Journal of the Marine Biological Association of the United Kingdom, page 1 of 11.
doi:10.1017/S0025315417000212

Inter-annual variability in otolith chemistry of catfish *Genidens barbus* from South-western Atlantic estuaries

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The catfish Genidens barbus *is a commercial species from South America. The aim of the present study was to examine the inter-annual variability in estuary-specific chemical signatures of otolith cores (Ba:Ca, Mg:Ca, Mn:Ca, Sr:Ca and Zn:Ca ratios) for three estuaries from Argentina and Brazil where adults were collected over multiple years. Secondarily, we evaluated whether the percentages of classification of individuals to their natal origin place are affected by the grouping of several cohorts. Most element:Ca ratios were not significantly different among year cohorts. Results from PERMANOVA revealed significant differences in the multi-element signatures of the otolith core between cohorts for the Plata River estuary (PR) (P = 0.006) and the Patos Lagoon (PL) (P = 0.03), while no significant differences (P = 0.9) were found for Paranaguá Bay (PB). The percentages of spatial classification (discriminant function analyses) decreased to between 15.5 and 25% for PR and PL when cohorts were grouped. This work makes it clear that the temporal variation in the chemical signature of the adult catfish otolith core can greatly affect the percentages of spatial classification.*

Keywords: Ariidae, Inter-annual variability, otolith chemical signatures, nursery area

Submitted 21 November 2016; accepted 1 January 2017

INTRODUCTION

Chemical fingerprints recorded in fish otoliths have the potential to resolve outstanding questions about fish stock and migration dynamics of a wide variety of species. Otoliths are structures composed mainly of calcium carbonate deposited as aragonite, vaterite or calcite crystals in a protein matrix (otoline), and small quantities of other chemical elements (Campana *et al*., 1997). These elements are deposited in the otolith during ontogeny and as the material is not re-absorbed, their concentrations may reflect the life history of the fish (Schuchert *et al*., 2010; Mai *et al*., 2014; Avigliano *et al*., 2015a, b; Phung *et al*., 2015; Reis-Santos *et al*., 2015). In particular, divalent elements such as strontium (Sr) and barium (Ba), replacing Ca in the matrix of the otolith, have been widely used to study displacements in salt gradients (Doubleday *et al*., 2014), with the premise that Sr in the otolith is positively related to salinity, while Ba shows the opposite behaviour (Bath *et al*., 2000; Secor & Rooker, 2000; Kraus & Secor, 2004; Martin & Thorrold, 2005; Avigliano & Volpedo, 2013). Moreover, other elements such as Li, Zn, Mn and Mg deposited in the otolith are influenced by different factors, such as water, diet, temperature etc. (Ranaldi & Gagnon, 2008; Bouchard *et al*., 2015). Thanks to these characteristics, otolith chemistry has been a useful tool for studying the life history of fish (Nordlie, 2012; Lin *et al*., 2014; Mai *et al*., 2014), discriminating stocks (Campana *et al*., 2000; Ferguson *et al*., 2011; Avigliano *et al*., 2015a) and identifying nursery areas (Brown, 2006; Vasconcelos *et al*., 2008; Leakey *et al*., 2009; Avigliano *et al*., 2015a; Bailey *et al*., 2015).

In general, to discriminate stocks, only the edge of the otolith is used, since it represents the final stage of life of the fish (Schuchert *et al*., 2010; Fairclough *et al*., 2013). Whereas to identify nursery areas, the chemistry core is used because it corresponds to the larval stage (Rooker *et al*., 2008; Bouchard *et al*., 2015). Unlike the study of chemicals of the otolith core in juvenile fish, this same analysis made in adult fish allows inferring about ontogenetic movements, homing behaviour, etc. For example, the existence of significant differences in the chemical signature of the otolith core in adult fish is an indicator that they use their birth areas as breeding sites without mixing (Gillanders *et al*., 2003; Rooker *et al*., 2008; Avigliano *et al*., 2015a). Finally, by making comparisons between the chemistry of the edge and that of the core, it is possible to estimate, in retrospect, the site of origin of fish using different analytical methods (Bradbury *et al*., 2008; Rooker *et al*., 2008; Avigliano *et al*., 2016).

The catfish *Genidens barbus* (Lacépède 1803) is distributed between latitudes 17°00′S and 40°32′S (López & Bellisio, 1965;

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Avigliano & Volpedo, 2015) and was one of the most important fishery resources in estuaries and rivers of south-western South America (Reis, 1986a; Tavares & Luque, 2004; Velasco *et al.*, 2007; Minagro, 2016). Baigún *et al.* (2012) classified the species as vulnerable for Uruguayan and Argentinean waters due to its complex life cycle (low fecundity, oral incubation and the critical status of the species fishery). For the same reason, the species was included in the Red List of endangered species in Brazil, with capture, transport and trade prohibited since 2015 (MMA, 2014; Di Dario *et al.*, 2015).

