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Fin spine chemistry as a non-lethal alternative to otoliths for stock discrimination in an endangered catfish

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ABSTRACT: Trace element:Ca (Ba:Ca, Mg:Ca, Mn:Ca and Sr:Ca) ratios in water and last growth intervals (outer 45 µm) of dorsal spine and otolith edges from *Genidens barbus* collected in 3 study areas (Brazil, Argentina–Uruguay and Patagonia) were compared to determine whether the spine can provide a non-lethal natural marker alternative to otoliths for this endangered species. We found an association between calcified structures and the availability of some element:Ca ratios in water. Among the measured element:Ca ratios, a strong correlation in Ba:Ca between outer edges of fin spines and otoliths was found ($r^2 = 0.87$, $p = 0.0001$), whereas Mg:Ca, Mn:Ca and Sr:Ca ratios were weakly correlated between structures ($0.09 < r^2 < 0.20$, $0.0006 < p < 0.02$). Several ratios were significantly different among sites for otolith and spine edge ($p < 0.05$). Permutational multivariate ANOVA ($p < 0.05$) and quadratic discriminant analysis (QDA) proved highly effective for characterizing differences in otolith and spine edge compositions between sampling sites (mean classification rates: 84.2 and 90.1% for otolith and spine edge, respectively), suggesting that both structures can be used as habitat markers. To identify the possible contribution of multiple stocks to the different areas, we performed QDA for the complete last year of a fish's life. Classification rates were high for both structures, averaging 79.6 and 81.2% for otolith and spine, respectively, suggesting the existence of new stocks in Patagonian waters. Spine chemistry seems to be an acceptable non-lethal advantage over otoliths to study different biological aspects of catfish.

KEY WORDS: *Genidens barbus* · Anadromous · Microchemistry · Southwestern Atlantic · Calcified structures · Indicator · Segregation · LA-ICP-MS · SXFM

1. INTRODUCTION

In recent decades, many fish species of commercial and ecological value have become endangered due to combined loss of habitat and overexploitation, leading to the collapse of fisheries around the world (FAO 2018). Efficient management strategies for fisheries require accurate understanding of population structure and habitat use among exploited species, in order to protect endangered stocks from over-

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fishing and allow for sustainably managed future populations (Cadrin et al. 2013). For some species, the problem has exceeded fisheries management policies and has become a conservation issue. This is the case for the anadromous white sea catfish *Genidens barbus*, for which there has been a constant and marked decrease in catches due to poor management policies and over-exploitation. In response, the catfish has been included on the Red List of endangered species in Brazil (MMA 2014) and was classified as vulnerable in Argentina (Baigún et al. 2012). Thus, its capture, transport and commercialization have been prohibited since 2015 in the major fishing areas (MMA 2014). This species is especially vulnerable because of its late sexual maturation age (8–9 yr) and particular reproductive strategy, whereby males incubate a few eggs inside their oropharyngeal cavity for months (Reis 1986).

Otolith microchemistry has been widely used as a natural tag of water chemistry in different environments (Cadrin et al. 2013). Because otolith calcium carbonate (aragonite) accretes continually and is not subsequently resorbed or otherwise altered (Casselman 1990), core-to-edge chemical time series for otoliths provide insights into marine and freshwater habitats experienced during the life histories of fish (e.g. Radigan et al. 2018). Particularly in *G. barbus*, otolith microchemistry has been a useful tool for stock classification (Avigliano et al. 2015a, 2017a), identification of nursery habitats (Avigliano et al. 2015a, 2016) and other aspects of its life history (Avigliano et al. 2015b, 2017a) throughout the southwestern Atlantic coastal area. The catfish ecology is complex, due to the presence of several migratory patterns such as freshwater residency, cyclic semi-amphidromy, amphidromy and anadromy (Avigliano et al. 2017a). However, Avigliano et al. (2016) suggested the presence of natal homing behavior because catfish use their birth areas as breeding sites and tend not to mix among large nursery areas. This particular aspect of its ecology enables the discrimination of populations of this species using otolith chemistry.

Nevertheless, due to the vulnerable state of the species, non-lethal methods are preferred to evaluate stocks. In this regard, several authors have used fin ray or spine chemistry as a non-lethal alternative for the study of endangered fish (Clarke et al. 2007, Allen et al. 2009, Smith & Whitledge 2010, Jari et al. 2011, Phelps et al. 2012, Wolff et al. 2013, Rude et al. 2014). Similar to bone, fin spines are mainly composed of hydroxyapatite (calcium phosphate) and collagen (Tzadik et al. 2017). Although fin spine resorption through life is well documented, chemical stability in the marginal area has been observed, making it a suitable candidate for reconstructing past aquatic habitats (Clarke et al. 2007, Tzadik et al. 2017).

Here, we evaluated the chemical composition of the first dorsal fin spine of *G. barbus* as a potential non-lethal natural marker alternative to otolith chemistry. Study objectives included: (1) determining the relationship between spine edge Sr:Ca, Ba:Ca, Mg:Ca and Mn:Ca ratios and water chemistry of 3 different catch sites; (2) evaluating the chemical stability of the spine relative to otoliths in relation to incorporation of elements into the tissue; (3) assessing whether spine edge composition, with and without paired otolith composition, can differentiate stock populations; and (4) simultaneously using spines and otoliths to evaluate new potential stocks in the Patagonian region.

