Experimental assessment of the effect of temperature and salinity on elemental composition of otoliths using laser ablation ICPMS

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Abstract: Laser ablation – inductively coupled plasma mass spectrometry (LA-ICPMS) is a new technique that can be used for the multielemental analysis of otoliths at specific loci. This method was used to sample the otoliths of Atlantic croaker (*Micropogonias undulatus*), reared under different constant regimes of temperature and salinity, to determine whether the elemental composition of otoliths changes ontogenetically. Each otolith was sampled at a number of loci, beginning at the center and then every 500 μm along the longest axis to near the edge; of 23 isotopes measured simultaneously at each locus, 18 were standardized to ^{48}Ca and included in analyses. The elemental composition at otolith centers and near their edges differed significantly amongst treatments, with the effect of temperature a stronger influence. Elemental composition also varied across otoliths from within treatments, indicating endogenous effects. Ontogenetic patterns differed amongst treatments, indicating that endogenous control was mediated by the external environment. Otoliths of fish from one tank where the physical conditions were switched, showed greater variation in the multielemental signal than that resulting only from ontogenetic change. All analyses indicated that otolith formation is the product of numerous interactive exogenous and endogenous processes, including water temperature, salinity, and ontogeny.

Résumé : La spectrométrie de masse avec plasma inductif et ablation au laser est une nouvelle technique qui peut servir à l'analyse multi-élémentaire des otolithes sur des sites précis. Nous avons employé cette méthode pour échantillonner les otolithes de tambours brésiliens (*Micropogonias undulatus*), élevés selon divers régimes constants de température et de salinité, afin de déterminer si la composition élémentaire des otolithes changeait pendant l'ontogenèse. Chaque otolithe a été échantillonné à un certain nombre de sites, tous les 500 μm le long du grand axe, depuis le centre jusque près du bord; sur les 23 isotopes mesurés simultanément à chaque site, nous en avons étalonné 18 par rapport au ^{48}Ca et inclus ceux-ci dans les analyses. La composition élémentaire des otolithes au centre et près des bord différait de façon significative entre les divers régimes, l'influence la plus forte étant celle de la température. La composition élémentaire variait entre les otolithes soumis à un même traitement, ce qui indique l'existence d'effets endogènes. Les profils ontogénétiques différaient entre les traitements, ce qui indique que les effets endogènes étaient régulés par le milieu extérieur. Chez les poissons d'un aquarium dont les conditions physiques avaient été changées, les otolithes présentaient une variation plus grande du signal multi-élémentaire que ce qu'on pouvait attendre du simple changement ontogénétique. Toutes les analyses ont indiqué que la formation des otolithes est le produit d'un grand nombre de processus exogènes et endogènes, notamment la température de l'eau, la salinité et l'ontogenèse.

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Introduction

Current techniques for analysis of the microchemistry of otoliths can be categorized into those for use on whole otoliths and those that sample at specific loci. The former provide a chemical record integrated over a fish's life, whilst the latter provide elemental information for minute parts of an otolith, to address hypotheses relating to age, growth rate, ontogeny, and environmental variation (Radtke 1984, 1989; Kalish 1989, 1991; Gallahar and Kingsford 1992). Unfortunately, the most popular and accessible technique for point sampling otoliths, i.e., X-ray analysis using an electron microprobe, can provide information on only the most abundant elements. It does not have the sensitivity to sample the many trace elements now known to occur in otoliths (Gallahar and Kingsford 1992; Gunn et al. 1992; Campana and Gagne 1994).

A new technique for point sampling solid materials, which may circumvent the limited sensitivity of X-ray analysis is laser ablation – inductively coupled plasma mass spectrometry (LA-ICPMS) (Gray 1985; Denoyer et al. 1991). Here, a high-powered, pulsed laser beam is focused onto a selected position of an otolith. With activation of the laser, causing conversion of photon energy to thermal (kinetic) energy, some of the otolith is vapourized and swept by a flow of argon gas into the plasma where it is atomized and ionized, from which the analyte ions are extracted and analysed by a mass spectrometer (Denoyer et al. 1991; Hall 1992). The otolith surface and the process of ablation can be viewed safely by a remote video camera, whilst electronic stepper motors associated with the stage can be used to focus on different loci.

This technique combines the benefits of point sampling with the sensivity of ICPMS, and avoids the sample preparation by acid dissolution used for solution-based ICPMS (Hall 1992). The technique has proven particularly useful in carbonate analysis, giving accurate estimates of elemental concentrations over several orders of magnitude (Perkins et al. 1991; Pearce et al. 1992). Such success with inorganic carbonates suggested that the technique may also be useful for analysis of biogenic carbonates. Indeed, LA-ICPMS analysis of the nuclei of otoliths from Atlantic cod (*Gadus morhua*), provided "elemental fingerprints" that successfully discriminated amongst different stocks (Campana et al. 1994). Laser ablation has also been used in association with mass spectroscopy to provide estimates of strontium concentration across scales of striped bass (*Morone saxatilis*), successfully discriminating between estuarine and marine fish (Coutant and Chen 1993).

The present study expands upon our earlier analysis wherein we determined that the multielemental composition of whole otoliths was affected by water temperature, salinity, and the growth rate of the fish (Fowler et al. 1995). Here, we assess whether otoliths from the same fish also reflect ontogenetic variation, and determine whether there is an interaction between ontogeny and environmental regimes, by sampling otoliths at particular loci using LA-ICPMS. The specific aims of our analyses here were to determine (1) if the elemental composition at age-specific locations on otoliths varies amongst fish reared in different regimes of temperature and salinity; (2) if the elemental composition varies at different positions on the same otoliths, and whether such ontogenetic patterns differ amongst treatments; (3) whether a switch in environmental conditions causes an identifiable change to otolith elemental composition.

Materials and methods

Juvenile Atlantic croaker (*Micropogonias undulatus*) were reared from hatching for several months under different levels of constant conditions of temperature and salinity. Full details of the spawning and rearing circumstances were presented by Fowler et al. (1995). The conditions of temperature and salinity of the treatments assigned randomly to two tanks each were as follows: treatment 1, 20°C and 26‰; treatment 2, 20°C and 35‰; treatment 3, 25°C and 26‰; treatment 4, 25°C and 35‰. These treatments were maintained for 71 d from 28 October 1992 (day 1) until 6 January 1993 (day 71) under their assigned conditions.