Recently, Avigliano *et al.* (2016) and (2015a) have shown the existence of different nursery areas of catfish and have suggested the presence of homing behaviour in Patos Lagoon (Brazil) and Plata River (Argentina–Uruguay) using the chemical composition from otolith cores. Therefore, the quality of the nursery areas is critical, and understanding the connectivity between nursery sources and adult populations can lead to more effective management and rebuilding of the fishery in the south-western Atlantic Ocean (Beck *et al.*, 2001; Vasconcelos *et al.*, 2011). Although this prior work comprehensively addresses the spatial variability in these chemical tracers, inter-annual variability has not been thoroughly investigated for these systems. The stability in the chemical composition of the otolith core is a basic requirement for monitoring nursery areas, especially when studying or comparing different cohorts of fish (Hamer *et al.*, 2003; Patterson *et al.*, 2008; Walther & Thorrold, 2009; Tournois *et al.*, 2013). In this regard, it is important to determine whether there is inter-annual variation so as not to misinterpret the conclusions (Patterson *et al.*, 2008; Walther & Thorrold, 2009; Reis-Santos *et al.*, 2012; Tournois *et al.*, 2013). Moreover, the existence of inter-annual variation would make it impossible to use otolith chemistry to retrospectively identify juvenile habitat use.

Ideally the temporal variability assessment would be made using juveniles collected in different years before there is significant opportunity for mixing. However, it has been shown that connectivity (spatial variability) among the studied estuaries is very low or null, especially among estuaries of Brazil (Avigliano *et al.*, 2016). Via quadratic discriminant function analysis of otolith core chemistry, Avigliano *et al.* (2016) obtained percentages of classified back to natal nursery areas with 100% cross-validation classification accuracies in the cohorts 2002, 2003, 2006 and 2007 (except 2002 cohort among PR and PL with 80–96% classification accuracies). This indicates that otolith core chemistry of adults *G. barbus* represents a good habitat marker for the study and monitoring of nursery areas. Then, the primary and fundamental assumption made here is that there was no mixing of fish between estuaries between juvenile and adult stages.

Here, we expand on our previous work to examine inter-annual variability in otolith chemical signatures. In this sense, our objective was to examine the inter-annual variability in estuary-specific signatures for three estuaries where adults were collected over multiple years. Secondarily, we evaluate whether the percentages of classification of individuals to their natal origin place are affected by the grouping of several cohorts. For this, we assessed the performance of quadratic discriminant functions to spatially discriminate nursery areas using grouped or separated cohorts. These data are used to discuss potential errors that could arise if migrants are not classified using baseline signatures from the appropriate cohort.

MATERIALS AND METHODS

Study area

Samplings were conducted in the Paranaguá Bay (PB), Patos Lagoon (PL) and Plata estuary (PE) (Figure 1) from north to south. The Paranaguá Bay estuary (PB) is 601 km^2 in area (Figure 1) and is connected with the adjacent continental platform by the channels Galheta and Sudeste (Angulo & Araújo, 1996). This system is located in the mountain subtropical rainforest ecoregion (25°28′53.21″S–48°24′41.06″W). Temperature water varied from 18–25°C in winter to 23–30°C in summer while salinity ranged from 12–29 in winter to 20–34 in summer (Lana *et al.*, 2001). The Patos Lagoon (PL) is a large coastal lagoon located on the coast plain of Río Grande do Sul (Brazil) (31°29′59.31″S–51°40′46.24″W), with a total area of 10 360 km^2 and is connected to the Atlantic Ocean by a narrow channel that is 4 km long and 740 m wide (Figure 1). The southern part of the system is occupied by an estuary (10% of the lagoon area). Temperature range is 12°C in winter to 27.5°C in summer (Muxagata *et al.*, 2012) and shows changes in salinity over hourly periods, but the average salinity (0–32) follows a seasonal pattern (dry summer and wet winter) influenced primarily by rainfall and wind direction (Seeliger *et al.*, 1997). Plata River estuary (PR) is the most important coastal ecosystems from Plata Basin (Argentina and Uruguay), with a total area of 10 360 km^2 and an average discharge of 23 000 m^3 s^{-1} towards the Atlantic Ocean (Guerrero *et al.*, 1997). This system is located in the Pampan plain (35°48′6.07″S–6°14′52.92″W) and shows variation of temperatures between 8–24°C (Guerrero *et al.*, 1997), highly variability salinity (0–32) and stratification during the year (Acha *et al.*, 2008).

Sampling

Adult catfish were caught in three estuaries (Figure 1) with hooks, longlines and gillnets, at depths ranging from 8 to 33 m between November 2010 and May 2015. Fishes between 7–12 years (total length range: 42–84 cm) were selected for analysis (total N = 107) (Table 1). The total length (in cm) was recorded and both *lapilli* otoliths were removed, rinsed with ultrapure water and cleaned of any remaining tissue with a plastic toothbrush. *Lapilli* otoliths were used rather than *sagittae* otoliths because they were larger and allowed less measurement error.

Age determination and sample preparation

In this study, the core of the otolith representing 0+ age was isolated following the methodology (manual micromilling) proposed by Avigliano *et al.* (2016). This age corresponds to the early months of life that occur between the fresh water or estuary, where the species spawns (Reis, 1986b; Araújo, 1988; Pereyra *et al.*, 2016).