2. MATERIALS AND METHODS

2.1. Study area and sampling

Water samples and fish were collected between November 2017 and January 2018 from 3 regions along the southwestern margin of South America: from north to south, the Paranaguá estuarine complex (PEC) in Brazil; the Paraná River Delta (PRD) in Argentina–Uruguay; and the Negro River (NR) in southern Argentina (Patagonia) (Fig. 1). Fish specimens from PEC and PRD comprise 2 well-defined stocks (Avigliano et al. 2015a, 2016, 2017b), whereas Patagonian fish represent a previously unstudied stock. Surface water temperatures decrease southward in each of the sampled ecoregions, from PEC (18–30°C, mountain subtropical rainforest; Lana et al. 2001), to PRD (8–24°C, temperate flooded savanna; Guerrero et al. 1997) and NR (6–15°C, Andes-sourced water; Luchini 1984).

In total, we collected 57 fish (20 for PEC, 16 for PRD and 21 for NR), using hooks and longlines. For each sampling site (1 sampling site for water and fish), 0.5 l water sample duplicates were manually collected in polyethylene-terephthalate bottles from a depth of 0.5 m. Salinities were measured on site using a Horiba U-52 salinity probe. Both fish and water samples were refrigerated (4°C) during transport to the laboratory. In the laboratory, fish were weighed, measured (total length, TL), and lapilli otoliths and dorsal spines were extracted. Otoliths and spines were cleaned of any remaining tissue using a plastic

Fig. 1. Study area along the southwestern margin of South America. Red arrows show the sampling sites for *Genidens barbus*. PEC: Paranaguá estuarine complex; LPRE: La Plata River Estuary

toothbrush that was rinsed with distilled water and dried between specimen processing. Otoliths were weighed on an analytical balance. Specimen lengths (cm, ±SD) for the localities averaged 48.4 ± 5.2 (range: 37.0–58.0) for PEC, 55.8 ± 8.2 (37.0–67.0) for PRD and 40.8 ± 4.2 (32.3–49.1) for NR.

2.2. Elemental analysis of water

Water samples were filtered under vacuum using cellulose acetate filters (0.45 µm) and acidified to 0.2% (v/v) (pH < 2) with nitric acid (Merck Pro-Analysis) (APHA 2012). Concentrations of Ba, Ca, Mg, Mn and Sr were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500 equipped with a Micro Mist nebulizer (Glass Expansion) and a quartz spray chamber; other elements such as Li, Cu, Pb and Zn were below detection limits in the otolith or spine. The water samples for trace element determination were analyzed in triplicate. Recoveries for replicates of reference materials NIST1640a (trace elements in freshwater, National Institute of Standards and Technology) and SLEW-3 (trace elements in estuarine water; National Research Council Canada) were within 6% of certified values, with an overall mean variation of ±2.8%. Limits of detection (µg l^{-1}) were 0.04 for Mg, 0.2 for Ca, 0.01 for Mn, 0.03 for Sr and 0.03 for Ba.

2.3. Preparation and elemental analysis of calcified structures

Left otoliths (n = 57) and dorsal spines (n = 57) were decontaminated 3 times with 3% hydrogen peroxide, 3 times with 2% HNO_3 (Merck) (Avigliano et al. 2017b) and rinsed 5 times with Milli-Q water (resistivity of 18.2 mOhm cm^{-1}), and dried in a laminar-flow hood for 24 h. Otolith and dorsal spine samples were embedded in epoxy resin and sectioned transversely using a Buehler Isomet low speed saw equipped with twin diamond-edge blades to obtain central sections 700 µm in thickness (Fig. 2). In preparation for elemental analysis, the calcified structure sections were fixed to glass slides using clear epoxy resin, polished using 9 µm grit sandpaper and ultrasonically cleaned (4 min) followed by rinsing with Milli-Q water and drying in a laminar flow hood at environmental temperature. In order to account for the possible effect of fish age on last-growth compositions, otolith rings (annuli) were counted using a stereomicroscope (Leica EZ4-HD) at 40× magnification. Annual periodicity of otolith ring formation in catfish has been validated by Reis (1986).