This work also involved one other experimental treatment not previously described. Here, for one tank the environmental conditions were altered through the rearing period, which was longer than for the other four treatments. The initial conditions for treatment 5 were: 25°C and 35‰ maintained from 28 October until 1 December 1992 (day 35) when they were changed to 20°C and 26‰. These were maintained until the 29 December, (day 63) when they were changed back to the original conditions, and subsequently maintained until 27 January 1993 (92 d), when the fish were killed.

Unfortunately, the total loss of fish from both tanks of treatment 4 eliminated the high-temperature, high-salinity treatment. Furthermore, the total loss of fish from one tank of treatment 2 (20°C and 35‰ salinity) reduced the level of replication of this treatment. The otoliths from all fish that died prior to the end of their assigned rearing periods were not considered in the elemental analyses.

Otolith preparation

At the end of the rearing period the surviving fish were killed and measured (standard and total length). The otoliths were removed, washed, air-dried, and weighed (Fowler et al. 1995). One sagitta from each fish was glued to a microscope slide, then ground and polished in the sagittal plane so that the exposed surface was as close as possible to the otolith primordium. This was done by hand using two grades of Imperial lapping film (30 and 3 μm, respectively). Polished otoliths were then sonicated in Super Q water for 3 min, air-dried in a positive flow fume hood, and stored in paper envelopes until ready for analysis.

Laser ablation ICPMS

The LA-ICPMS analyses were done in four blocks of time over a 2-d period (subsequently named in analyses as Block). Within each block of time, otoliths were processed one at a time, taking one from each tank sequentially. Each otolith section was placed within the sample cell, and the laser focused onto the central core. At this time, an argon gas blank was read to determine the current background level for each isotope, which was later used in the blank correction process. The sample site was then hit with two

energy pulses from the laser with a 1-s interval, after which the ablated material was processed by the ICPMS. The pulsed Nd:YAG laser (1064 nM) was run in Q-switched mode at 640 v using a nominal beam diameter of 30 μm. The argon flow rate to the ICPMS was 1.5 L·min^{-1}. The ICPMS was run with 20 channels per atomic mass unit, 7-s peak integration, and a dwell time of 320 μs per channel.

The isotopes sampled were ^{10}B, ^{24}Mg, ^{25}Mg, ^{27}Al, ^{46}Ca, ^{48}Ca, ^{57}Fe, ^{58}Ni, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{69}Ga, ^{85}Rb, ^{86}Sr, ^{87}Sr, ^{88}Sr, ^{118}Sn, ^{121}Sb, ^{137}Ba, ^{138}Ba, ^{207}Pb, and ^{208}Pb. These were chosen from a more extensive list on the basis of a small pilot study involving one otolith. From this test, isotopes that gave low natural levels relative to background or that were confounded with interference molecular ions (Date 1991; Hall 1992), were not subsequently sampled. ^{69}Ga and ^{121}Sb were included as indicators of contamination from the sectioning process, as we had previously determined that they occur at comparatively high levels in the lapping film.

Each otolith was first sampled at the central core and then at intervals of 500 μms along the longest axis, towards the edge. Since the otoliths varied considerably in size (Fowler et al. 1995, Table 2), those from the different treatments were sampled at a different number of loci. The small otoliths from treatments 1 and 2 were sampled at only two positions, those from treatment 3 were sampled at three positions, whilst those from treatment 5 were sampled at four. Otoliths were sampled sequentially taking one per tank, so that sequence effects could subsequently be corrected for.

Table 1. Mean count, SE, and mean count of Ar blanks run before each otolith was assayed, of 23 isotopes sampled from the otoliths of juvenile Atlantic croaker using LA-ICPMS.

Isotope	Mean count	SE	Mean blank count
^{10}B	390	17	217
^{24}Mg	38 865	3 760	503
^{25}Mg	5 872	568	154
^{27}Al	38 756	10 587	11 923
^{46}Ca	6 658	293	1 114
^{48}Ca	283 358	14 422	4 533
^{57}Fe	7 273	158	5 101
^{58}Ni	5 565	226	3 850
^{59}Co	1 663	54	1 370
^{60}Ni	2 073	158	1 237
^{65}Cu	324	58	103
^{66}Zn	1 264	351	180
^{69}Ga	202	57	82
^{85}Rb	272	21	31
^{86}Sr	89 758	5 095	807
^{87}Sr	63 838	3 800	23
^{88}Sr	606 245	31 771	65
^{118}Sn	71	9	51
^{121}Sb	87	10	43
^{137}Ba	244	17	18
^{138}Ba	1 481	112	17
^{207}Pb	167	21	47
^{208}Pb	358	47	85

Note: Data are calculated from all sample positions on all otoliths (n = 202). Only one blank record was recorded prior to sampling each otolith (n = 77).

Data analysis

Output from LA-ICPMS were counts per second for the 23 isotopes indicated above. The counts were corrected for the blank values recorded before each otolith was sampled. In some cases this resulted in negative values where blank values were higher than counts from the otolith. As this presented problems when transforming the data in later analyses, a constant corresponding to the largest negative value + 1 was added to all counts of any isotope where a negative value was generated by the blank-correction process. Finally, since preablation could not be used on small otoliths to ensure the absence of surface contamination (Campana et al. 1994), our data were subjected to outlier analysis to identify potentially spurious values caused by contamination (Tukey 1977). Any value greater than 3 interquartile distances from the 25th or 75th percentiles was rejected (19 of a total of 202 records from all records).

The design of the experiment was originally conceived as a 2 × 2 factorial approach, with temperature and salinity as orthogonal factors. However, the loss of one treatment forced an alteration of the design to a mixed model, with treatment as a fixed effect and tanks nested within treatments. Therefore, the generalized linear model for all hypothesis testing was

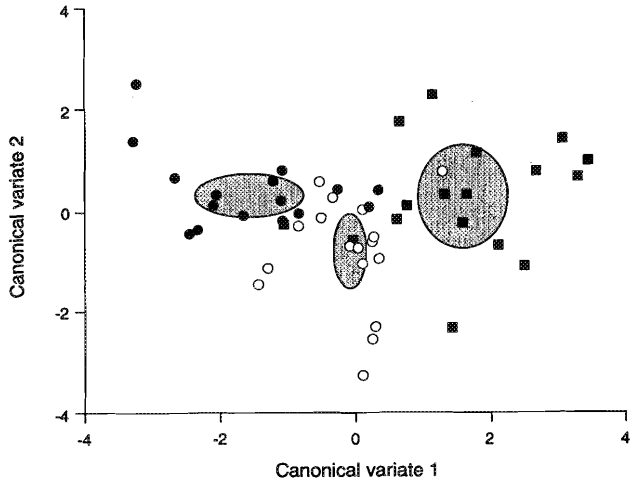
$$[1] \quad Y_{ijklm} = \mu + a_{il} + b_{ij} + c_{ijk} + d_m + \varepsilon_{ijklm}$$

where Y_{ijklm} represents the observation from the lth position on the otolith of the kth fish, from the jth tank nested within the ith treatment; μ is the grand mean; a_{il} measures the fixed effect of position l under the ith treatment; b_{ij} measures the random effect of the jth tank nested within the ith treatment; c_{ijk} measures the random effect of the kth fish within the jth tank under the ith condition; d_m represents the fixed effect of the mth block in which the particular otolith was analysed during the laser ablation process (and assumes no interaction between the block term and any other factor); and ε_{ijklm} represents the random measurement errors, which are assumed to be independent and normally distributed.

Multivariate analysis of variance (MANOVA) was used to test for differences in elemental fingerprints among treatments at particular loci within the otoliths. There were insufficient degrees of freedom to consider tank effects as a nested term in the multivariate model and so individual fish were pooled across tanks with treatment as a fixed factor. The laser sampling was done in four time periods over two consecutive days. Instrument drift across time periods was taken into account by treating these periods as blocks in the MANOVA model (d_m in Eq. 1).

When the MANOVA detected significant treatment effects, canonical discriminant analysis (CDA) was used to display these data in reduced space. The effect of block was removed from the CDA analyses by using the residuals of one-way ANOVAs incorporating the block term as the main factor for each of the isotopes. To determine where

Fig 1. Plot of the first two canonical variates from a CDA comparing the elemental composition at the center of the otoliths. Treatment 1, 20°C and 26‰ salinity (open circles); treatment 2, 20°C and 35‰ salinity (solid squares); treatment 3, 25°C and 26‰ salinity (solid circles).



significant differences among treatments lay, bootstrapped 95% confidence ellipses were calculated on class means on the first and second canonical variates. Canonical scores on both first and second variates were resampled 998 times with replacement, for each of the three treatments (Efron and Gon 1983). When confidence intervals were asymmetric around class means, the largest of the two intervals was used to produce a conservative and symmetric 95% confidence ellipse.

Univariate analyses were performed on each of the isotopes to determine those contributing to the differences detected in the MANOVA. The univariate model was that stated in Eq. 1 with the *l*th term dropped from the model as individual positions on the otoliths were analysed separately. The tank mean square was used as the error term in the test for significance of treatment and type III sums of squares were used throughout because of unequal sample sizes. To allow a more powerful test of treatment effects, tank and residual errors were pooled when the tank effect was not significant with $p > 0.2$. Where significant differences among treatments were detected, means were compared using Tukey's honestly significant difference, applying Kramer's correction for unequal sample sizes (Quinn and Day 1989).

To test for a treatment effect on ontogenetic variation of elemental composition we calculated differences between centers and edges and compared amongst treatments. It was important to use differences in the statistical analyses since assays of multiple ions within a given otolith would be expected to be correlated. Differences were calculated as

$$[2] \quad z_{ijkm} = yc_{ijkm} - ye_{ijkm}$$

where z_{ijkm} represents the difference between the center and edge positions on the otolith of the *k*th fish from the *j*th tank nested within the *i*th treatment and sampled with the laser unit during the *m*th time block, yc_{ijkm} represents the observation from the center position (*c*) and ye_{ijkm} represents the observation from the edge position (*e*). Substituting from Eq. 1 for *y*:

$$[3] \quad z_{ijkm} = (\mu + a_{ic} + b_{ij} + c_{ijk} + d_m + \varepsilon_{ijkmc}) - (\mu + a_{ie} + b_{ij} + c_{ijk} + d_m + \varepsilon_{ijkme})$$

$$[4] \quad = a_{ic} - a_{ie} + \varepsilon_{ijkmc} - \varepsilon_{ijkme}$$

$$[5] \quad = \delta_i + \varepsilon_{ijkm}'$$

Therefore, tank, fish and block effects dropped from the model, and the differences (δ_i) among treatments were compared using a one-way ANOVA.

A similar approach was used when comparing the effects of the switched treatment on otolith microchemistry. Differences were again calculated between adjacent positions on the otolith. On this occasion, however, samples were taken at the center and then every 500 μm out to the edge. As outlined above (Eq. 3), the difference (z_1) between the center (*p*) and the adjacent position 500 μm from the center (*q*) is

$$[6] \quad z_{1ijkm} = a_{ip} - a_{iq} + \varepsilon_{ijkmp} - \varepsilon_{ijkmq}$$

$$[7] \quad = \delta_{1i} + \varepsilon_{ijkm}'$$

Similarly, the difference (z_2) between the 500 μm position (*q*) and the next position (*r*) is

$$[8] \quad z_{2ijkm} = a_{iq} - a_{ir} + \varepsilon_{ijkmq} - \varepsilon_{ijkmr} = \delta_{2i} + \varepsilon_{ijkm}''$$

In this case ε_{ijkm}' and ε_{ijkm}'' from the same fish are correlated, while errors from different fish are not. Therefore, a MANOVA approach to repeated measures was used to determine if the switched treatment had an effect on otolith composition. Differences (δ_{1i} and δ_{2i}) were treated as dependent variables in the MANOVA with treatment as the main factor.

Multivariate statistical analyses share common assumptions of normality of errors (or multivariate normality) and homogeneity of variance (or equal variance–covariance matrices). Consequently, all elemental ratios to ^{48}Ca were transformed ($y = \log_e x$), after which they were tested by residual analysis and found to conform to the assumptions of the univariate ANOVA. We assumed that this would also ensure the validity of the multivariate tests.

Results

Output from ICPMS after laser ablation at each sample point on each otolith were counts per second for each of the 23 isotopes representing 16 elements. These ranged from <100 to >500 000 counts·s^{-1} encompassing five orders of magnitude (Table 1). ^{69}Ga, ^{118}Sn, and ^{121}Sb consistently gave the lowest values; ^{10}B, ^{46}Ca, ^{57}Fe, ^{58}Ni, ^{65}Cu, ^{85}Rb, ^{137}Ba, ^{138}Ba, ^{207}Pb, and ^{208}Pb had counts in the range of 10^2–10^3·s^{-1}; ^{24}Mg, ^{25}Mg, ^{27}Al, ^{86}Sr, and ^{87}Sr had counts in the range of 10^4–10^5·s^{-1}; only ^{48}Ca and ^{88}Sr were present at levels of >10^5 counts·s^{-1}. The three isotopes ^{27}Al, ^{69}Ga, and ^{121}Sb were excluded from subsequent analyses as they occurred in high concentrations in either the lapping film, glue or glass slides. ^{118}Sn was excluded as it did not exceed background levels.

Table 2. ANOVA table examining treatment and tank effects ($\alpha = 0.05$) for each of the 18 isotopes considered in the analysis of otolith chemistry, at both the center and edge positions, using LA-ICPMS.

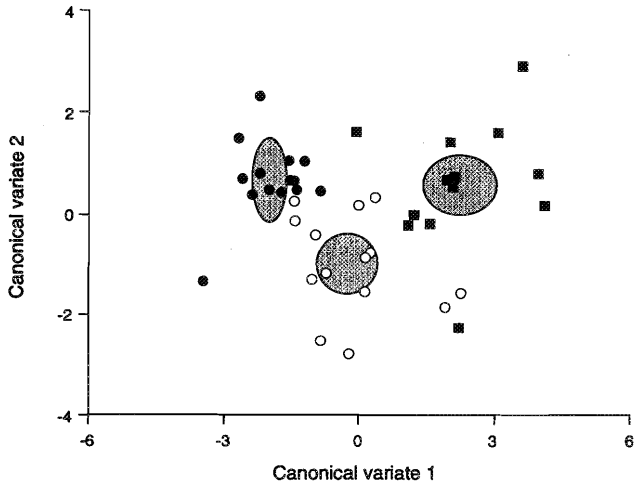
		Center				Edge			
Isotope	Source	MS	F	$p > F$	Pooled	MS	F	$p > F$	Pooled
^{10}B	Treatment	0.2608	1.27	0.4409	0.5807	1.0636	24.06	0.0399	
	Tank	0.2057	0.48	0.6223		0.0442	0.14	0.8693	
	Error	0.4293				0.3145			
^{24}Mg	Treatment	1.7115	19.68	0.0484		2.0902	5.58	0.1519	0.0225
	Tank	0.0870	0.14	0.8677		0.3743	0.69	0.5064	
	Error	0.6110				0.5412			
^{25}Mg	Treatment	4.2144	4.25	0.1906	0.0078	5.0457	14.24	0.0656	0.0005
	Tank	0.9926	1.32	0.2766		0.3543	0.65	0.5252	
	Error	0.7517				0.5418			
^{46}Ca	Treatment	0.0098	0.57	0.6356	0.7564	0.0891	2.56	0.2807	0.0363
	Tank	0.0172	0.40	0.6714		0.0132	0.11	0.8971	
	Error	0.0427				0.0278			
^{57}Fe	Treatment	0.5834	1.07	0.4830	0.2047	0.1133	0.34	0.7462	0.7557
	Tank	0.5450	1.33	0.2752		0.3330	0.97	0.3882	
	Error	0.4111				0.3441			
^{58}Ni	Treatment	0.4179	2.14	0.3189	0.7083	0.4337	0.8	0.5568	0.6510
	Tank	0.1957	0.18	0.8323		0.5448	0.58	0.5656	
	Error	1.0621				0.9432			
^{59}Co	Treatment	0.0851	0.18	0.8476	0.8315	0.9059	0.73	0.5777	0.4138
	Tank	0.4735	0.60	0.5545		1.2394	1.46	0.2428	
	Error	0.7939				0.8464			
^{60}Ni	Treatment	0.7899	0.89	0.5299	0.2874	0.7969	0.28	0.782	
	Tank	0.8905	1.09	0.3454		2.8581	5.03	0.11	
	Error	0.8192				0.5682			
^{65}Cu	Treatment	1.3853	0.88	0.533		0.6381	2.47	0.2886	0.1455
	Tank	1.5811	1.69	0.1950		0.2588	0.87	0.4279	
	Error	0.9347				0.2988			
^{66}Zn	Treatment	0.0165	0.07	0.9384	0.9562	2.2528	11.38	0.0808	0.3411
	Tank	0.2509	0.28	0.7566		0.1987	0.31	0.7377	
	Error	0.8943				0.6459			
^{85}Rb	Treatment	0.2897	1.74	0.3656	0.4609	0.2489	26.75	0.036	
	Tank	0.1669	0.44	0.6472		0.0093	0.05	0.9501	
	Error	0.3802				0.1815			
^{86}Sr	Treatment	0.0834	1.24	0.4462		0.0182	4.45	0.1835	0.5782
	Tank	0.0672	1.79	0.1784		0.0041	0.12	0.8836	
	Error	0.0376				0.0330			
^{87}Sr	Treatment	0.1310	0.84	0.5420		0.0950	47.07	0.0208	
	Tank	0.1551	4.73	0.0133		0.0020	0.05	0.9508	
	Error	0.0328				0.0400			

Table 2 (*concluded*).