The left otolith of each pair was weighed, washed with ultrapure water, dried and embedded in crystal epoxy resin. Only left otoliths were used to avoid possible differences in the chemical composition of right and left otholits (Loher *et al.*, 2008). Next, the otolith was sectioned transversely through the core to a thickness of 1000 μm using a Buehler® Isomet low speed saw equipped with twin diamond edge blades (15 HC series). Age was determined

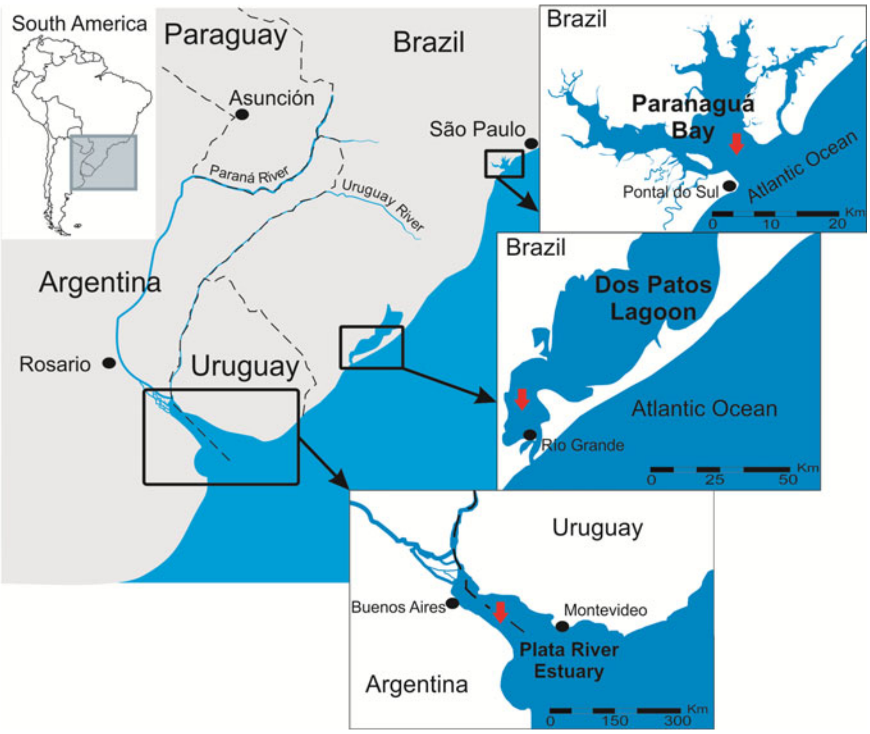

Fig. 1. Sampling sites of the catfish *Genidens barbus* (red arrows).

by counting of the rings in the otolith section immersed in ultrapure water, using a stereomicroscope (Leica® EZ4-HD, Singapore) at 8× magnification, by two independent observers. Age determination by counting the ring (*annuli*) number in *lapilli* otoliths of *G. barbus* was validated by Reis (1986a).

The precision of the age determinations between two readers was calculated by using the following equation of index of average percentage error (IAPE) (Beamish & Fournier, 1981):

$$\mathrm{IAPE}j = \frac{1}{N}\sum_{j=1}^{N}\left(\frac{1}{R}\sum_{i=1}^{R}\frac{|X_{ij} - X_j|}{X_j}\right) \times 100$$

where N is the number of fish aged, R is the number of times each fish is aged, X_{ij} is the age determination of the j fish, X_j is the mean age calculated for the j fish.

IAPE between the two independent readers was relatively low (1.31%).

Table 1. Number of specimens of *Genidens barbus* per group (birth year or year-class) and sampling site. PR, Plata River; PL, Patos Lagoon and PB, Paranaguá Bay.

Birth year	PR	PL	PB	Total
2002	18	6	–	24
2003	14	8	2	24
2004	12	9	–	21
2005	6	–	–	6
2006	9	–	19	28
2007	–	–	4	4
Total	59	23	25	107

According to Avigliano *et al.* (2016) and Holden & Raitt (1974), to avoid possible year-class or cohort effect in trace elements composition of the otoliths, the year of birth of fish was calculated by subtracting the number of annuli from the capture date. Then, the year-class or cohort represents the year fish were born (Table 1).

Otolith cores were exposed in the sections by polishing the rings from the margin to the core with a high speed lathe (Dremel 300) equipped with a diamond blade (Dremel EZ545-EZ), under a stereomicroscope (Leica® Zoom 2000 Z30 V) (Buffalo, NY, USA) in a laminar flow hood. Sections were washed with Milli-Q water (18.2 MΩ) (Millipore, Sao Paulo, Brazil) to remove dust contamination from the micromilling process. Once dry, the cored material was removed carefully from the section using forceps, washed in Milli-Q water and dried. Otolith cores were transferred to a sterile centrifuge tube and weighed using a Sartorius AG® ED2242 (Göttingen, Germany) microbalance to the nearest 0.0001 g. After weighing, cores were decontaminated with 1% HNO_3 (Arslan & Secor, 2008) and rinsed 5 times with Milli-Q to remove any contamination from weighing and micromilling, transferred to new sterile centrifuge tubes and dried overnight in a laminar flow hood.

Otolith chemistry

Otolith cores were digested with 10% nitric acid during 48 h (Avigliano *et al.*, 2015a) and the abundances of isotopes ^{137}Ba, ^{44}Ca, ^{24}Mg, ^{55}Mn, ^{88}Sr and ^{66}Zn were determined by inductively coupled plasma mass spectrometry (ICP-Ms), Agilent 7500 cx (Agilent, Waldbronn, Germany) equipped with a Micro Mist nebulizer for ^{44}Ca and ^{88}Sr and Cross Flow nebulizer for ^{137}Ba, ^{24}Mg, ^{55}Mn and ^{66}Zn. After the analysis of each sample, the instrument was cleaned with Milli-Q water and 5% nitric acid matrix to prevent memory effects.

Multi-element calibration standard 2-A (Quality Control Standard, Agilent Technologies® Pure, Japan) was used as an external standard while scandium, yttrium, terbium and holmium were used as internal standards. All measurements were performed in triplicate (RSD $< 4\%$).

Based on three times the standard deviation, the detection limits for water samples (LOD) in $\mu g\,l^{-1}$ of the blank signal ($N = 10$) were $0.2\ \mu g\,l^{-1}$ for Ba, Mg, Mn and Zn and $15\ \mu g\,l^{-1}$ for Sr and Ca. Otolith Certified Reference Material for trace elements (FEBS-1, National Research Council, Canada) was used to checked the digestion and analytical procedures. Analysis of these reference materials showed good agreement, with the following element recovery rates: 99% for Ba, 114% for Ca, 105% for Mg, 104% for Mn, 93% for Sr and 114% for Zn.

Trace elements concentration were expressed as molar ratios (mmol element:mol Ca) (Sinclair *et al*., 1998; Bailey *et al*., 2015).

Data analysis

TEMPORAL VARIABILITY

The element:Ca ratios did not fit the normal distribution and homogeneity of variance (Shapiro–Wilk, $P < 0.05$; Levene, $P < 0.05$) even after transformation log $(x + 1)$ or square root. Therefore, non-parametric statistics were used to compare variables between year-classes or cohorts. To ensure that differences in otolith weight (≈fish size) among samples did not confound any temporal patterns in elemental composition, the effect of otolith weight on element ratio was examined used analysis of covariance (ANCOVA, otolith weight as co-variate) (Longmore *et al*., 2010; Kerr & Campana, 2013). Mn:Ca ratio (only Patos Lagoon) correlated with otolith weight ($P < 0.01$, $b = -17.23$) and was corrected by subtracting the common slope in ANCOVA (Longmore *et al*., 2010; Kerr & Campana, 2013). This fit eliminated the effect of otolith weight on the Mn:Ca variable (ANCOVA, $P > 0.05$). Ba:Ca, Mg:Ca, Sr:Ca and Zn:Ca ratios were not significantly correlated ($P > 0.05$) with otolith weights and it was not necessary to correct any variable.

Each element:Ca ratio was compared among year cohorts using the Kruskal–Wallis test. Mardia's skewness and kurtosis tests showed multi-dimensional non-normality; hence, permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was used to evaluate the otolith core multi-elemental fingerprints and detect differences in core area from different year-classes for each sampling sites. The analysis was based on Gower distances and under 9999 permutations (Gower, 1966). Modified Gower dissimilarity matrix is more appropriate for dealing with multivariate heterogeneity of variance than the Bray–Curtis measure (Anderson *et al*., 2006).

Finally, discriminant function analyses (DA) were used to assess the ability of otolith elemental ratios to classify adults to their cohorts (Kerr & Campana, 2013; Bouchard *et al*., 2015). Because the assumption of homogeneity of variances-covariances matrices was not met (verified with Bartlett test), quadratic discriminant function analysis (QDA) was used. The expected classification accuracies was calculated based on chance alone given the number of groups and sample sizes (Ruttenberg & Warner, 2006; White & Ruttenberg, 2007). A randomization test was used to determine if the classification success rate was significantly different from random (Hayes, 1998; Ruttenberg & Warner, 2006; White & Ruttenberg, 2007).

Multicollinearity between variables was analysed, thus preventing a false outcome in the QDA analysis and the use of redundant variables in the study (Graham, 2003). All statistical tests were performed using the InfoStat 2015, Past 3.01 and SPSS 19 programs.

SPATIAL VARIABILITY

In this work we do not intend to describe the spatial differences, but to assess whether the temporal variation has a strong effect on the spatial classification of individuals. To this end, PERMANOVA and QDA with randomization test were performed for each estuary and cohort separately and, on the other hand, grouping the cohort (just classifying by the location). If there is not a strong temporal variation in some of the estuaries, we expect to obtain similar percentages of classification between both analyses. Previously, normality and homogeneity of variance assumptions were verified with ANCOVA, as well as the relationships between elemental concentration and otolith weight.

RESULTS

Temporal variability

The average values and deviations from element:Ca ratio are shown in Figure 2. Most element:Ca ratios were no significantly different among year cohorts. In the PR, the otolith Mn:Ca, Sr:Ca and Zn:Ca ratios were similar between year-classes ($P > 0.06$) while Ba:Ca and Mg:Ca were higher for 2005 and lower for 2003 cohort ($P < 0.01$). In the PL, the otolith Mn:Ca ratio was lowest for 2002, highest for 2003 and intermediate for 2004 ($P < 0.02$), while Ba:Ca, Mg:Ca, Sr:Ca and Zn:Ca were similar between year-classes ($P > 0.05$). No significant differences ($P > 0.05$) were found among year cohorts for PB in element:Ca ratios.

Results from PERMANOVA analysis revealed significant differences in the multi-element signatures of the otolith core between cohorts for the PR ($F = 2.9$, $P = 0.006$) and the PL ($F = 2.3$, $P = 0.03$) while no significant differences ($F = 0.2$, $P = 0.9$) were found for PB. The P values of the PERMANOVA tests among years are shown in Table 2. Specifically for PR, the chemical signature of the 2005 cohort differed significantly from 2002, 2003 and 2006 (Table 2). In the case of PL, 2003 cohort differed from the 2002 and 2004 ones (Table 2).

The relative importance of each signature in homogenizing multivariate signatures among year cohorts was shown by classification rates of QDAs (Table 3) and biplot (Figure 3). Classification rates were generally low for the PR and the PB, averaging 50 and 39%, respectively. In PR only a relative high percentage of individuals was properly classified to 2005 (75%) while for PB 2003 had 100% classification. For PL the assigned percentage of individuals was relatively high (81%) varying from 77.8 to 85.7% (Table 3). All the percentages of correctly classified individuals were significantly different from random ($P < 0.05$).

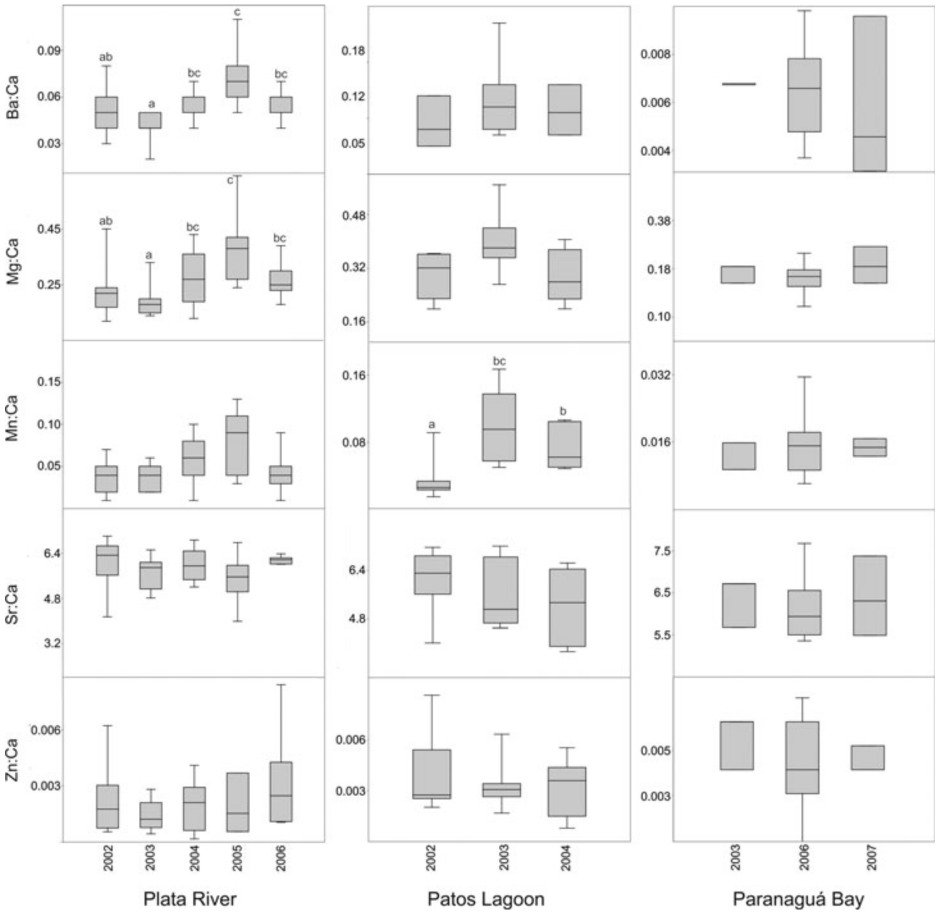

Fig. 2. Mean ± SE elemental ratio (mmol/mol) in core of otoliths from different sampling locations for year-classes. Different letters indicate statistical significant differences.

Cohort grouping and spatial variability

Results from PERMANOVA revealed significant differences in the multi-element signatures of the otolith core between sampling sites, taking into account the separated and grouped cohorts ($0.0001 < P < 0.04$). The percentage of individuals correctly classified was high when cohorts were analysed separately, averaging 93.1% for the PR, 89.1% for the PL and 100% for the PB (Table 4). However, the percentages of classification decreased between 15.5 and 25% for PR and PL when grouping cohorts, averaging 77.6 and 63.6% respectively (Table 4). Percentages of classification were significantly different from random ($P < 0.05$). The percentage of classification remained unchanged for PB when analyses were performed by grouping cohorts (Table 4).

Table 2. The *P* values of the PERMANOVA tests based on multi-elemental composition (Ba:Ca, Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca and Zn:Ca) of otolith core. PR, Plata River; PL, Patos Lagoon and PB, Paranaguá Bay. Bold text indicates a statistically significant correlation with $P < 0.05$). N = number of specimens.

Sampling site	Cohort (N)	2002	2003	2004	2005	2006
PR	2002 (18)	–	0.59	0.07	**0.00**	0.30
	2003 (14)	0.59	–	0.20	**0.02**	0.24
	2004 (12)	0.07	0.20	–	0.07	0.44
	2005 (6)	**0.00**	**0.02**	0.07	–	**0.02**
	2006 (9)	0.30	0.24	0.44	**0.02**	–
PL	2002 (6)	–	**0.04**	0.40	–	–
	2003 (8)	**0.04**	–	**0.04**	–	–
	2004 (9)	0.40	**0.04**	–	–	–
PB	2003 (2)	–	–	–	0.90	0.86
	2006 (19)	–	0.99	–	–	0.52
	2007 (4)	–	0.86	–	0.52	–

DISCUSSION

This work makes it clear that the temporal variation in the chemical signature of the adult catfish otolith core can greatly affect the percentages of spatial classification. Therefore, the group of cohorts used to study nursery areas could lead to a misinterpretation of results.

It is important to underline that this study was performed using cores of adult individuals and not juveniles. It is necessary to take this point into account because this method may lead to wrong conclusions, confusing temporal variation with variations caused by connectivity between estuaries. However, we have assumed that connectivity among estuaries is extremely low, based on reports of Avigliano *et al.* (2016) and the high levels of spatial classification obtained in this study (Table 4). Supporting this assumption, transects of Sr:Ca and Ba:Ca obtained by LA-ICPMS of the same otoliths

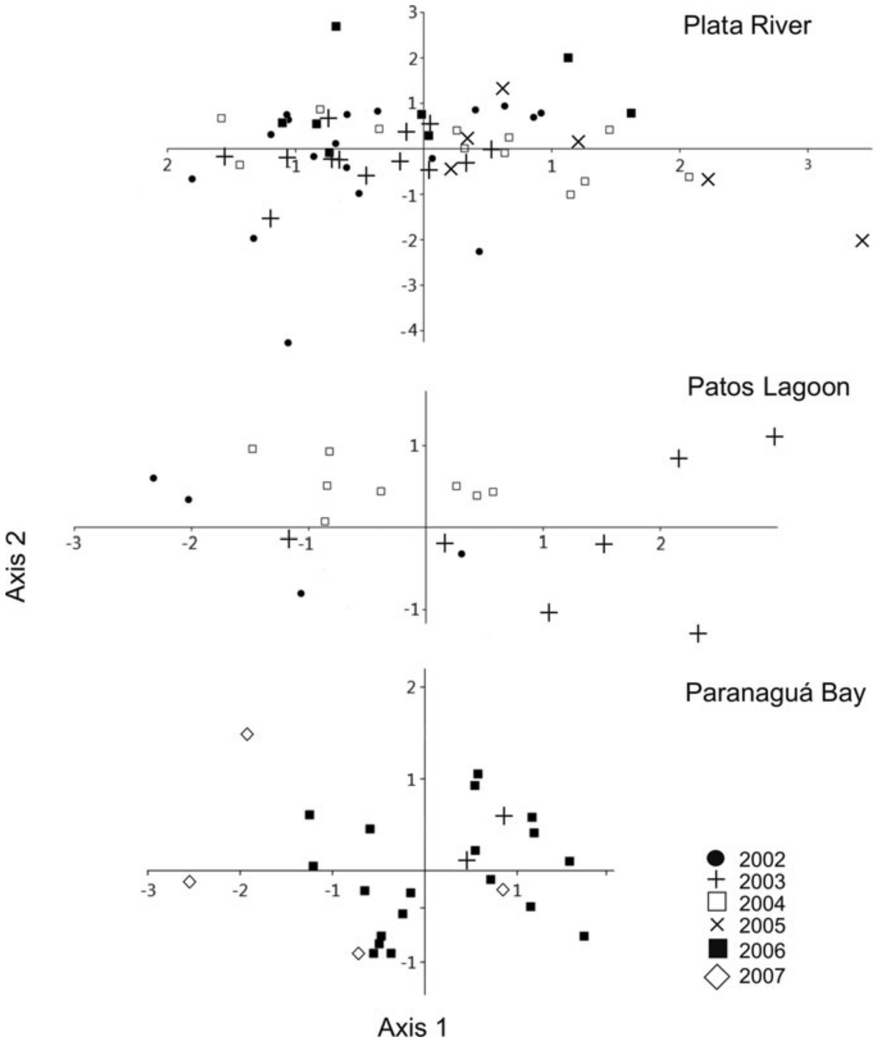

Fig. 3. Discriminant analysis of the elemental ratios in core of otoliths from different sampling locations for year-classes.

used in this study show that migrations occur mainly among freshwater and estuarine environments with the marine incursions being relatively rare and brief (Avigliano 2016, unpublished data). Previously, Avigliano *et al.* (2015b) have

Table 3. Percentage of adult fish classified to each year-class by quadratic discriminant function analyses based on multi-elemental composition (Ba:Ca, Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca and Zn:Ca) of otolith core. PR, Plata River; PL, Patos Lagoon and PB, Paranaguá Bay. Bold numbers indicate the percentage of correctly classified individuals. N = number of specimens.

Sampling site	Cohort (N)	2002	2003	2004	2005	2006	2007
PR	2002 (18)	**33.3**	0	38.9	22.2	5.6	–
	2003 (14)	38.5	**30.8**	30.8	0	0	–
	2004 (12)	9.1	0	**63.6**	18.2	9.1	–
	2005 (6)	0	0	25.0	**75.0**	0	–
	2006 (9)	0	0	37.5	12.5	**50.0**	–
PL	2002 (6)	**80.0**	20.0	0	–	–	–
	2003 (8)	14.3	**85.7**	0	–	–	–
	2004 (9)	11.1	11.1	**77.8**	–	–	–
PB	2003 (2)	–	**100**	–	–	0	0
	2006 (19)	–	55.6	–	–	**27.8**	16.7
	2007 (4)	–	33.3	–	–	0	**66.7**

Table 4. Percentage of adult fish classified to each region (separate and grouped cohorts) by quadratic discriminant function analyses based on multi-elemental composition (Ba:Ca, Mg:Ca, Mn:Ca, Sr:Ca, Ba:Ca and Zn:Ca) of otolith core. PR, Plata River; PL, Patos Lagoon and PB, Paranaguá Bay. Bold numbers indicate the percentage of correctly classified individuals. N = number of specimens.

Separate cohorts	Original region (N)	Predicted region		
		PR	PL	PB
2002	PR (18)	**94.4**	5.6	–
	PL (6)	20	**80**	–
2003	PR (14)	**78.6**	0	21.4
	PL (8)	12.5	**87.5**	0
	PB (2)	0	0	**100**
2004	PR (12)	**100**	0	–
	PL (9)	0	**100**	–
2006	PR (9)	**100**	–	0
	PB (19)	0	–	**100**
	Mean	**93.1**	**89.1**	**100**
Grouped cohorts (2002, 2003, 2005, 2006)				
	PR (53)	**77.6**	12.1	10.3
	PL (23)	27.3	**63.6**	9.1
	PB (21)	0	0	**100**

studied the habitat use of a few specimens caught in PR and PL and have found Sr:Ca ratios $\times 10^{-3}$ less than 6.5. For other diadromous species in the study area (*Centropomus parallelus*) a Sr:Ca ratio $\times 10^{-3}$ of 8–11 has been considered as reference value for seawater (Daros *et al.*, 2016). This suggests that the values lower than 6.5 reported by Avigliano *et al.* (2015b) for catfish correspond to the use of estuarine and freshwater environments, with marine incursions being rare. In short, the classification percentages obtained in this work would not be modified when using juvenile specimens instead of adult ones.

On the other hand, it is very difficult to collect a representative sample size of juveniles to make this type of study. First, because of mouthbrooding by males which lasts about 3 months, during which they do not feed and are not captured by fishing gear with hook (Araújo, 1988). Even today, it is difficult to collect adult specimens, due to local unrest among fishermen and academic and political fields, generated by the prohibition of use of the resource (Di Dario *et al.*, 2015).

Retrospective determination of natal source depends on the premise that chemical signatures are sufficiently stable across time to allow for accurate classification (Rooker *et al.*, 2003). To date, several studies have examined the issue of temporal stability of otolith chemistry and results indicate that chemical signatures vary among year cohorts (Patterson *et al.*, 1999, 2008; Campana *et al.*, 2000; Gillanders, 2002, 2005; Hamer *et al.*, 2003; Walther & Thorrold, 2009; Carson *et al.*, 2013; Walther *et al.*, 2014). Long-term stability of otolith chemical signatures is not evident in any study to date, suggesting that chemical signatures may serve only as short-term natural tags (1–3 years) (Rooker *et al.*, 2003, 2008; Schaffler *et al.*, 2014). As in the present work, Schaffler *et al.* (2014) compared the efficiency of the classification of juvenile from Atlantic Menhaden *Brevoortia tyrannus* by grouping cohorts. However, these authors found that errors increased only 7–10% by grouping different cohorts, concluding that on short time scales (a few years), a discriminant function built from several year-classes may be able to accurately predict group membership. Rooker *et al.* (2003) have found that the spatial variation between nursery areas of bluefin tuna (*Thunnus thynnus*) is so strong that the classification of individuals in their respective areas of origin is not affected by the existing temporal variation. This allowed performing further studies gathering year-class without compromising the interpretation of results (Rooker *et al.*, 2008). Campana *et al.* (2000) found that the otolith chemistry of Atlantic cod *Gadus morhua* was stable between years but not over longer time frames (4–13 years). Similarly, Schaffler & Winkelman (2008) found that a discriminant function based on 3 years of data for striped bass *Morone saxatilis* was able to predict group memberships with only 4–6% increased error for two cohorts, but for a third year-class the overall error rate increased to 51%.

In the present study, otolith chemical signature of *G. barbus* differed between year-classes in the PR and PL. However, only age class 2005 in PR and 2003 in the PL were significantly different from the rest, which explains the relatively high percentage of well classified individuals for those years (75 and 85%, respectively) in the analyses of inter-annual variation. On the other hand, univariate contrasts for all five elements were statistically similar in all sampling sites, except in the Ba:Ca and Mg:Ca ratios for the PR and Mn:Ca ratio for the PL. These differences in inter-annual variation were enough to adversely affect the space qualifying percentage for PR and PL (overall errors increased 15.5–25%, respectively) when year-classes were grouped, indicating spatial differences were not sufficient to counter temporal variability. Furthermore, we recognize the limitations to the use of such a small number of fish from PB (2003 and 2007) which could affect the power of statistical tests.

Other authors have reported a higher univariate variation between different cohorts than that observed in this study. However, the percentages of classification obtained from the discriminant functions are dissimilar between studies and depend on the environment and biological characteristics of the species. For example, Walther & Thorrold (2009) obtained percentages of inter-annual classification between 8–38% in *Alosa sapidissima*, while Rooker *et al.* (2003) obtained percentages between 71–80% for *Thunnus thynnus* when in both works they found univariate temporary differences in several elements. The percentages of inter-annual classification found for *G. barbus* were intermediate in relation to those reported in previous works.

It is clear that with the obtained data it is not possible to relate directly the inter-annual variability in the otolith fingerprint with the environmental or physicochemical variations. However, water chemistry and several environmental factors (temperature, salinity, diet, genetics) may generate variability in otolith chemistry (Campana, 1999; Brown & Severin, 2009; Tabouret *et al.*, 2010; Sturrock *et al.*, 2012; Avigliano & Volpedo, 2013; Reis-Santos *et al.*, 2013; Bouchard *et al.*, 2015). Depending on the species, the incorporation of Mn, Mg and Zn in the otolith can be influenced (or not) by environmental or physiological factors (Martin & Thorrold, 2005; Ranaldi & Gagnon, 2008). Moreover, in diadromous fish the incorporation of elements such as Sr and Ba would be related mainly to salinity (Secor & Rooker, 2000; Kraus & Secor, 2004; Martin & Wuenschel, 2006; Brown & Severin, 2009; Tabouret *et al.*, 2010; Sturrock *et al.*, 2012). Walther & Thorrold (2009) have found inter-annual differences in the chemical form of other fish in several rivers in the North-west Atlantic, and these differences have been related to major river changes. Patterson *et al.* (2008) also has explained the inter-annual differences in otolith chemistry of red snapper *Lutjanus campechanus* with variations in environmental or hydrodynamic conditions of the study area.

Nevertheless, differences in spatial otolith chemistry were expected because hydrography (temperature, water fluxes, salinity, bathymetry and ecoregion) differ between the estuary studies. The temporal variation observed in 2005 for the PR and in 2003 for the PL would also be related, at least in part, to environmental variations. Particularly in the transition from 2002 to 2003 the PL experienced extreme hydrological changes due to the higher rainfall peak recorded in the period 1986–2008 (more than 2000 mm per year) and the increase in the volume of river discharge into the estuary in the period 1964–2004 (Marques & Möller, 2008; Möller *et al.*, 2009; Abreu *et al.*, 2010). In 2003 the highest peak of chlorophyll in the last 30 years also was registered ($18\ \mu g\ l^{-1}$) (Abreu *et al.*, 2010). These changes were associated with the greatest decrease of salinity (<2 UPS) and increase in water temperature (up to 30°C) in the period 1986–2008 (Abreu *et al.*, 2010) which severely affected some fisheries, such as the pink shrimp (Möller *et al.*, 2009). On this site, a non-significant trend to the increase of the Ba:Ca ratio for 2003 was observed, consistent with the low salinity and the increase of flux/rainfall recorded for that year.

On the other hand, in 2005 a cell of cool water was observed in the PR coast with temperatures 2–4°C cooler than the surrounding waters (Simionato *et al.*, 2010). Again, variations of this kind could affect the incorporation of elements such as Ba and Mg in the PR, although we cannot relate it directly.

The absence of univariate significant differences in the element:Ca ratios found in PB (2003, 2006 and 2007) suggests a relative stability in the factors that have influence on the incorporation of the different elements in the otolith. In this sense, the grouping of cohorts for the resource study might be plausible only in this site, at least in the cohorts studied.

In conclusion, this paper attempts to address the issue of temporal (inter-annual) variability in site-specific otolith chemistry signatures. Significant temporal variation was found for some sites in the Ba:Ca, Mg:Ca and Mn:Ca ratios as well as multivariate differences (PR and PL).

The variations were sufficient to significantly reduce the power of discrimination between the three nursery areas. This shows evidence of the importance of testing the spatial variability before grouping cohorts deliberately to study the resource by using the core of the otoliths.

This is a worthy goal, and one that deserves more attention in the otolith chemistry literature, particularly because temporal variability may interfere with the ability to classify natal origins of adult fishes if the wrong baseline signature is used to parameterize the classifying function. For this reason, although it is difficult to obtain large sample sizes of *G. barbus*, it is not recommended to use a cohort group for monitoring and management of this species, unless trace elements and year-classes do not show temporal variation.

ACKNOWLEDGEMENTS

We thank Marta Bavio and Estefania Videla for excellent technical assistance. We also thank the anonymous reviewers for helpful comments on the manuscript.

FINANCIAL SUPPORT

The authors are indebted to CONICET (PIP 112-20120100543CO), ANPCyT (PIP 2010-1372), Universidad de Buenos Aires (UBACYT 20620110100007), Universidad Nacional de San Martín, Instituto de Oceanografía from Universidade Federal do Rio Grande (FURG) and Centros Asociados para el Fortalecimiento de Posgrados Program (CAFP BA 43/13 Brazil/Argentina) for financial support.

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