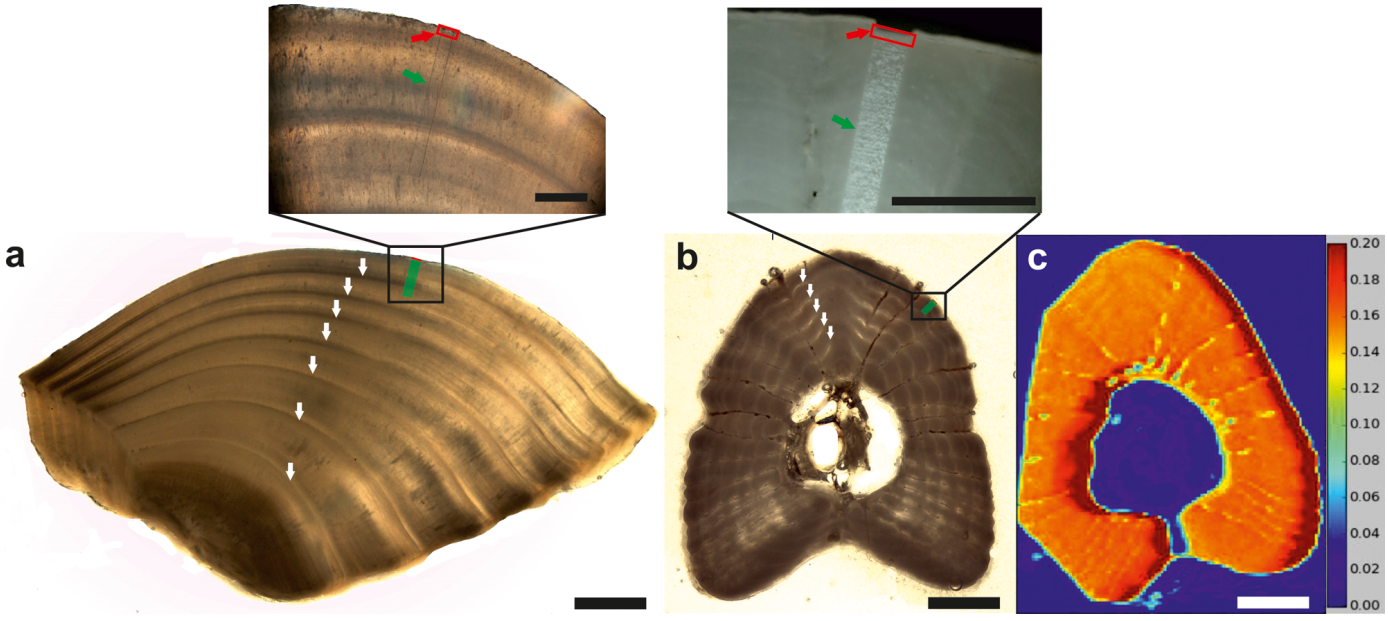

Fig. 2. Calcified structure section of *Genidens barbus* from the Paraná River Delta: (a) otolith, (b) spine and (c) spine calcium map analyzed by scanning X-ray fluorescence microscopy. The scale represents the calcium concentration in mg kg^{-1}. The green rectangle shows the growth in the fish's last year; the red rectangle indicates the last 45 µm of growth; white arrows indicate annuli. Scale bars = 400 µm

To compare elemental compositions (Ba, Mg, Mn and Sr) for recent phases of growth, exterior regions (~600 µm) of otolith and spine sections were analyzed by laser ablation (LA) ICP-MS at the Department of Geosciences, University of Texas at Austin (USA), using an ESI NWR193-UC excimer LA system (193 nm, 4 ns pulse width) coupled to an Agilent 7500ce ICP-MS. The laser system is equipped with a large format 2-volume ablation cell that enabled all samples and standards to be analyzed in 2 separate loadings (otoliths and spines). Laser transects for both otoliths and spines were transverse to growth banding from interior to external edges, using a 15 × 80 µm rectangular aperture while scanning at 5 µm s^{-1} at a repetition rate of 20 Hz. To minimize spatial aliasing, the long axis of aperture was maintained parallel to growth banding in all scans. Laser energy densities (fluences) averaged 2.21 ± 0.03 and 2.91 ± 0.03 J cm^{-2} (±SD) for otoliths and spines, respectively. The ICP-MS instrument was operated at a radio-frequency power of 1600 W with an argon carrier gas flow rate of 900 ml min^{-1}. Oxide production rates, as monitored by ThO/Th on NIST 612, averaged 0.36 ± 0.04% over the analysis periods. Sample analysis intervals (40–60 min) were bracketed by replicate (2 or 3) analyses of standards, including USGS MACS-3, USGS MAPS-4 and NIST 612. Baseline intensities before each standard and sample analysis were determined from 45 s gas blank measurements.

Measured intensities were converted to elemental concentrations (µg g^{-1}) using Iolite software (Paton et al. 2011), with ^{43}Ca as the internal standard and Ca index values of 38.3 weight% for otoliths (aragonite) (Yoshinaga et al. 2000). Unlike aragonitic otoliths, the Ca content of hydroxyapatite in skeletal structures can vary and could not be reasonably assumed based on stoichiometry (Tzadik et al. 2017). Accordingly, spine Ca concentration was independently quantified by scanning X-ray fluorescence microscopy (SXFM) at the Cornell High Energy Synchrotron Source (NY, USA). SXFM employs high-energy X-rays generated by a synchrotron, as described by Limburg et al. (2011). Briefly, a focused X-ray beam was rastered across the sample to produce elemental maps that were quantified by PyMCA software library (http://pymca.sourceforge.net) (Solé et al. 2007). The SXFM-derived Ca content of 17 weight% (Fig. 2) was then assigned as the Ca internal standard concentration for quantifying corresponding Ba, Mg, Mn and Sr and concentrations from LA-ICP-MS analysis. For otoliths, USGS MACS-3 (trace elements in synthetic calcium carbonate) was used as the primary calibration standard and NIST 612 (trace elements in glass) was used as an external reference standard. For spines, USGS MAPS-4 (trace elements in synthetic calcium phosphate) was the primary calibration standard and USGS MACS-3 and NIST 612 were external reference standards. Moreover, an in-house

standard (powdered otoliths pressed into a pellet) was used for SXFM (Limburg et al. 2011). Analysis of reference materials showed good agreement with the following element recovery rates for otoliths and spines, respectively: 97 and 100% for ^{88}Sr, 95 and 93.6% for ^{138}Ba, 85 and 92% for ^{24}Mg, 89 and 82% for ^{55}Mn. Limits of detection (LOD, µg g^{-1}), calculated in Iolite as a measure of the standard deviation of the estimated baseline intensity during bracketing gas blank intervals, for respective otoliths and spines were: 0.10 and 0.02% for ^{88}Sr, 0.17 and 0.04% for ^{138}Ba, 0.34 and 0.09% for ^{24}Mg, 0.99 and 0.16% for ^{55}Mn. Molar element:Ca ratios, derived from the quantified elemental concentrations in water and calcified structures, are reported and discussed in the sections below.

2.4. Statistical analysis

Spatial element:Ca variations in water composition and last-growth (outermost 45 µm) intervals of otoliths and fin spines among sample localities were statistically analyzed to evaluate environmental correspondence.

Element:Ca ratios of the calcified structures were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests, respectively. Only the otolith and spine Sr:Ca ratio met both normality and homogeneity (Shapiro-Wilk and Levene's, $p > 0.05$). The otolith Mg:Ca and Mn:Ca and spine Mn:Ca met both normality and homogeneity assumptions after Log $(x + 1)$ or $\sqrt{x}$ transformation. The otolith Ba:Ca and spine Ba:Ca and Mg:Ca ratios did not meet homogeneity (Levene's, $p < 0.05$) (normality was met; Shapiro-Wilk, $p > 0.05$), even after being Log $(x + 1)$ or $\sqrt{x}$ transformed. To ensure that differences in fish age, total length or otolith weight did not confound spatial patterns in otolith elemental composition, we examined the effect of these factors (co-variables) on the elemental ratios by using analysis of covariance (ANCOVA) (Cadrin et al. 2013). ANCOVA is robust to violations of the assumption of homogeneity of variance (Olejnik & Algina 1984). Covariance between fish age, total length and spine elemental ratios were also tested. Only the spine Sr:Ca ratio showed a significant correlation with age ($p < 0.05$). This age effect was corrected by subtracting the common slope ($b = -0.063$) in ANCOVA (Cadrin et al. 2013).

Univariate non-parametric statistics (Kruskal-Wallis test) were used to compare otolith Ba:Ca and spine Ba:Ca and Mg:Ca ratios between sampling sites, while ANOVA was used to contrast otolith Mg:Ca, Mn:Ca and Sr:Ca and spine Sr:Ca and Mg:Ca ratios.

Linear regression was used to characterize the relationship between otolith and spine element:Ca ratios (last 45 µm of the otolith edge) and between these structures and water (data were log transformed to homogenize variances). No correlations were made between the calcified structures and water because we only had 3 sampling sites for water.

Permutational multivariate analysis of variance (PERMANOVA) was used to detect differences in the chemistry of calcified structures between sampling sites. Two analyses were performed based on Mahalanobis distances (Anderson 2006) with 9999 permutations for otolith and spine (last 45 µm of the otolith edge) separately. After testing the multicollinearity between variables, 2 (last 45 µm of the otolith edge) quadratic discriminant function analyses (QDAs) was used to assess the ability of elemental ratios to sort fish into specific catch areas (otolith and spine separately). Calculations for expected prior probability classification were based on sample sizes and group numbers (White & Ruttenberg 2007).

Avigliano et al. (2017b) evaluated element:Ca ratios of otoliths in the last year of life of *Genidens barbus* as a potential method for stock identification. Because this catfish migrates annually (Avigliano et al. 2017b), stock differentiation is only possible by comparing element:Ca ratios for the last year of life (~300–600 µm). Moment of capture element:Ca ratios (last 45 µm of the otolith edge) will not include information on possible annual displacements between populations. In this sense, 2 QDAs were performed for both otoliths (~last year) and spines (~last complete ring) separately, using the statistical treatment previously described.

Statistical tests were performed using the Systat 13 and SPSS 19 programs.

3. RESULTS

3.1. Water chemistry

The salinity of the sampling sites was ~27 PSU for PEC and ~0 PSU for PRD and NR. Water Ba:Ca and Mn:Ca ratios tended to be high for NR, intermediate for PRD and low for PEC (Fig. 3). The Ba:Ca ratio ranged from 0.066 (PEC) to 0.41 (NR) mmol mol^{-1}, while the Mn:Ca ratio ranged from 0.017 (PEC) to 1.71 (NR) mmol mol^{-1}.

Opposite patterns were found for water Sr:Ca and Mg:Ca ratios, where the highest values were ob-

served in PEC and the lowest in NR and PRD (Fig. 3). Water Sr:Ca ratios varied from 1.46 to 9.25 mmol mol^{-1}, while Mg:Ca ratios ranged from 372 to 4115 mmol mol^{-1} for PEC and NR, respectively.

3.2. Univariate analysis of calcified structures

Differences in elemental ratios for otoliths and spines among the 3 sampling localities are shown in Fig. 3. The otolith edge Ba:Ca ratio was high for NR, intermediate for PRD and low for PEC (H = 39.6, p < 0.0001). The otolith Mn:Ca ratio was significantly higher for PRD and NR than for PEC ($F_{2,54}$ = 7.2, p < 0.0001), while the otolith Sr:Ca ratio was higher for PEC than for PRD and NR ($F_{2,54}$ = 13.1, p < 0.0001). No significant differences ($F_{2,54}$ = 1.2, p = 0.3) were found between sites for otolith Mg:Ca ratios.

Mg:Ca and Mn:Ca ratios were higher in spines than otoliths by factors of 20 to 180, while both structures had the same order of magnitude of Ba:Ca and Sr:Ca values. Spine and otolith Ba:Ca ratios showed similar spatial trends, with PRD significantly lower than PRD and NR (H = 6.9, p < 0.02) (Fig. 3). By comparison, spine and otolith Mg:Ca ratios differed significantly among the 3 sampling sites (H = 22.8, p < 0.001), while spines showed different ranges, with PRD having the highest and PEC the lowest ratios (Fig. 3). Similar to otoliths, the spine Mn:Ca ratios were significantly higher for PRD and NR than for PEC ($F_{2,54}$ = 14.0, p < 0.001). Spine Sr:Ca ratios were lowest for NR ($F_{2,54}$ = 4.29, p = 0.02), with no signifi-

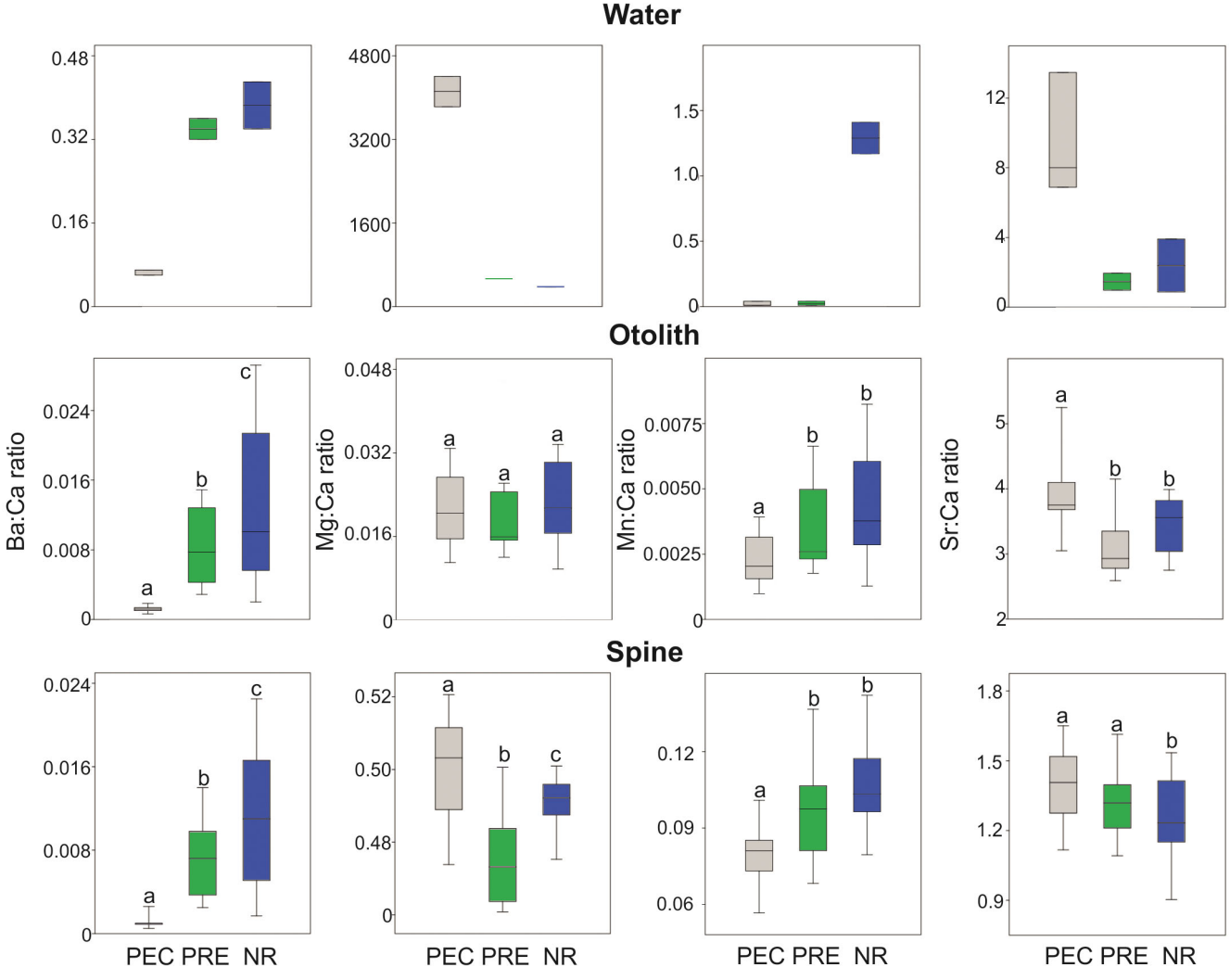

Fig. 3. Elemental ratios (mean ± SD, mmol mol^{-1}) in water and in *Genidens barbus* otoliths and spines from different sampling locations. Different letters indicate statistically significant differences (p < 0.05) between sampling sites. PEC: Paranaguá estuarine complex (Brazil); PRD: Paraná River Delta (Argentina–Uruguay); NR: Negro River (Patagonia, Argentina)

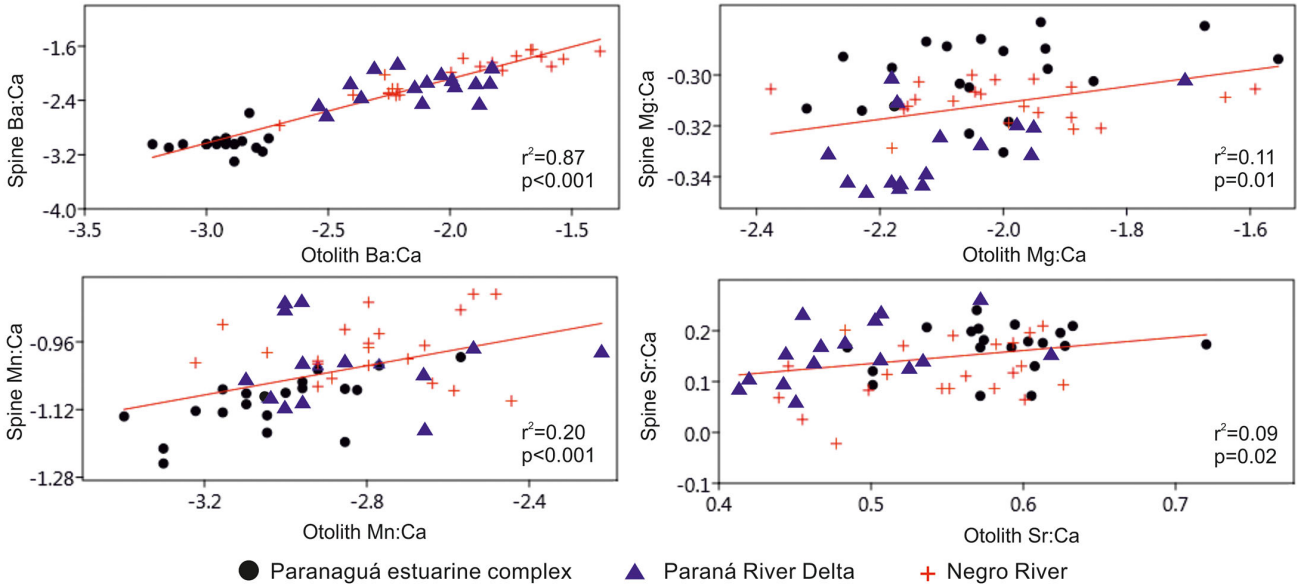

Fig. 4. Linear regressions of *Genidens barbus* spine element:Ca ratios versus otolith element:Ca ratios. All variables were log–log transformed

cant differentiation ($p > 0.05$) between PEC and PRD ($F_{2,54} = 0.7$, $p > 0.05$).

3.3. Relationships between otolith and spine

Dorsal fin spine edge Ba:Ca ratios strongly correlated with edge otolith Ba:Ca ($r^2 = 0.87$, $p = 0.0001$), whereas Mg:Ca ($r^2 = 0.11$, $p = 0.01$), Mn:Ca ($r^2 = 0.20$, $p = 0.0006$) and Sr:Ca ($r^2 = 0.09$, $p = 0.02$) ratios were only weakly correlated between the 2 structures (Fig. 4).

3.4. Multivariate analysis of calcified structures

For otolith and spine samples, PERMANOVA and QDA (Table 1, Fig. 5a,b) were effective for discriminating between sampling sites when the last 45 µm (the moment of capture) of otolith and spine edges were used. PERMANOVA revealed significant multivariate differences among all comparisons of the 3 sampling sites for otolith ($F = 6.0$, $0.002 < p < 0.04$) and spine ($F = 9.1$, $0.0001 < p < 0.0008$). Considering the expected prior probability classification (0.35 for PEC, 0.28 for PRD and 0.37 for NR) based on sample sizes and group numbers, rates of QDA were high for both otolith (mean = 84.2%) and spine (mean = 90.1%). For PEC, the percentage of well classified individuals obtained was 95 and 100% for otolith and spine, respectively. Between PRD and NR, the percentage of correctly classified individuals ranged from 75.0 to 76.2% and 68.8 to 95.2% for otolith and spine, respectively.

When the fingerprint of the last year was used (Table 1, Fig. 5c,d), QDAs were highly effective in detecting differences in otolith and spine chemistry between sampling sites, suggesting the existence of spatial segregation. Classification rates of QDA were very similar between structures, averaging 79.6 and 81.2% for otolith and spine, respectively (Table 1). It is interesting to highlight that all cases of misclassifi-

Table 1. Cross-classification matrix of the quadratic discriminant analysis. Numbers represent the classification percentage on the basis of multi-elemental composition of otolith and spine edge (last 45 µm of the edge and the last year of the fish's life). N: sample size. PEC: Paranaguá estuarine complex (Brazil); PRD: Paraná River Delta (Argentina–Uruguay); NR: Negro River, Patagonia (Argentina)

	N	Last 45 µm			Last year		
		PEC	PRD	NR	PEC	PRD	NR
Otolith							
PEC	20	**100**	0	0	**100**	0	0
PRD	16	0	**81.3**	18.8	0	**62.5**	37.5
NR	21	0	28.6	**71.4**	5	19.0	**76.2**
Mean				84.2			79.6
Spine							
PEC	20	**100**	0	0	**100**	0	0
PRD	16	0	**75.0**	25.0	0	**62.5**	37.5
NR	21	0	4.8	**95.2**	0	19.0	**81.0**
Mean				90.1			81.2

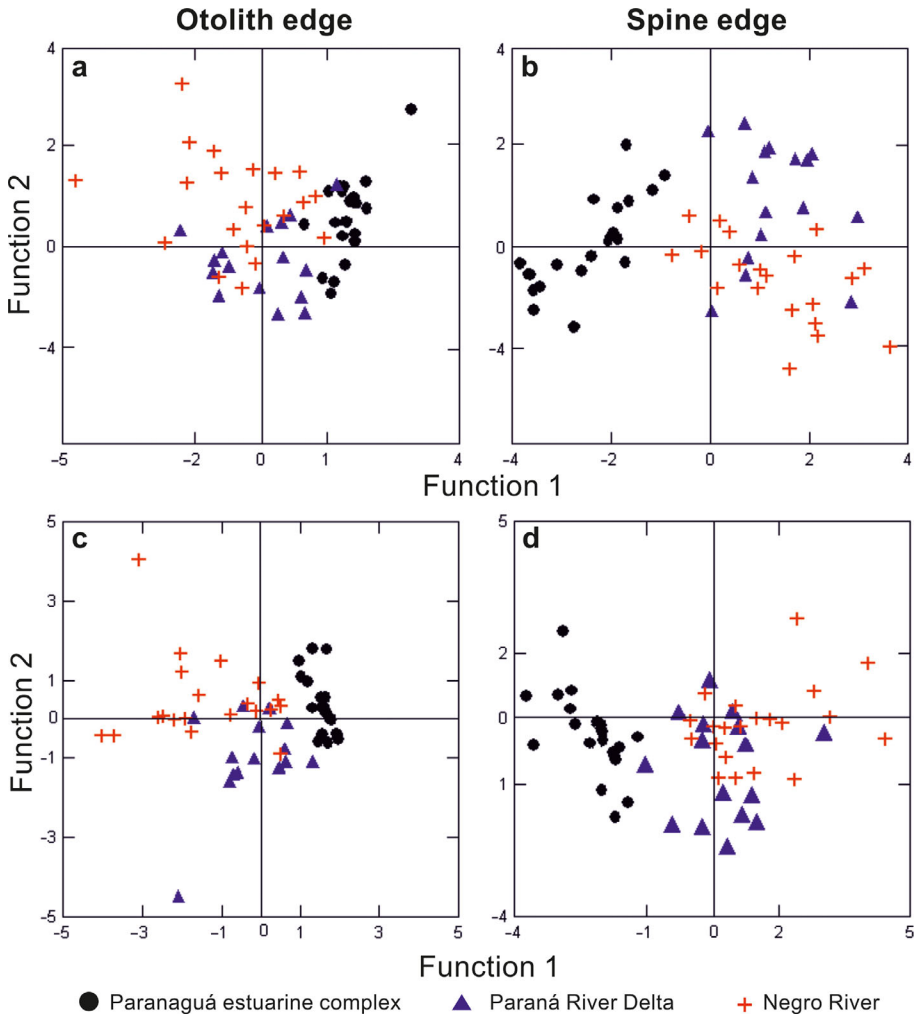

Fig. 5. Quadratic discriminant analysis of *Genidens barbus* otolith and spine element:Ca ratios. (a,b) Means of the last 45 µm (see Fig. 2); (c,d) means of the last year of a fish's life

cation, except 1 fish (last year analysis for otoliths from PEC), were associated to the adjacent sampling site.

4. DISCUSSION

Unlike for otoliths, resorption and vascularization of calcified material in fin spines is well documented (Drew et al. 2006, Tzadik et al. 2017). Such processes begin in the interior of fin spines and proceed outward during spine growth, in some cases causing the loss of annuli (Drew et al. 2006, Tzadik et al. 2017). Nevertheless, primary chemical compositions of spines are sufficiently retained during growth in some species (especially at the edge of the structure) such that they may provide useful tracers of aquatic environments (Clarke et al. 2007, Allen et al. 2009, Smith & Whitledge 2010, Jari et al. 2011, Phelps et al. 2012, Wolff et al. 2013, Rude et al. 2014). The results of this study support the fact that *Genidens barbus* spine composition can effectively track water mass characteristics. Although spines are metabolically active structures, their extraction is not lethal. Thus, fin spines may provide a new, non-lethal alternative to otoliths for discriminating among anadromous populations of this endangered catfish species.

Trace element incorporation in otoliths is species-specific and might be influenced by a diversity of factors such as environment (salinity, temperature) (Bath Martin & Thorrold 2005), genetics (Barnes & Gillanders 2013), hypoxia (Limburg et al. 2011), physiology (growth rates, metabolic changes) (Sturrock et al. 2014) and diet or the chemical composition of the surrounding water (Webb et al. 2012).

Although salinity and water chemistry are thought to influence trace element incorporation in fin spines (Clarke et al. 2007, Phelps et al. 2012, Tzadik et al. 2017), trace element uptake has been poorly studied. In this work, we examined the chemical composition of the otolith and spine in an area with a strong latitudinal environmental gradient (Avigliano et al. 2016), in terms of temperature, salinity and water chemistry, considering 2 clearly differentiated populations (PEC and PRD) (Avigliano et al. 2016, 2017b) and a potential new stock (NR).

For several fish species, the Sr:Ca ratios of otoliths and ambient water are correlated with salinity, and thus the otolith Sr:Ca ratio has been a useful indicator of habitat salinity (Secor & Rooker 2000, Avigliano & Volpedo 2013). The otolith Ba:Ca ratio of some species may be negatively related to salinity, and a negative relationship has been reported with the otolith Sr:Ca ratio (Fowler et al. 2016, Avigliano et al. 2017b). Specifically, otolith Sr:Ca and Ba:Ca ratios have previously been used to study the life history of *G. barbus* in salinity gradients (Avigliano et al. 2015b, 2017a). In our paper, patterns of Sr:Ca and Ba:Ca ratios observed among water, otolith and spine samples (Fig. 3) also correspond with salinity differences of the sampled localities, which were higher in PEC. However, the association among salinity and spine Sr:Ca ratios was less evident for PEC and PRD (Fig. 3). On the other hand, water chemistry of large estuaries may exhibit spatio-temporal variations, which could affect the reactions studied here.

According to Fig. 3, water and spine Mg:Ca ratios tended to be higher in fish from PEC than in fish from PDR and NR; otolith Mg:Ca ratios seemed to have a similar but less significant relationship (Fig. 3). Factors affecting the incorporation of Mg into otoliths are highly variable among species and require experimental studies for validation. Gaetani & Cohen (2006) reported a negative effect of temperature on Mg incorporation in aragonite, whereas Miller (2011) reported a positive correlation with temperature and somatic growth rate in the anadromous Chinook salmon *Oncorhynchus tshawytscha*. Mg incorporation into the otolith was not strongly related to the effect of temperature or water Mg:Ca ratio in euryhaline species such as spot *Leiostomus xanthurus* and chinook salmon (Bath Martin & Thorrold 2005, Miller 2011). Bath Martin & Thorrold (2005) suggested that Mg incorporation into otoliths is under strong biological regulation.

Otolith Mn:Ca ratios tended to be low in PEC, intermediate in PRD and higher in NR for water, spine and otolith (Fig. 3). Field studies have reported an apparent association between water and otolith Mn:Ca ratios (Dorval et al. 2007, Mohan et al. 2012). However, experimental trials to investigate the relationships between otolith and water Mn:Ca ratios have been inconclusive (Bath Martin & Thorrold 2005, Miller 2009). An association between hypoxia and high concentration of Mn in otoliths has been reported (Limburg et al. 2011). In our work, the highest values of Mn in otoliths and spines corresponded to relatively cold waters (lower probability of hypoxia). In this sense, there seems to be an apparent association between calcified structures and the availability of Mn in the environment.

The correlation between structures suggests the same way of accumulation and relative stability in the spine. However, unlike the Ba:Ca ratio, the correlations for Sr:Ca, Mn:Ca and Mg:Ca ratios were weak and showed a high data dispersion. This could be because the distance used (last 45 µm) could represent different periods of time between the structures. Smaller sections for analysis (perhaps the last 10 µm) or better age resolution that allows for determining which parts of the spine and otolith correlate could contribute to improve correlations between elements. On the other hand, fish move actively through marked salinity gradients (0–34 PSU, Avigliano et al. 2017a) that cover a few kilometers, and it is possible that the time of incorporation of elements is different between structures. In this sense, there could be a delay between the timing of environmental exposure and the subsequent incorporation of linked elemental signatures into respective otolith–spine pairs.

The QDAs corresponding to the last 45 µm of spine and otolith growth suggest that fish could be effectively classified into their catch areas, indicating that both structures are potentially useful habitat markers. The relatively low/moderate percentage of individuals incorrectly classified (18.8–28.6% for otolith and 4.8–25% for spine) between PRD and NR (Fig. 5a,b) suggest some difficulty of the model in discriminating between these 2 sites (e.g. element:Ca ratios in calcified structures could be less spatially variable for low-salinity waters).

Similar results were obtained when the chemical time-series for the last year of life was used (~300–600 µm). Classification success rate of 100% obtained between the populations from PEC and PRD (Table 1) agree with those previously reported in studies which have used different chemical approaches to evaluate the segregation between these 2 areas (Avigliano et al. 2016, 2017b). For example, Avigliano et al. (2017b) used the otolith edge Ba:Ca, Mg:Ca, Mn:Ca, Na:Ca, Sr:Ca and Ba:Ca ratios to dis-

criminate stock of *G. barbus* between PEC and PRD, while Avigliano et al. (2016) employed the otolith core Li:Ca, Mg:Ca, Mn:Ca, Sr:Ca and Zn:Ca ratios to identify nursery areas. This evidence suggests that the PEC and PRD populations remain isolated throughout their entire life history.

According to prior probabilities of the QDAs (last year), the percentages of correctly classified individuals were also high for PRD and NR (62.5–76.2% for otolith and 62.5–81.0% for spine; Fig. 5c,d), even when the elemental ratios of the last ~45 µm showed some difficulty in separating these sites (Fig. 5a,b). This strongly suggests the presence of a new stock in the extreme south of the species' distribution.

In conclusion, element:Ca ratios of spine and otolith edges were effective for discriminating between the different populations. Investigating catfish spine chemistry seems to be an acceptable means to study different biological aspects of catfish, providing a non-lethal method for monitoring possible recovery of the species. Governments and scientists should collaborate to establish spine chemistry as a fisheries management tool. In this sense, the spine chemistry could be used in the future to evaluate the connectivity between different estuaries. Spine and otolith chemistry suggest the presence of a distinct group of catfish in the Patagonian region with relatively high segregation. This highlights the need to study the conservation status of this possible new stock located at the southern end of the species' distribution. Finally, although encouraging, further paired otolith–spine studies are required to validate their respective utility to proxy environmental characteristics not directly related to salinity or water chemistry (e.g. temperature). This information could be used in the future to reconstruct the life history of catfish.

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