		Center				Edge			
Isotope	Source	MS	F	$p > F$	Pooled	MS	F	$p > F$	Pooled
^{88}Sr	Treatment	0.0241	1.62	0.3813	0.4187	0.0425	7.89	0.1114	0.1768
	Tank	0.0149	0.53	0.5938		0.0053	0.22	0.8052	
	Error	0.0282				0.0245			
^{137}Ba	Treatment	0.3772	2.53	0.2836	0.4800	0.0460	0.42	0.7026	0.8832
	Tank	0.1493	0.28	0.7541		0.1086	0.29	0.7528	
	Error	0.5259				0.3798			
^{138}Ba	Treatment	2.1439	11.64	0.0791	0.0113	0.1673	16.13	0.0584	0.5851
	Tank	0.1842	0.42	0.6617		0.0104	0.03	0.9693	
	Error	0.4424				0.3324			
^{207}Pb	Treatment	0.3967	0.84	0.5438	0.5492	0.9834	0.84	0.5440	
	Tank	0.4729	0.56	0.5746		1.1733	2.25	0.1176	
	Error	0.8437				0.5208			
^{208}Pb	Treatment	1.1794	2.09	0.3232	0.1223	1.5865	0.92	0.5198	
	Tank	0.5633	0.84	0.4363		1.7172	2.87	0.0678	
	Error	0.6676				0.5982			

Note: The "pooled" column in each case shows the probability for treatment effects when the tank and residual mean squares were pooled when the probability of a tank effect was >0.2, otherwise left blank. Degrees of freedom: treatment, 2; tank, 2; error, 48 (center) and 42 (edge).

Fig. 2. Plot of the first two canonical variates from a CDA comparing the elemental composition at the position sampled closest to the edge. Treatment 1, 20°C and 26‰ salinity (open circles); treatment 2, 20°C and 35‰ salinity (solid squares); treatment 3, 25°C and 26‰ salinity (solid circles).



Positional variation amongst treatments

Otolith centers

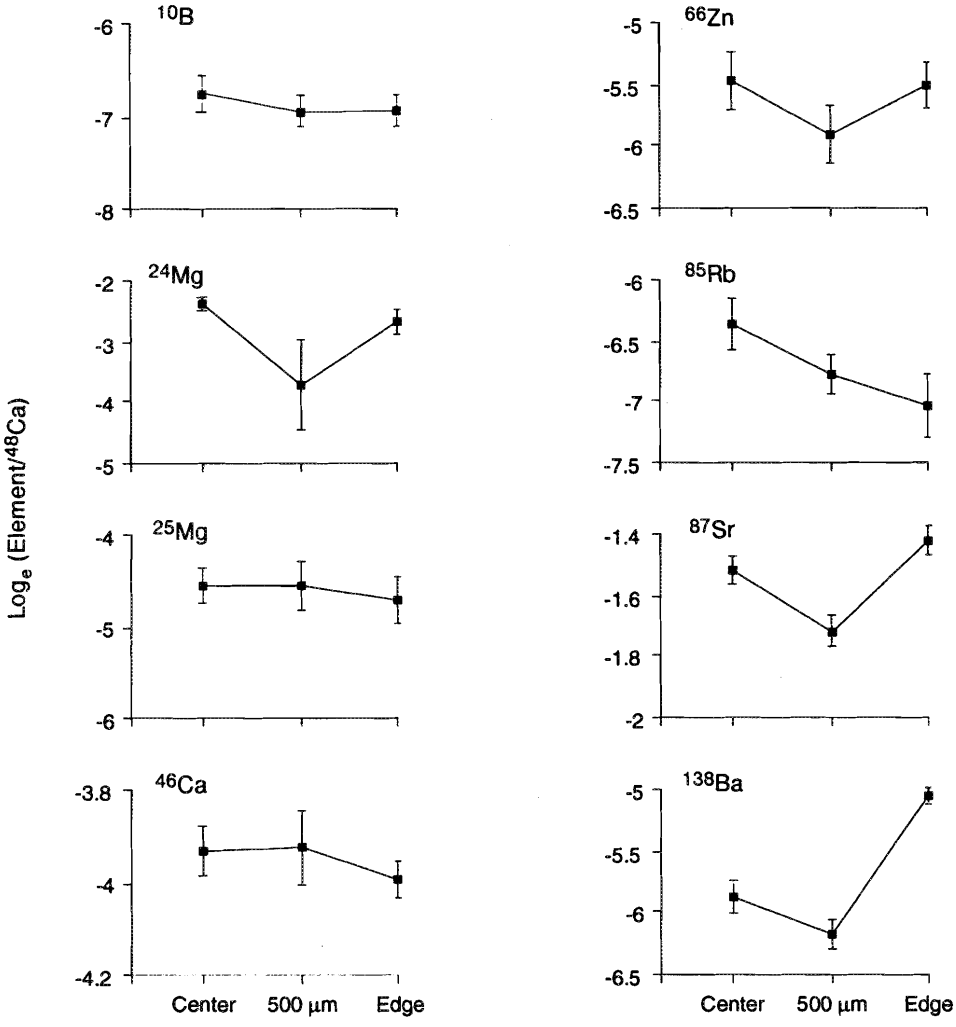
Elemental composition of otolith centers differed significantly amongst treatments (MANOVA, Pillai's trace = 1.0108, F = 1.76, df = 36, 62, p = 0.025), and the CDA plot indicated considerable separation amongst all the three treatments (Fig. 1). Class means of the three treatments differed significantly as none of the 95% confidence intervals overlapped. These means were spread evenly along the first canonical variate, indicating that there were influences of both temperature and salinity loading on this variate. The confidence ellipses overlapped on the second variate suggesting little separation in this plane.

Univariate ANOVAs detected significant differences among treatments for ^{24}Mg, ^{25}Mg, and ^{138}Ba (Table 2). For each of these the standardized values were higher in the low-temperature treatments than the high-temperature treatment, although Tukey's HSD was not powerful enough to distinguish among treatments. There was little difference between the means for treatments 1 and 2, attributable to the salinity regimes.

Otolith edges

Elemental fingerprints from the otolith edges also differed significantly amongst treatments (MANOVA, Pillai's trace = 1.3913, F = 3.17, df = 36, 50, $p < 0.0001$). The CDA plot showed clearer separation amongst all three treatments than was achieved for the center position (Fig. 2). Furthermore, there was less within-treatment variability in elemental fingerprints, reflected in the smaller confidence ellipses. The relationships among treatments were similar to those for the center position. The three treatments showed some separation along the first canonical variate, with treatment 1 falling between treatments 2 and 3, suggesting that the first canonical variate was influenced by both temperature and salinity. Canonical variate 2 also separated treatment 1 from the other two treatments, perhaps reflecting

Fig. 3. Comparison of the means (±SE) of the transformed, standardised data for eight isotopes at the three positions sampled in otoliths from treatment 3.



an interactive influence between temperature and salinity. The univariate analyses indicated significant differences for five isotopes: ^{10}B, ^{24}Mg, ^{25}Mg, ^{46}Ca, and ^{66}Zn (Table 2). The first four of these were more abundant in the otoliths from low-temperature treatments, and no obvious differences amongst means were attributable to salinity.

Ontogenetic variation amongst treatments

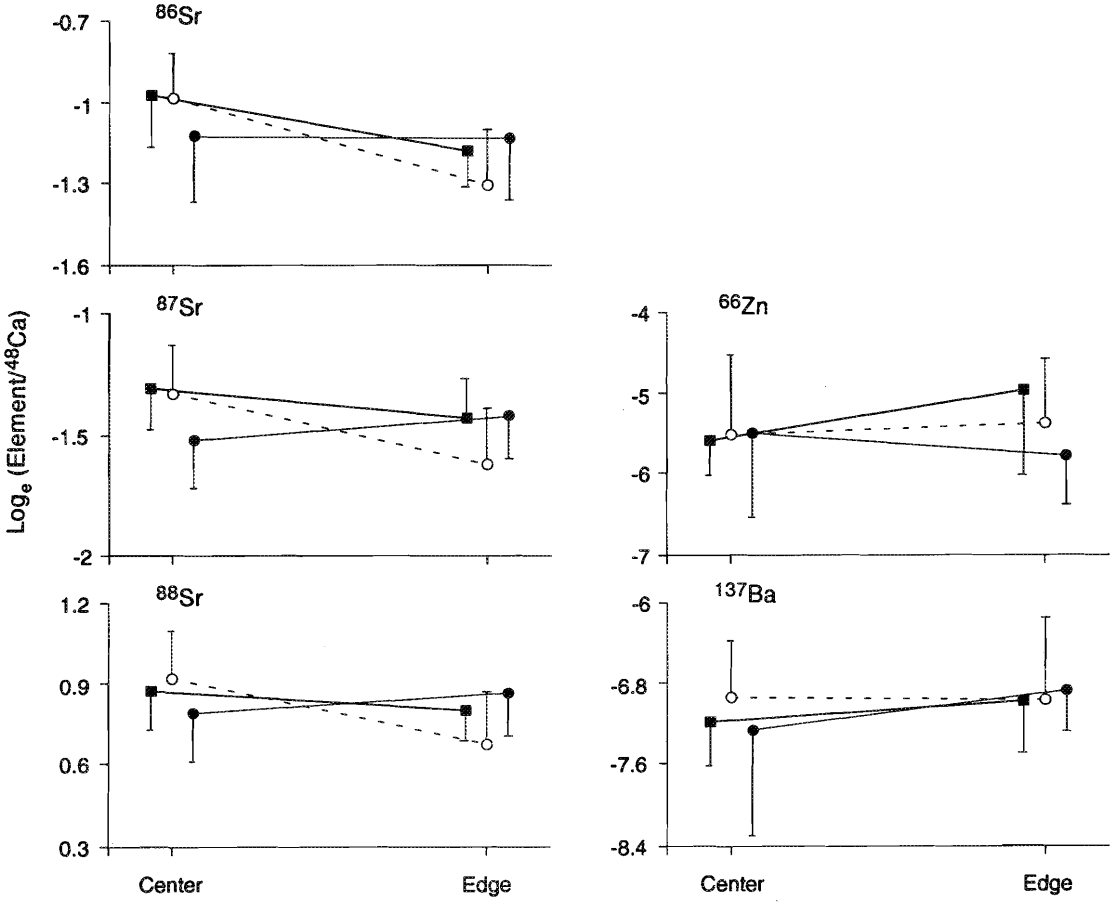
Elemental composition varied amongst sample loci within the same otoliths. For example, the standardized amounts of eight isotopes recorded at the three sample positions for fish from treatment 3 are shown in Fig. 3. Whilst three isotopes show only a minor reduction between the center and edge, four isotopes decreased between the center and 500-µm position and subsequently increased at the edge. ^{85}Rb presented an alternative ontogenetic pattern by decreasing consistently from the center to the edge.

Ontogenetic variation differed amongst treatments. To determine whether this was statistically significant, we compared the differences between the centers and edges for each isotope amongst treatments using one-way ANOVAs. Five isotopes gave significant differences: ^{66}Zn (F = 3.25, df = 2, 41, p = 0.049), ^{86}Sr (F = 6.34, df = 2, 41, p = 0.004), ^{87}Sr (F = 8.87, df = 2, 41, p = 0.0006), ^{88}Sr (F = 6, df= 2, 41, p = 0.0052) and ^{138}Ba (F = 8.7, df = 2, 41, p = 0.0007). Multiple comparisons amongst means generally stressed that treatment 3 was different from treatments 1 and 2, implicating water temperature as the stronger treatment effect on ontogenetic patterns (Fig. 4).

Analysis of switched environmental conditions

For one of the rearing tanks the environmental conditions were switched through its rearing period to determine whether this altered the elemental composition of the otoliths sufficiently to be detectable by LA-ICPMS. On the basis of our earlier results (Figs. 3 and 4), it is likely there would be considerable ontogenetic variation across these large otoliths. Consequently, the effect of the switch in environmental conditions could only be assessed relative to such ontogenetic variation. Here, we used the results from treatment 3 as a control to assess the extent of change across otoliths from treatment 5. Fish from treatment 5 were

Fig. 4. Comparison of the mean (±SE) levels for the five isotopes whose difference between the centers and edges differed significantly amongst treatments. Treatment 1, 20°C and 26‰ salinity (open circles); treatment 2, 20°C and 35‰ salinity (solid squares); treatment 3, 25°C and 26‰ salinity (solid circles).



older and larger than those from treatment 3, although their otoliths had grown at the same average rate (0.035 mm·day^{-1}), suggesting that it would be unlikely that any difference in elemental composition was attributable to a difference in otolith growth rate.

Principal components analysis was used to reduce the dimensionality of the data, and to assess the extent of change in elemental composition across otoliths. The first principal component accounted for 36.2% of the variability, whilst the first and second together accounted for 54.3%. Variation in both principal components across the sample positions for otoliths from both treatments reinforced the significance of ontogenetic variations (Fig. 5). Also, both principal components show clear separation between treatments, particularly at 500 μm from the center, the position where we expected the effect of the switch in environmental conditions to be manifested. Both principal components were compared independently between treatments using a MANOVA. No significant difference was found for the first principal component (p = 0.7240), but there was a significant difference for the second principal component (p = 0.0336). This is likely to be at least partly a consequence of the switch in treatment, whose effect was concentrated between the center and the 500-μm position.

To isolate which isotopes contributed most to the treatment effects, the difference between the 500-μm position and the center, and between 500 and 1000 μm for each otolith were calculated, and compared independently between the two treatments using a MANOVA. Of the 18 isotopes, only ^{86}Sr (F = 6.1689, df = 2, 22, p = 0.0075) and ^{87}Sr (F = 4.7563, df = 2, 22, p = 0.0192) differed between treatments. The differences between the center and 500-μm position were greater from the switched treatment than from treatment 3 (Fig. 6). This suggests that the relative amounts of ^{86}Sr and ^{87}Sr in the otolith matrix changed because of the switch in conditions from 25°C and 35‰ to 20°C and 26‰.

Discussion

The usefulness of studies of microelemental composition of otoliths for retrospective determination of the early life history of wild fish depends on the extent to which the chemical signal can indicate when and how long fish were associated with different water masses. However, numerous factors such as water temperature, salinity, and growth rates complicate this by affecting the rate of deposition of elements in otoliths (Kalish 1991; Radtke and Shafer

1992). We previously quantified variation in trace element inclusion rates due to temperature, salinity, and growth rate by rearing larval Atlantic croaker under constant physical regimes (Fowler et al. 1995). Here, the second otolith from each of the same fish was sampled using LA-ICPMS to point sample age-specific loci, to test hypotheses relating to the ontogenetic variation across otoliths, and the interaction of ontogeny and environmental conditions.

Factors affecting otolith microchemistry

At the center of the otoliths the multielemental signal differed amongst the three treatments. Here the broad beam of the laser (30 μm) clearly sampled otolith material deposited over numerous days, thus swamping the fact that all fish had spent their first 24 h in the same environmental conditions. A multivariate pattern similar to the otolith centers was also obtained for the edges, although these latter results were inherently less variable than the former. In both cases, different temperature and salinity regimes resulted in subtle differences for particular isotopes, which nevertheless combined in the multivariate comparisons to give obvious differences. Canonical discriminant analyses did not indicate a stronger influence of either temperature or salinity, but the differences amongst treatments identified for several isotopes using univariate techniques indicated that the larger effects were attributable to the temperature difference. This reinforces our observation made from analyses of whole otoliths by solution-based ICPMS that the effect of temperature was stronger than that of salinity (Fowler et al. 1995).

The otolith centers (the material from the youngest stage) and the edges (the oldest stage) presented similar multivariate patterns. This indicated that, from the initiation of otolith formation through until near the end of the rearing period, the different environmental conditions influenced the rate of ionic inclusion. Such treatment effects were evident despite intraotolith variation, as elemental composition of otoliths is known not to be homogeneous with respect to age (Kalish 1989).

By point sampling different loci on the same otoliths we identified significant variation in the elemental composition across otoliths. Because environmental conditions were constant through the rearing period this variation must have resulted from intrinsic ontogenetic influences under endogenous control. Previous studies have documented cross-otolith patterns for Sr/Ca ratios (Radtke 1984, 1987, 1989; Radtke et al. 1990; Gallahar and Kingsford 1992). However, all such analyses were done on otoliths from wild fish, making it impossible to distinguish ontogenetic variation from that related to natural environmental influences. Indeed, for most of the above-mentioned studies the Sr/Ca ratios were interpreted to be the direct consequence of variation in water temperature. The possible effect of natural ontogenetic variation was not considered.

Ontogenetic patterns varied amongst different rearing conditions, indicating that the endogenous control referred to above must be mediated by exogenous influences from the environment. This idea of an interactive effect between ontogeny and the environment on elemental composition of otoliths was strongly supported by the results from our "switched" treatment tank, where an alteration in environmental conditions exacerbated the variation in otolith elemental composition beyond the level due to ontogeny alone.

Fig. 5. Comparison of the first and second principal components amongst sample positions from treatment 3 (shaded squares) and treatment 5 (open circles).

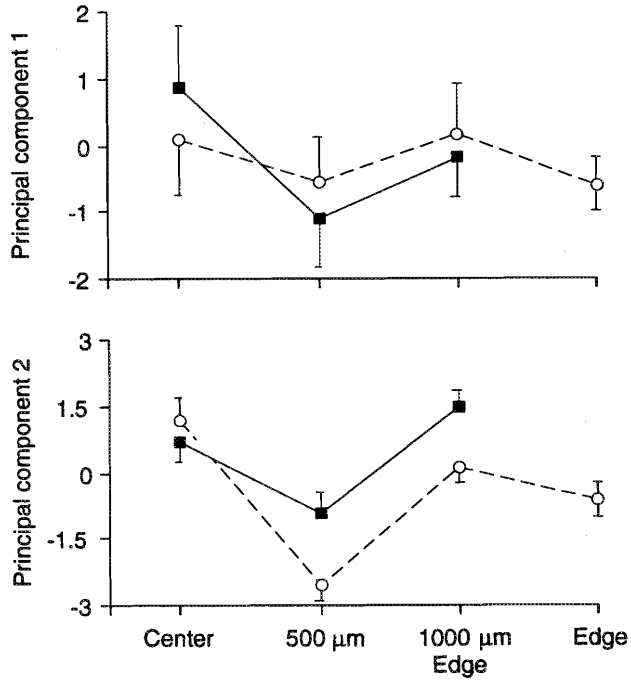
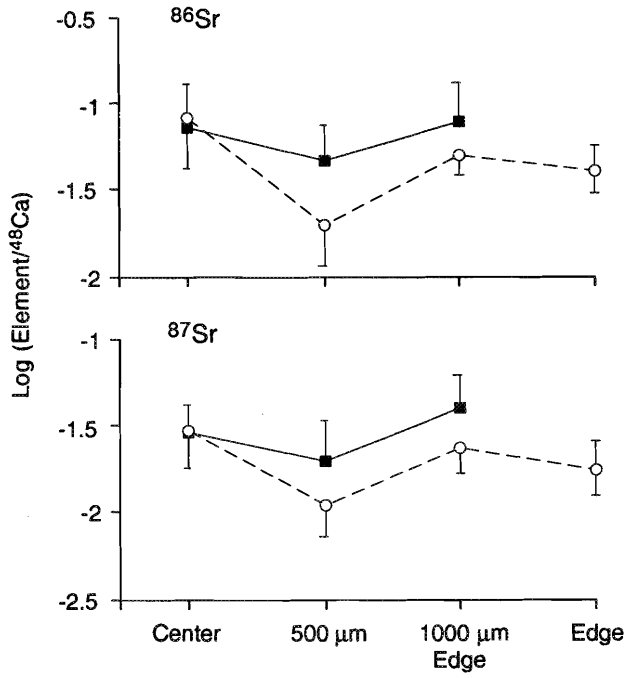


Fig. 6. Comparison of the amounts of ^{86}Sr and ^{87}Sr at the different sample loci across otoliths from treatments 3 (shaded squares) and treatment 5 (open circles).



In our previous paper we suggested that the salinity effect was related to the different ionic concentrations determined by the level of dilution with deionized water (Fowler et al. 1995). We cannot tell whether the relative isotopic concentrations in otoliths are proportional to the isotopic concentrations in the water, or whether those in the otoliths reflect a biological response to these concentrations.

Determining how water temperature influences the elemental composition of otoliths is difficult. Numerous studies have suggested that Sr/Ca ratios are the direct consequence of water temperature, suggesting that the rate at which Sr replaces Ca in the aragonite matrix is temperature dependent (Radtke 1984, 1987, 1989; Radtke et al. 1990; Radtke and Shafer 1992; Townsend et al. 1989). Alternatively, Kalish (1989, 1991) proposed a "physiological" model to account for Sr/Ca ratios that relates physiological changes to changes in the chemistry of blood plasma, which affects the chemical composition of the endolymph and ultimately that of the elemental composition of the otolith. This model has been supported by relationships between Sr/Ca ratios and otolith growth for different species (Sadovy and Severin 1992, 1994; Gallahar and Kingsford 1992).

For us, determining the influence of temperature is even more difficult for several reasons. Firstly, we have identified that many ions contaminate the otoliths of Atlantic croaker, down to trace levels of concentration. However, the mechanisms of inclusion of such ions and where they are located within the otolith microstructure are unknown (Radtke and Shafer 1992; Fowler et al. 1995). Furthermore, the effect of temperature is correlated with and confounded by otolith growth rate. As we suggested previously (Fowler et al. 1995), the elemental differences may be at least in part a consequence of their different sizes and growth rates. We proposed a model that related the amounts of different ions to the relative growth rates of the crystalline incremental and proteinaceous matrix zones of the diurnal bipartite increments of otoliths. Since the otoliths from our high- and low-temperature treatments differed in size, the widths of their daily increments must also have been different. Perhaps, also the relative widths of the different zones comprising the daily increments vary, affecting the potential contamination rates by the ions that contaminate the different zones.

Advantages and constraints of LA-ICPMS

As a new point-sampling method to describe otolith microchemistry LA-ICPMS has some advantages over the electron microprobe (cf. Gunn et al. 1992). Here, preparation of otoliths was simple, involving only hand grinding, polishing, and ultrasonic cleaning. The sampling process itself, which involved placing the otolith into the sample cell, followed by laser ablation and processing was fast and easy. We sampled 77 otoliths at a total of more than 200 sample loci, in four sessions over 2 d, obtaining estimates of 23 isotopes per locus. In comparison, preparation of sections for microprobe analysis is critical and time-consuming as the otolith surface must be as smooth and clean as possible because of the sensitivity of the technique to topographic irregularities (Gunn et al. 1992). Processing time also is likely to be longer because of the requirement for a specimen chamber vacuum. Finally, the electron microprobe has sensitivity to sample only those elements that are major constituents, such as Ca, Sr, and Na that occur in otoliths at concentrations of greater than about 300 ppm. It cannot measure the many elements that occur in otoliths at trace levels or discriminate amongst isotopes of a given element (Edmonds et al. 1991; Campana and Gagne 1994; Campana et al. 1994).

There are, however, disadvantages of LA–ICPMS. Since the laser beam has a diameter of approximately 30 μm, it operates at a relatively large lateral spatial scale, providing an "elemental fingerprint" that integrates over many days. The laser beam was also quite destructive, restricting its use to only two pulses per sample locus, separated by distances of 500 μm across the otolith surface. For many of our small otoliths this still caused substantial damage, sometimes blasting large sections of the otoliths from the surrounding resin. This was not a problem for the larger otoliths. By comparison the spatial resolution and destructiveness of the electron microprobe is far less, allowing elemental analysis to be resolved on a much finer temporal scale (Kalish 1989; Gallahar and Kingsford 1992; Gunn et al. 1992).

This study supported the view that control of otolith microchemistry is complex, and the consequence of numerous interactive factors (Kalish 1991; Radtke and Shafer 1992). Through these analyses we clearly identified the influence of exogenous factors, i.e., water temperature and salinity and the endogenous effect of ontogeny. To these can be added the within-treatment growth rate effect, that we identified from solution-based analyses (Fowler et al. 1995). The complexity is best exemplified by the extent to which ontogenetic patterns were controlled by different physical regimes. These studies have answered numerous questions about otolith microchemistry, but in so doing have also highlighted a number of areas where our knowledge is lacking. These include dissociating the effect of temperature and growth rate and determining where and how contaminant ions attach in the otolith microstructure. Perhaps the refinement of laser techniques, through the reduction of beam width and destructiveness (Hall 1992; Huang et al. 1993), will allow a finer level of resolution of analysis, to help address these questions.

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