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Otolith elemental fingerprint and scale and otolith morphometry in *Prochilodus lineatus* provide identification of natal nurseries

Esteban Avigliano[a,b,*], Alejandro Domanico[c,d], Sebastián Sánchez[e], Alejandra V. Volpedo[a]

[a] Instituto de Investigaciones en Producción Animal (INPA), Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, CONICET, UBA, Av. Chorroarín 280 (C1427CWO), Buenos Aires, Argentina
[b] Centro de Ecología Aplicada del Litoral (CECOAL), CONICET, Ruta 5, km 2.5 (3400), Corrientes, Argentina
[c] Dirección de Pesca Continental, Subsecretaría de Pesca y Acuicultura, Ministerio de Agroindustria, Alférez Pareja 125, Buenos Aires, Argentina
[d] Comisión de Investigaciones Científicas (CIC), Calle 526 s/n, La Plata, Argentina
[e] Instituto de Ictiología del Nordeste, Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste, CONICET, UNNE, Santiago Cabral 2139 (3400), Corrientes, Argentina

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ABSTRACT

The dentification of nursery areas is a basic requirement for fishing management in large rivers. Morphometry (circularity, ellipticity, form factor, rectangularity and roundness indices) and chemistry (Sr:Ca, Ba:Ca and Zn:Ca ratios) of *lapilli* otolith, and geometric morphometry of scales of juveniles *Prochilodus lineatus*, were compared in three sites in the Plata Basin, in order to evaluate their applicability to identify possible nursery areas. Otolith microchemistry based on ICP-OES found significant differences in the Ba:Ca and Zn:Ca ratios among sampling sites. When all the combined techniques were considered, the quadratic discriminant analysis (QDFA) showed the highest classification success (89.5–92.9%), in relation to separate techniques classification. Otolith microchemistry, individually considered, appears to be a good and effective tool to identify individual fish from different locations (77.8%–84.2%). Otolith morphometry found significant differences in the ellipticity, circularity and form factor indices between sites. Otolith morphological indices supported results from the elemental study with a success in the allocation of 63.2–78.6%. When considering all variables for scale geometric morphometry, discriminant analysis showed a good percentage of the classification of the individuals (58.3–82.8%). These results indicate that the otolith microchemisty and morphometry and scale morphometry are acceptable markers of habitat and represent a potential tool (in combination or individually) for the identification of streaked prochilod nursery areas.

1. Introduction

The streaked prochilod (*Prochilodus lineatus* Valenciennes, 1836) is one of the most important commercial freshwater species in South America. This species, distributed in the Plata Basin, is exploited by Argentina, Brazil, Bolivia, Paraguay and Uruguay (Espinach Ros and Fuentes, 2000). The mentioned basin has a natural flooding pulse regime (Neiff and Malvárez, 1999) associated to the reproductive cycle of the streaked prochilod, involving migrations upstream, followed by spawning in open river waters coupled to the flooding periods as a mechanism of dispersion of eggs mechanism (Espinach Ros and Sánchez, 2006; Sverlij et al., 1993). The streaked prochilod migrations for food or reproduction have a distance of more than 1000 km (Bayley, 1973; Espinach Ros et al., 2008). Even though there is a lack of statistics on catching of this species for consumption, it is of common knowledge that some countries, such as Argentina, have exported 36,000 t/year of streaked prochilod captured only in the lower region of the basin (Minagro, 2013, 2004).

In the last decades, studies on the species have been intensified, but most research efforts were made in the middle and low section of the Paraná River in Argentina (Fig. 1) (Espinach Ros and Sánchez, 2006; Espinach Ros, 2008; Espinach Ros et al., 2012; Sverlij et al., 1993). It is suggested that this region represents the primary

* Corresponding author at: Instituto de Investigaciones en Producción Animal (INPA), Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, CONICET, UBA, Av. Chorroarín 280 (C1427CWO), Buenos Aires, Argentina.
E-mail addresses: estebanavigliano@conicet.gov.ar (E. Avigliano), aadomanico@gmail.com (A. Domanico), sebasanchez58@hotmail.com (S. Sánchez), avolpedo@fvet.uba.ar (A.V. Volpedo).

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Fig. 1. Study area. The circles indicate the streaked prochilod (*Prochilodus lineatus*) collection sites.

breeding area and the most important catching region of the Plata Basin. However, in spite of the socio-economical importance of the resources of the region, othersub-basins such as Uruguay River (Argentina, Brazil and Uruguay border) (Fig. 1) are scarcely studied and managed (Baigún et al., 2012; Bayley, 1973; CARU, 2014, 2010). In recent years, the presence of eggs and larvae of this species has been found in Uruguay River, downwards and upwards of the Salto Grande hydroelectric dam (Argentina-Uruguay), suggesting both areas as a breeding region (CARU, 2014, 2010).

Identification of nursery areas is a very important tool to generate strategies that may ensure the efficiency of sustainable management of fisheries (Beck et al., 2001; Colloca et al., 2009). In this sense, preservation and management of nursery areas promote the maintenance of fishery resources, avoiding their decay to irreversible values. This decline could not only compromise the fisheries continuity, but also affect the productive socioeconomic sector that depends on them (Beck et al., 2001). The chemical composition and morphometry of fish otoliths are valuable natural tags of habitual use, due to specific otolith properties. Even though physiological factors can affect the incorporation of trace elements in the otolith, salinity and temperature are among the most relevant environmental factors regarding the incorporation of elements in this structure (Bouchard et al., 2015; Campana, 1999; Elsdon and Gillanders, 2002; Martin and Thorrold, 2005; Secor and Rooker, 2000; Sturrock et al., 2012). The predominant source of several elements as Sr and Ba to otoliths is the surrounding water (Kerr and Campana, 2013). Likewise, otolith morphometry is also related to environmental factors including salinity, temperature, depth, among others (Avigliano et al., 2014; Lombarte, 1992; Lombarte et al., 2010; Reichenbacher and Reichard, 2014). Lately, due to the strong relationship between chemistry and morphometry of fish otolith with different environmental features these tools have been widely used to identify nursery areas of different commercially important species (Avigliano and Volpedo, 2016; Avigliano et al., 2015a; Bailey et al., 2015; Bouchard et al., 2015; Gillanders et al., 2003; Rooker et al., 2001; Tanner et al., 2013; Tournois et al., 2013; Vasconcelos et al., 2008, 2007). On the other hand, the use of geometrical morphometry of scales has been recently performed to identify populations (Staszny et al., 2012) and could also be a good tool to discriminate nursery areas.

Based on the above considerations, the present study tests the applicability of the otolith elemental fingerprint and scale and otolith morphometry in *Prochilodus lineatus* to identify possible nursery areas. For this purpose, morphometry (ellipticity, circularity, form factor, rectangularity, and roundness indices) and microchemistry (Sr:Ca, Ba:Ca and Zn:Ca ratios) of *lapilli* otolith, and geometric morphometry of scales were compared between three sampling sites from the Plata Basin.

2. Material and methods

2.1. Study area and sample collection

The Plata Basin, with an area of 3,170,000 km^2, is among the largest basins in the world. The most important rivers are the Paraná (4000 km long) and the Uruguay (1800 km long) (Fig. 1) (Guerrero et al., 1997). The Plata Basin goes through 5 South-American countries (Argentina, Bolivia, Brazil, Uruguay and Paraguay). Paraná and Uruguay rivers headwaters are the mountains of the Atlantic forest of southeast Brazil, then becoming the international boundary between Argentina, Brazil, Paraguay and Uruguay (Fig. 1). The Plata Basin discharges into the Río de la Plata estuary (30,362 km^2) with an average discharge of 23,000 m^3/s towards the Atlantic Ocean (Guerrero et al., 1997).

Fish samples were collected between April 2010 and November 2010 by using multifilament three-layer nets in the Uruguay River, upstream (UpUR) and downstream (DoUR) of the hydroelectric dam of Salto Grande (Corrientes and Entre Ríos provinces, Argentina-Brazil international boundary), and in the Paraná River (Corrientes province, Argentina-Paraguay international boundary) (Fig. 1). Fish were transported to the laboratory at 4 °C where they were measured (standard length = SL) and the *lapilli* otoliths were extracted. We preferred to use lapillus otoliths rather than sagittal or asteriscus otoliths because they were larger and allowed less measurement error (Assis, 2005; Avigliano et al., 2015e). Scales were removed from the shoulder region in front of the 1st dorsal fin ray above the lateral line and stored dry in paper envelopes, according to Ibañez et al. (2007).

2.2. Selection of samples

Fish of 0+ years old were selected for the study. The otoliths were washed with ultrapure water and dried. The left otolith of each pair was sectioned transversely through the core by using a rotary saw equipped with a diamond blade (Dremel® 250 and 300) and they were burned directly onto a Bunsen burner (Christensen, 1964). The number of rings in the otolith section was counted through the use of a stereomicroscope (Leica® EZ4-HD, Singapore) at 30× magnification. Age determination by counting the ring number in *lapillus* otoliths of *P. lineatus* was validated by Espinach Ros et al. (2008). In total, 19 individuals from UpUR site (SL mean ± SD and range: 15.9 ± 2.4; 11.0–19.1 cm), 20 individuals from DoUR (15.9 ± 3.0; 11.5–20.0 cm) and 29 from Paraná River (18.9 ± 2.4; 12.0–20.2 cm) were selected for the analysis.

2.3. Otolith microchemistry

The right otoliths (age – 0+) were washed in Milli-Q water and once dry, they were transferred to a sterile centrifuge tube and weighed by using a Sartorius AG® ED2242 (Göttingen, Germany) microbalance to the nearest 0.0001 g. Then, otoliths were decontaminated 3 times with 1.7% HNO_3 and finally rinsed 5 times with

Milli-Q to remove any contamination from weighing, transferred to new sterile centrifuge tubes and dried overnight in a laminar flow hood.

The otolith were digested with 30% nitric acid during 24 h (Avigliano et al., 2015e). The concentrations of Sr, Ba, Zn and Ca were determined (in triplicate) by sing an inductively coupled plasma optical emission spectrometer (ICP-OES, PerkinElmer® Optima 2000 DV, Überlingen, Germany), equipped with a cross-flow nebulizer, Scott chamber, and quartz torch (method 200.7) (USEPA, 1994), according to Avigliano et al. (2015e). The samples were introduced into the equipment with a PerkinElmer® AS-90 Plus autosampler. External calibrations were performed in all cases with a PerkinElmer® Pure Quality Control Standard 21 (QCS 21, USA). Every 10 samples, a blank and a sample of known concentration prepared from the QCS 21 standard were analyzed to determine whether interference or cross-contamination had occurred. The efficiency of the otolith digestion process was verified by means ofcertified reference materials (FEBS-1, National Research Council, Canada) and an acceptable recovery percentage was obtained (111% for Ba, 90% for Ca, 105% for Sr and 99% for Zn). The detection limits (LOD) in μg/L based on ten times the standard deviation of the blank signal was 8 for Ba, 15 for Ca, 10 for Sr and 12 for Zn.The water used throughout the study was obtained from a Milli-Q water purification system (Millipore, Sao Paulo, Brazil) with a resistivity of 18.2 M Ohm/cm.

The results were examined and assessed in relation to the known concentration. The reported results were corrected, based on a control blank. Concentrations of trace elements were expressed as molar ratios (element:Ca in mmol/mol) to account for fluctuations in the amount of analyzed material material analyzed and the loss of material during the preparation process (Bailey et al., 2015; Sinclair et al., 1998).

2.4. Otolith morphometry

Prior to digestion, right *lapilli* otoliths were photographed under a stereoscopic microscope (Leica® EZ4 HD). The following morphometric variables were recorded on the images using an image processor (Image-ProPlus® 4.5): otolith length (OL), otolith width (OW), and otolith perimeter (OP), in millimeters; and otolith area (OA) in square millimeters. The following shape indices were then calculated: circularity (OP^2/OW), ellipticity ($OL - OW/OL + OW$), form factor ($[4\pi \times OA/OP^2]$), rectangularity ($OA/[OL \times OW]$) and roundness $(4A)(\pi\ OL^2)^{-1}$. The nomenclature of the indices used was taken from Tuset et al. (2003b). Circularity provides information on the complexity of the otolith contour (Tuset et al., 2003b). Ellipticity reflects the similarity to an ellipse, with values close to 0 indicating a tendency towards circularity (Tuset et al., 2003b). The form factor is a dimensionless value that indicates the similarity of the otolith contour to a circle; its values range from 0 to 1, with a value of 1 corresponding to a perfect circle (Tuset et al., 2003b). Rectangularity gives information about the approximation to a rectangular or square shape, with a value of 1 indicating a perfect rectangle or square (Tuset et al., 2003b). The roundness is the ratio between the actual area and the area of a circle of the same length. Namely, this factor is larger if and when the shape of otolith is more circular (Ponton, 2006).

2.5. Scale geometric morphometry

Scales were cleaned with distilled water, dried and photographed by using a stereoscopic microscope (Leica EZ4 HD). Only one scale per fish was used for the analysis (Ibañez et al., 2007). Knowing that the scales may be lost and regenerate, we selected unregenerate scales.

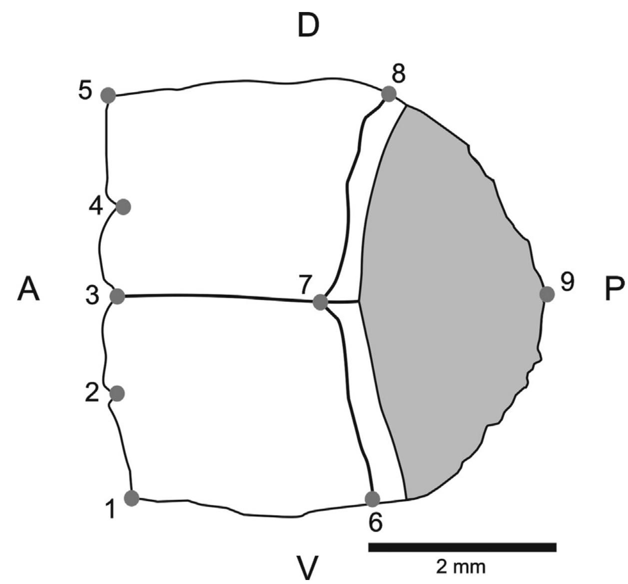

Fig. 2. Landmark definitions used in the fish scales.

These variables were based on 9 landmarks according to Ibañez et al. (2007). Landmarks 1 and 5 are the ventro and dorso lateral end of the anterior portion of the scale, respectively; landmark 3 is in the center of the anterior edge of the scale; landmark 2 is located between landmarks 1 and 3; landmark4 is between landmarks 3 and 5; landmarks 6 and 8 are at the boundary between the anterior area with circuli and the posterior area covered by cteni (spine-like ornamentations) respectively; landmark 7 is the focus of the scale; and landmark 9 is positioned at the tip of the posterior portion of the scale (Ibañez et al., 2007) (Fig. 2). The configurations of landmark coordinates were scaled, translated and rotated by a generalized Procrustes analysis (GPA) using MorphoJ, TPS util, TPS relw and TPS dig programs (Klingenberg, 2011; Rohlf, 2001). Details of the framework of geometric morphometrics using landmarks can be found in Zelditch et al. (2012).

2.6. Data analysis and statistic

One-way analysis of variance (ANOVA) followed by the Bonferroni test (Sokal and Rohlf, 2012) were used to explore individual elemental fingerprint differences between sampling sites. Previously, normality and homogeneity of variance were tested (Shapiro-Wilk test, $p > 0.05$ and Shapiro-Wilk test, $p > 0.05$, respectively). Moreover, relationships between elemental concentration and otolith weight were tested with analysis of covariance (ANCOVA, otolith weight as co-variate) (Burke et al., 2008; Campana et al., 2000; Galley et al., 2006; Kerr and Campana, 2013; Longmore et al., 2010). Element: Ca ratios were not significantly correlated with otolith mass (ANCOVA, $p > 0.05$). Multivariate analysis of variance (MANOVA) was used to evaluate the otolith multi-elemental fingerprints and to detect differences in the multi-elemental composition between different sampling sites. Post-hoc multivariate pairwise comparisons between locations were performed by using the Hotelling T-square test. A quadratic discriminant function analysis (QDFA) was performed in order to obtain the cross classification matrix and to determine the capacity of these variables to identify the site of origin of the fish (Longmore et al., 2010; Silva et al., 2011). The expected classification accuracies was calculated based on chance alone given the number of groups and sample sizes (Ruttenberg and Warner, 2006; White and Ruttenberg, 2007). Randomization test was used to determine if the classification success rate was significantly different from random (Hayes, 1998; Ruttenberg and Warner, 2006; White and Ruttenberg, 2007). Multicollinearity between element:Ca ratios was analyzed, thus preventing a false outcome in the QDFA analysis and the use of redundant variables in the study (Graham, 2003).

Two sample tests were applied in order to compare the shape indices from the selected sites. After testing normality and homoscedasticity assumptions, original data series of ellipticity, circularity and roundness were compared with ANOVA, followed by the Bonferroni test, while rectangularity and form factor were compared with a Kruskal-Wallis test. ANCOVA was then used to correct the effect of SL on the studied variables (Longmore et al., 2010; Sadighzadeh et al., 2012). ANCOVA is robust to violations of the assumption of homogeneity of variance (Olejnik and Algina, 1984). It was only necessary to correct the form factor ($p < 0.01$, constant = −0.0004). The indices were assessed in relation to the variation by allometric growth and corrections were not necessary (Bani et al., 2013; Lleonart et al., 2000; Lombarte and Lleonart, 1993).

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 to detect differences in core area from different sampling sites. The analysis was based on Gower distances with 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). After testing multicollinearity, QDFA and randomization test were performed through the use of the morphometric variables in order to obtain the cross classification matrix and to determine the capacity of these variables to identify the site of origin of the fish.

This geometric analysis was performed on the ten Cartesian coordinates or variables of 9 landmarks, reconstructed from distance measurements among the landmarks. Shape variables generated from the landmark analysis were considered to be invariant regarding mathematical differences in translation, rotation, and scale (Márquez et al., 2010). The data matrix was checked and corrected by allometric effects. We used the multivariate regression of shape; size was computed as centroid size (CS), the square root of the sum of squared distances from each landmark to the

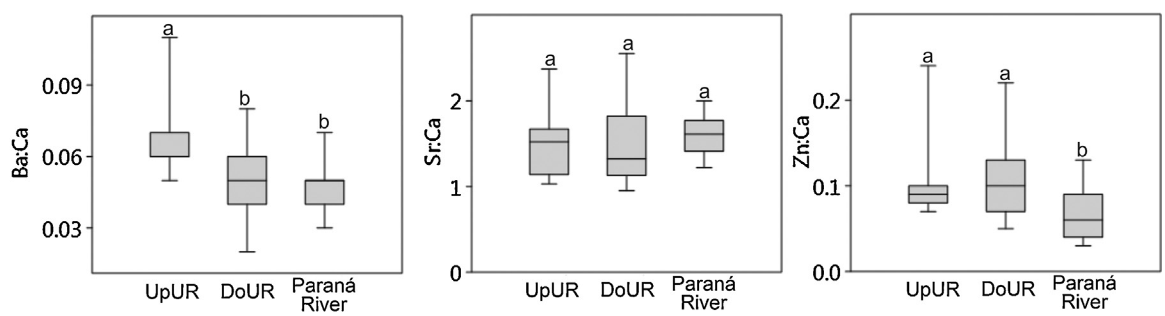

Fig. 3. Mean element:Ca ratios (mmol/mol) of the otolith. Different letters indicate statistical significant differences between sampling sites ($p < 0.05$).

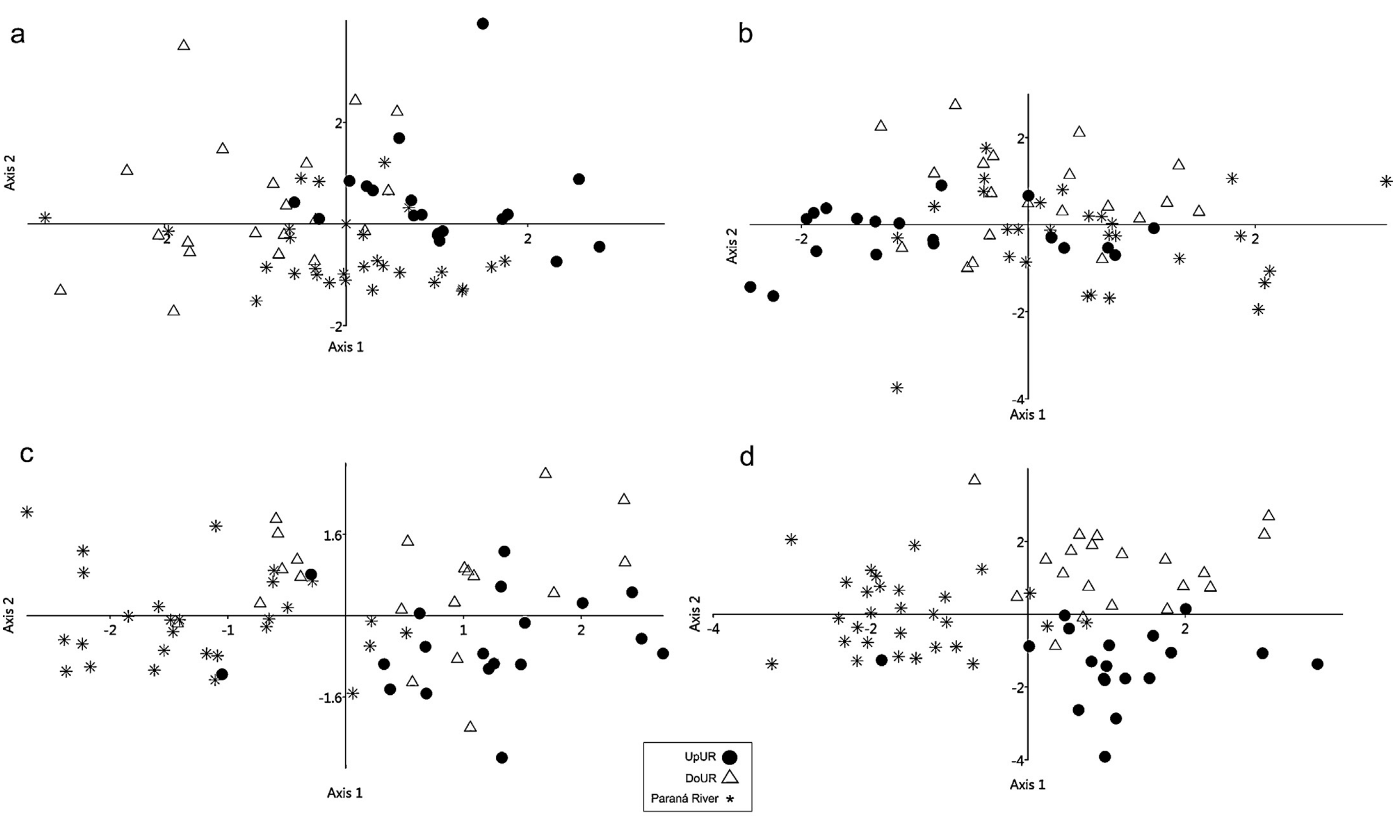

Fig. 4. