arrow) that corresponds to the alizarin complexone mark and the beginning of the experiment. Measurements of otolith growth were made near the sulcus, as the distance from the alizarin mark to the edge of the otolith. The measurement axis (line with dot-ends) was oriented perpendicular to the growth bands, which were visible with transmitted light as well as in the electron images.

before analysis. Initially, we analyzed water samples with inductively coupled plasma mass spectrometry (ICPMS), but due to analytical problems, it was necessary to mix and collect additional water samples (using the same procedures as those in the laboratory experiments and caging study). Comparison of calcium concentrations measured with and without internal standard revealed divergent results from ICPMS, suggesting that the estuarine water sample matrix may have interfered with calcium detection (this was not the case when we measured pure groundwater samples). The best method for compensating for matrix effects is to use an estuarine water reference material that is certified for calcium, but none was available. To overcome problems with the ICPMS analyses, the subsequent water samples were analyzed with atomic absorption spectrometry (AAS). Consequently, note that for all of our experiments (including the salinity experiment) water chemistry was quantified from samples taken after the experiments ended. This situation precluded direct estimation of the partition coefficients, D_{Sr}, but still permitted us to order the otolith chemistry results according to the characteristic patterns of the mixing curves in the laboratory and field experiments. AAS does not typically have interference problems when measuring calcium; therefore, a Perkin-Elmer machine, model 2380, was used for all Sr and Ca water chemistry analyses, with the exception of Sr/Ca reported for pure groundwater samples. Strontium in groundwater was below detection limits of AAS, and because ICPMS calcium measurements in groundwater were considered to be reliable (no matrix effects were indicated in comparison of measurements with and without internal standard), we used ICPMS to measure calcium and strontium in groundwater samples. For other samples, ICPMS and AAS gave similar strontium concentrations (Pearson: $r = 0.99$).

To characterize mixing curves of Sr/Ca in the laboratory and in the Patuxent River estuary, replicate assays were performed on different occasions (15 May, 24 June 2002 for both the dilution experiment and the Patuxent River, and additionally on 27 August 2002 for the Patuxent River). The water samples for the laboratory mixing curve were generated by combining groundwater with water supplied to our laboratory from the CBL pier. In samples generated in May, pier water salinity was 15.4, and in June, pier water salinity was 12.5. Note that this introduced between-replicate variability to the mixing curve and that the salinity of the pier water during the dilution experiment was 13.5.

2.3. Statistical analyses

In all four experiments, otolith Sr/Ca response values were log-transformed to satisfy normality and variance homogeneity assumptions of performed tests. Standard error estimates from log-transformed data cannot be transformed back onto a meaningful scale (see Sokal and Rohlf, 2000); therefore, we characterized the uncertainty in our estimates by plotting and reporting asymmetric 95% confidence intervals of the mean. For the caging experiments, uncertainty of cage means was characterized by 95% confidence intervals plotted in the figures. Confidence intervals of least-squared means are plotted for the dilution experiment, and the raw cage and tank means for all experiments are reported in tables. Residuals were inspected graphically to determine whether assumptions were valid and an experiment-wise significance level of alpha = 0.05 was selected prior to conducting statistical testing.

Otolith Sr/Ca responses from both the dilution and salinity experiments were analyzed using tank means. The dilution experiment was analyzed as a randomized block ANOVA with four salinity treatments. This same analysis was also used to test for treatment effects on other potential confounding responses: otolith precipitation (proxied by otolith increment width), consumption, and mortality. Multiple comparisons between treatment levels were conducted post-hoc using Tukey adjusted p-values. To test for treatment effects on somatic growth, final mean weights were analyzed with the randomized block ANOVA by adding initial weight as a covariate. While five perch were initially subsampled from each tank for microprobe assays, three otolith sections (out of 80) were determined to be unsuitable due to preparation flaws, and these were excluded from the analyses (see Table 2). The salinity experiment was analyzed as a fully factorial 2-way ANOVA with salinity level, Sr/Ca level, and their interaction.

In the caging experiments, as in the laboratory experiments, we treated cage means as the response. In the 2001 caging experiment, a single factor ANOVA was used to test for differences in otolith Sr/Ca between freshwater and brackish habitats. Possible confounding responses, otolith precipitation rate (proxied by otolith increment width) and temperature, were also analyzed with the same single factor ANOVA. Similar to the dilution experiment, this analysis was modified by incorporating initial mean weight as a covariate in a test of final weight as another confounding response. In the 2002 caging experiment, cage means of otolith Sr/Ca from each period were analyzed as a fully factorial 2-way ANOVA with treatment (freshwater or brackish), period (I, II, or III), and their interaction. Correlation between repeated measurements on the same cage was modeled by using a first-order autoregressive covariance structure (see Littell et al., 1996).

Table 2
Dilution experiment results where juvenile white perch were exposed to varying levels of salinity (*S*)

S	Block	*T*	Initial size	Final size	*C*	*N*	Increment	Otolith Sr/Ca	*n*
0.8	2	25.7	1.6	3.3	275.6	10	89	3.98	4
1.0	4	25.7	1.8	3.5	322.2	10	85.7	4.13	5
1.1	1	25.7	1.1	3.0	187.5	7	84	3.39	5
1.4	3	25.8	1.9	3.6	323.6	10	85.5	3.90	5
3.0	1	25.8	2.0	3.6	289.5	10	76.9	3.35	5
3.1	2	25.9	1.7	3.1	331.8	10	79.4	3.50	5
3.3	3	25.9	1.7	3.1	337.8	10	79.3	3.69	5
3.3	4	26.0	2.3	4.0	325.2	10	79.1	3.65	5
7.3	3	26.3	1.3	2.6	213.1	8	83.2	3.62	5
7.7	2	26.4	1.7	3.2	335.7	10	99.6	3.42	5
7.8	1	26.4	1.3	2.9	282.6	10	91.4	2.99	5
7.9	4	26.5	2.1	4.0	369.4	10	80.7	3.69	5
12.8	3	27.0	2.1	3.8	321.1	9	89.6	3.38	5
13.1	1	27.1	1.3	2.6	249.4	8	82.6	3.04	4
13.2	2	27.2	1.8	3.3	344.9	10	91.9	3.15	4
13.2	4	27.1	2.1	3.9	369.1	10	85.2	3.28	5

Mean values in each tank (row) are shown for temperature (*T*, in °C), size (in g), and marginal otolith increment (in μm). Total amount of chironomid larvae consumed (*C*) during the experiment was measured in grams (fresh weight), and the mean otolith value of Sr/Ca (in mmol mol^{-1}) is based on a subsample (*n*) of perch that survived the experiment (*N*).

This analysis tested our ability to detect movements between freshwater and brackish habitats using Sr/Ca measurements from individual otoliths.

Mixing curves between aqueous Sr/Ca and salinity were estimated as power curves (see Surge and Lohmann, 2002). For the Patuxent River and in the laboratory, data were fitted using linear regression of log-transformed salinity and Sr/Ca values. Confidence intervals around the mean of the mixing curves were estimated by treating water samples as repeated measurements from a given month. This approach provided a characterization of the mixing curve. To compare otolith Sr/Ca responses with the water chemistry patterns, we used salinity to match otolith values with least-squares estimates of means from the mixing curves. Therefore, we implicitly assumed that while the absolute level of the mixing curve can fluctuate, its shape remains reasonably constant. This approach should be viewed as constructing a scenario, where given a particular mixing curve we examine the expected association between chemistry of the otolith and the water.

3. Results

3.1. Dilution experiment

There was an inverse relationship between otolith Sr/Ca and salinity (Fig. 5, upper panel), and a significant treatment effect was detected ($F_{0.05,3,9} = 19.14$, $p < 0.001$). This contrasted with the general expectation of a positive correlation between salinity and otolith Sr/Ca, which has heretofore dominated the literature (see review by Secor and

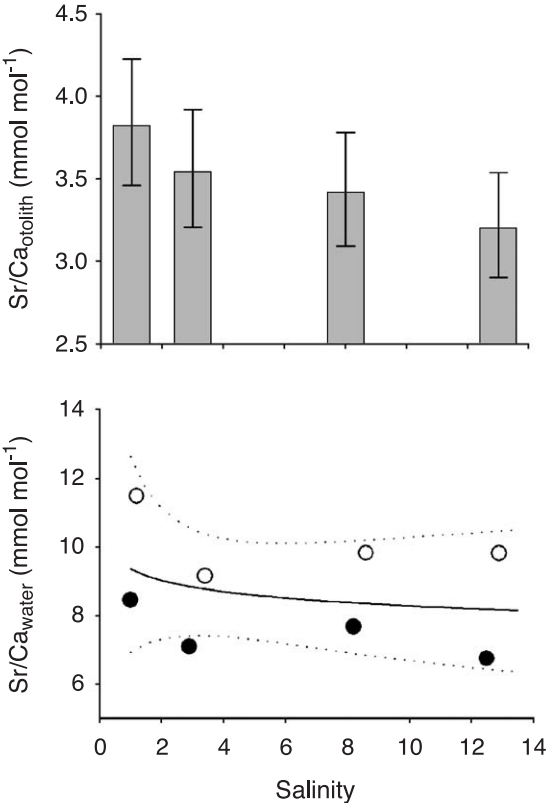

Fig. 5. Comparison of otolith Sr/Ca means and expected Sr/Ca mixing curve in white perch dilution experiment. Least-squares means of otolith Sr/Ca with 95% confidence intervals are plotted versus salinity (upper panel). The mixing curve results were generated by diluting water from the CBL pier with groundwater supplied to our laboratory (lower panel). The black dots and open circles represent samples that were formulated in two different months, and the dotted curves are 95% confidence intervals of the least-squares mean mixing curve.

Rooker, 2000). Still, this inverse trend was consistent with the inverse mixing curve between Sr/Ca and salinity observed in the laboratory water (Fig. 5, lower panel). While the mean relationship was inverse, the slope of this mixing curve was not significant, but the intercept was highly significant (regression: $t = 18.2$, $df = 6$, $p < 0.001$), demonstrating that aqueous Sr/Ca was high and constant across the salinity range investigated (Fig. 5). In addition, while we did not rear any fish in pure groundwater from our lab, the value of Sr/Ca in this freshwater end value was several-fold higher than brackish water Sr/Ca levels (due to low calcium), varying between 22 and 27 mmol mol^{-1}. This supported both the inverse nature of the mixing curve as well as the expected high aqueous Sr/Ca values in the dilution experiment. Thus, while the dilution of water from the Patuxent River with groundwater produced an unexpected mixing curve, a direct relationship between the chemistry of the otolith and the mean mixing curve estimated for the laboratory water was still observed (Fig. 6, filled circles).

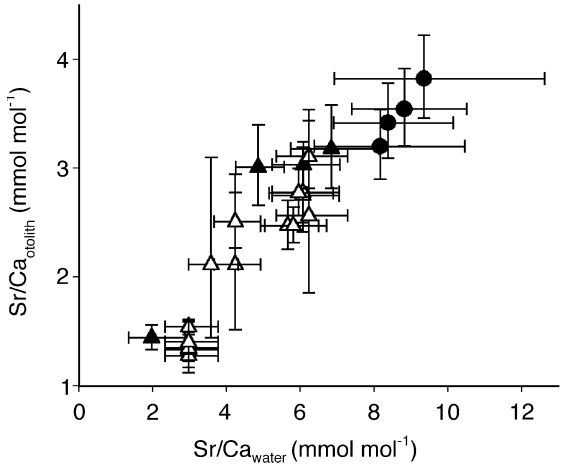

Fig. 6. Relationship between Sr/Ca in white perch otoliths and aqueous Sr/Ca. Though paired measurements from the otolith and the water were not available, the water chemistry values are estimated from the mean mixing curve relationships (shown in either Fig. 5 or Fig. 7). The error bars demarcate 95% confidence intervals along both axes. Data from both caging experiments (triangles) and the dilution experiment (dots) are shown. Open and filled symbols represent data from years, 2002 and 2001, respectively.

Both somatic growth and otolith precipitation rates may affect otolith Sr/Ca, and we used tank means of initial and final weight (g) and marginal increment width to test for possible confounding interactions (Table 2). A significant treatment effect on marginal increment width was observed, ($F_{0.05,3,9} = 4.63$, $p = 0.032$) and Tukey multiple comparisons revealed only one significance difference ($p = 0.039$) between salinity 3 and 8 treatments. In the ANCOVA of final weight, salinity ($F_{0.05,3,5} = 9.44$, $p = 0.017$), initial weight ($F_{0.05,1,5} = 361$, $p < 0.001$), and their interaction ($F_{0.05,3,5} = 6.8$, $p0.033$) were significant. The results indicated that growth declined with salinity for perch that were initially small, and that growth increased with salinity for larger perch. Growth in total length (10 mm month^{-1}) observed during the experiment was equivalent to that estimated by length frequency analysis of wild juvenile fish in the Patuxent estuary for the period 1989–2001 (unpublished seine survey data, Maryland Department of Natural Resources, Annapolis).

Other potentially measurable influences on the Sr/Ca response in perch otoliths during this experiment included consumption (direct) and mortality (indirect). We detected no treatment effects on consumption, even when tank 2, which was biased towards a low consumption value due to mortality of three individuals, was excluded. In addition, the small amount of mortality that we observed overall (8 fish out of 160) was not associated with any particular treatment or block of tanks (Table 2).

3.2. Caging experiment 2001

A positive association between Sr/Ca in the otolith and salinity was observed in the 2001 caging experiment (Fig. 7, upper panel). A significant difference ($F_{0.05,1,3} = 1136$, $p < 0.001$) in otolith Sr/Ca was observed between brackish (Sr/Ca = 3.08 mmol mol^{-1}) and

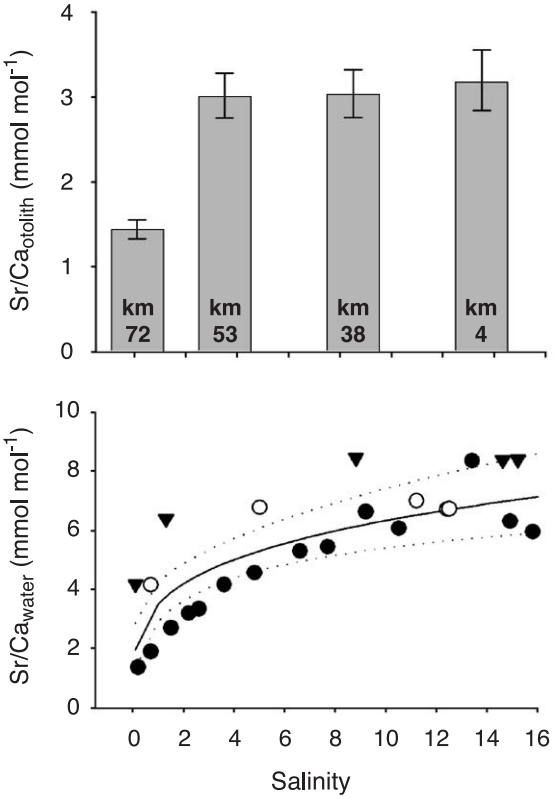

Fig. 7. Comparison of otolith Sr/Ca means and expected Sr/Ca mixing curve in the 2001 white perch caging experiment. Mean values of otolith Sr/Ca that were observed at the four locations in the caging experiment are plotted with 95% confidence intervals versus salinity (upper panel). Bars are labeled by location (river km). The mixing curve results (lower panel) were generated from samples collected in three different months (different symbols). Dotted curves represent 95% confidence intervals of the least-squares mean mixing curve.

freshwater (Sr/Ca = 1.45 mmol mol^{-1}). In addition, marginal increment values of field-collected juvenile perch from these same salinity regimes in the Patuxent (brackish: Sr/Ca = 2.72 mmol mol^{-1}, $n = 16$, S.E. = 10^{-5}; freshwater: Sr/Ca = 1.13 mmol mol^{-1}, $n = 6$, S.E. = 10^{-6}) were similar (albeit slightly lower) than in the caging experiment. Mean marginal increment widths were significantly higher in the brackish habitat ($F_{0.05,1,3} = 321$, $p = 0.015$), and no differences in mean temperature or growth were detected between habitats. The mixing curve between Sr/Ca in the water and salinity showed a curvilinear increase (Fig. 7, lower panel), and, as in the dilution experiment, a direct relationship is present between the mean mixing curve estimate for the Patuxent River and Sr/Ca in the otolith (Fig. 6, filled triangles). The slope of the mixing curve was found to be highly significant ($t = 5.5$, $df = 22$, $p < 0.001$).

All of the mortality in the 2001 caging experiment occurred at the start, as no mortality was observed between the mid-term cage inspection and the end of the experiment. There

was intermediate growth and high survival at the intermediate sites (river km 53 and 38, see Table 3), probably due, in part, to high biofouling of the cages with encrusting bryozoans, hydroids, and tunicates, which attracted abundant prey items (e.g., gammarid amphipods, polychaete worms, and *Palaeomonetes* sp. shrimp). At river km 72, no additional biofouling occurred after the cage was transferred from CBL, and low growth and survival in the cages was related to a probable low encounter rate of benthic invertebrates, small planktonic organisms, and other prey items. In contrast, the cage at river km 4 (CBL) experienced the highest biofouling and somatic growth of all the cages; therefore, lack of prey did not explain the low survival. Instead, high salinity at this site may have been a lethal stress for some individuals, as evidenced by the lack of wild juvenile fish at and above salinities recorded at this site (unpublished seine survey data, Maryland Department of Natural Resources, Annapolis).

3.3. Caging experiment 2002

The caging experiment results in 2002 supported the ability of our assay to detect movements of juvenile perch between freshwater and brackish habitats recorded in the Sr/Ca chronologies of otoliths. As expected, a significant treatment effect of freshwater versus brackish salinity ($F_{0.05,1,4} = 32.34$, $p = 0.005$) was detected along with a significant period effect ($F_{0.05,2,6} = 87.22$, $p = 0.001$). No interaction between treatment and period was detected ($F_{0.05,1,6} = 0.19$, $p = 0.679$), indicating that time or ontogenetic effects on otolith Sr/Ca are negligible. Mean Sr/Ca in the FW treatment was slightly higher in period II than in period I (Table 1), but this was due to differences in salinity. Note in period I freshwater salinity was assumed to be 0.5, whereas salinities in the freshwater treatment for period II in fact ranged between 1 and 3. Variable mortality and the complete loss of some cages were due to various factors. In one instance, a large floating tree branch pierced one of the mesh panels on a cage, releasing all of the fish inside. At another location, a cage was completely filled with mud and silt, suffocating all of the fish inside. Freshwater sites seemed to be associated with lower survival during period II, and this may be partly due to the slower rate of biofouling at these sites. Regardless, the survivors of the experiment provided observations from the steepest part of the mixing curve, salinities between 1 and 3. Based upon the approximate salinities for each period in each cage and

Table 3
Juvenile white perch caging experiment results from 2001

Cage	Location (river km)	*S* (CV)	*T* (CV)	Initial size	Final size	*N*	Otolith increment	Otolith Sr/Ca
1	72	0.1 (18%)	26.4 (12%)	2.3	2.7	6	77.1	1.45
2	72	0.1 (18%)	26.4 (12%)	2.2	2.8	3	85.6	1.44
3	53	3.5 (25%)	27.5 (5%)	2.0	3.9	10	97.9	3.01
4	38	8.4 (11%)	28.6 (6%)	1.5	3.1	9	98.3	3.03
5	4	14.1 (2%)	26.5 (9%)	1.4	3.7	6	96.9	3.18

Initially, 10 juveniles were placed in each cage and the cages were deployed for a month. Mean values from each location/cage are shown for salinity (*S*), temperature (*T*, in °C), size (in g), and marginal otolith increment (in μm). The mean otolith value of Sr/Ca (in mmol mol^{-1}) is based on analysis of individuals that survived the experiment (*N*).

Table 4
Salinity experiment results where juvenile white perch were held in a factorial design with two aqueous Sr/Ca levels and two salinity levels

Aqueous Sr/Ca	Salinity	*T*	Initial size	Final size	*N*	Otolith increment	Otolith Sr/Ca
Low	0.1	20.1	3.8	3.9	4	27.3	2.63
Low	0.1	20.1	3.0	3.3	5	27.8	2.68
Low	7.0	20.0	5.9	6.4	4	29.2	3.04
Low	7.0	20.1	3.6	3.7	5	26.7	2.96
High	0.1	20.0	4.9	5.5	5	27.7	4.33
High	0.1	20.0	3.3	3.6	3	29.4	4.31
High	7.0	20.2	4.9	6.8	4	23.0	4.37
High	7.0	20.1	5.3	5.1	4	24.8	5.64

Means for temperature (*T* in °C), size (in g), mean marginal otolith increment (in μm), and Sr/Ca (mmol mol^{-1}) in the otolith are based on the surviving individuals (*N*). Initially, each tank (row) had five fish. Aqueous Sr/Ca treatment levels are described in the text and correspond to low and high levels of dissolved strontium (calcium was held constant).

least-squares mean values of otolith Sr/Ca for each experimental period in each cage (Table 1), we used the mean mixing curve relationship (Fig. 7, lower panel) to order the results according to the water chemistry (Fig. 6, open triangles). While this provides only a qualitative assessment of the proportionality between the otolith and the water chemistry, a direct relationship is again indicated across a broad range of estuarine conditions.

3.4. Salinity experiment

The salinity = 14 treatment was fully lethal, providing additional evidence for sublethal salinity effects observed in cages at river km 4. Thus, our results are based on the low (0) and intermediate (7) salinity levels (Table 4). We detected a significant difference in otolith Sr/Ca between strontium treatments ($F_{0.05,1,4} = 53.38$, $p = 0.002$) and a positive, but nonsignificant, association between otolith Sr/Ca and salinity ($F_{0.05,1,4} = 3.28$, $p = 0.145$). There was no significant interaction between salinity and aqueous Sr/Ca on otolith Sr/Ca ($F_{0.05,1,4} < 0.01$, $p = 0.981$). The significant increase in otolith Sr/Ca from the low to high strontium treatments was expected, and the nonsignificant effect of salinity had low power (< 0.5; comparing tank means between salinities at either Sr/Ca level with a *t*-test), indicating that minor salinity effects might be detectable with a larger sample size. Somatic growth and survival were generally lower than in the other two experiments (Table 4), and this was related to the larger initial size, lower temperature, and later starting date for this experiment.

4. Discussion

Our results strongly support a direct positive association between Sr/Ca in the water and in the otolith. The dilution and caging experiments provided an unexpectedly rigorous evaluation of this association. Despite contrasting mixing curves between the laboratory conditions and the Patuxent River (Figs. 5 and 7), a direct positive relationship was indicated between Sr/Ca in the otolith and that in the water (Fig. 6). The proportionality of

this relationship suggests that Sr/Ca in the otolith is roughly 40% of that in the water (similar to Wells et al., 2003), but direct measurement of a partition coefficient is needed. The salinity experiment indicated that the relationship between aqueous and otolith strontium was not strongly affected by salinity (or factors correlated with salinity, such as osmoregulation). Because aqueous Sr/Ca can vary substantially in freshwater and low salinity regions in estuaries, a primary consideration in interpreting patterns of otolith Sr/Ca within fluvial estuaries is the nature of the Sr/Ca mixing curve.

While recognized by some (Fowler et al., 1995; Thorrold et al., 1998; Bath et al., 2000; Milton and Chenery, 2001), the importance of nonlinear changes in water chemistry to chemical patterns in otoliths has often received inadequate attention. Further, no study has explicitly considered the importance of the freshwater end member (Sr/Ca) in interpretations of otolith strontium chronologies (though, strontium isotopic ratios have been studied; see, for example, Kennedy et al., 2000). As an example of this omission, consider the recent study of inferred dispersal patterns of bay anchovy (*Anchoa mitchilli*) in Chesapeake Bay, in which Kimura et al. (2000) observed that a moderate (but significant) amount of otolith Sr/Ca variability (54%) was associated with salinity, but did not characterize the mixing curve. Given that there is little expected change in Sr/Ca in the water across the range of salinity (from 5 to 28) in this system, the interpretation of gradual trends in otolith Sr/Ca chronologies (Kimura et al., 2000) is supported by the underlying gradual rate of change in aqueous Sr/Ca for this part of the estuarine mixing curve. Attempts to calibrate levels of otolith Sr/Ca as they relate to different salinity regimes have been documented when the expected positive association was observed (Secor et al., 1995, 1998; Tzeng, 1996; Kawakami et al., 1998; Kraus and Secor, 2003), but cases of negative or ambiguous results have been infrequent in the literature (but see Hoff and Fuiman, 1995). We speculate that such negative results may exist as unpublished data. We offer an example from our own laboratory in which young-of-the-year striped bass were held in conditions that mirrored our dilution experiment on white perch, using a flow through mixture of groundwater and brackish water from the CBL pier (Fig. 8; R. Kimura, unpublished data). Presumably, the mixing curve in the striped bass experiment was the same as in our dilution experiment, thus explaining the inverse trend observed between otolith Sr/Ca and salinity. In contrast, the positive relationship between salinity and otolith Sr/Ca reported for striped bass by Secor et al. (1995) is supported by a low freshwater Sr/Ca value (0.9 mmol mol^{-1}) in a separate rearing system (spring water in the upper Potomac River watershed).

The conservative incorporation of strontium into white perch otoliths across contrasting mixing curves raises a question about past interpretations of diadromous behavior inferred from otolith Sr/Ca patterns in other species. Evidenced by life-long Sr/Ca otolith chronologies, polymorphic diadromous behaviors have been observed in Japanese eels (Tsukamoto and Arai, 2001; Tsukamoto et al., 1998), European eels (*A. anguilla*; Limburg et al., 2003), and Baltic sea trout (*S. trutta*; Limburg et al., 2001). Interpretations of otolith Sr/Ca variability in these studies were based on the expectation that time spent in freshwater habitats would be reflected in the otolith as bands of low Sr/Ca. While many of the samples showed otolith Sr/Ca patterns that matched expected diadromous migrations between freshwater and seawater, some of the patterns suggested the existence of population contingents (sensu Secor, 1999), which never used

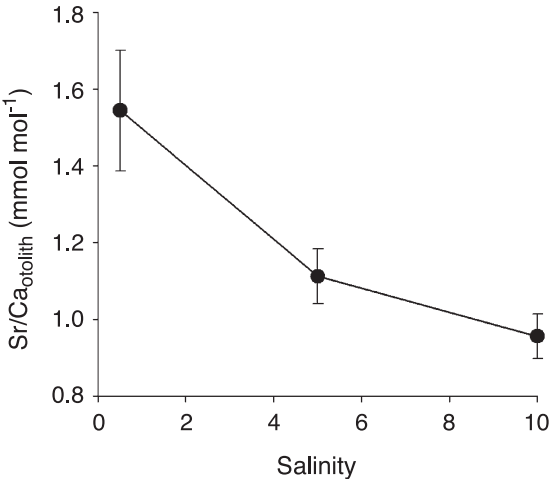

Fig. 8. Otolith Sr/Ca observed at three laboratory salinities for juvenile (41–50 d post-hatch) striped bass (*M. saxatilis*). The experiment was conducted at CBL in 1998 (R. Kimura, unpublished data, Japanese Fisheries Research Institute, Yokohama). Mean values and standard errors are shown. The data represent five individuals from each salinity treatment and were analyzed using the same electron microprobe assay that was used in our white perch experiments.

freshwater habitats. Could the apparent existence of facultative diadromy represent an artifact of high Sr/Ca in the freshwater end member of some estuaries? In cases where Sr/Ca in the freshwater end member is close that of seawater, it is evident from our experiments that migration between freshwater and seawater environments could be inferred as marine residency. Thus, water chemistry data are needed on a per-system basis to support the absence of a freshwater stage in eels or sea trout. In contrast to inferred patterns of marine residency, occurrences of freshwater contingents are conceptually supported since these contingents have lower otolith Sr/Ca levels than marine contingents (e.g., Secor et al., 2001). While the possibility of the preceding scenario is intriguing and should be considered in future studies, we suspect that it is probably not a major issue for most studies. This is because the available data (presented in the Introduction) on naturally occurring freshwater sources with high Sr/Ca indicate that such systems are in the minority.

In addition to changes in water chemistry due to estuarine gradients, temperature is known to be an important factor in the incorporation of strontium into otoliths. While we did not explicitly investigate temperature effects, previous work indicates that temperature effects on strontium incorporation in otoliths may be either positive or negative depending on the range of temperatures. Below 10 °C, temperature dependency is negative, and above this temperature the relationship is typically positive (see review by Campana, 1999). In either case (positive or negative), first order approximation of the magnitude of the response is around 0.1 mmol mol^{-1} change in Sr/Ca in the otolith with each one-degree change in temperature (Campana, 1999; Bath et al., 2000). In the Patuxent River estuary, temperature fluctuates annually from approximately 2.4 to 27.5

°C (Ritchie and Genys, 1975), but during any single period (up to 3 months), temperature variation within the spatial extent of juvenile white perch habitats is on the order of 2 °C (equivalent to that observed across treatments in the caging experiment). This temperature variation corresponds with an expected 0.2 mmol mol^{-1} change in otolith Sr/Ca, whereas changes due to water chemistry between different perch habitats (freshwater versus brackish) would be expected to result in otolith Sr/Ca differences of 1.5 mmol mol^{-1} or greater (Fig. 7, upper panel). Therefore, within a given life history stanza (e.g., juveniles), patterns in otolith Sr/Ca of wild perch moving between freshwater and brackish habitats will be primarily reflective of water chemistry rather than temperature. Within the dilution experiment there was small variation in aqueous Sr/Ca (but an inverse pattern, Fig. 5), and confounding temperature variation provides an alternate explanation of the observed treatment differences in otolith Sr/Ca. Still, when the dilution experiment results are compared to the caging experiments (Fig. 6), it is again clear that water chemistry rather than temperature, salinity (in the salinity experiment), or other factors (e.g., somatic or otolith growth) dominated patterns of otolith Sr/Ca in juvenile white perch.

In summary, for those estuarine fish that move between freshwater, estuarine and seawater environments, the underlying gradient of Sr/Ca in the water is the most important consideration in interpreting variability in otolith Sr/Ca. Based upon our crossover design experiment, our electron microprobe assay of otolith Sr/Ca can be used to infer migrations between brackish and freshwater habitats with the Patuxent River estuary or other estuaries with similar mixing curves. This is due to the curvilinear behavior of the mixing curve in which most of the variation occurs at salinities <8. Our findings have implications not only for studying dispersal of resident estuarine fish that move between freshwater and brackish habitats, such as white perch, but also for applying Sr/Ca tracers in otoliths to questions about diadromy in fish.

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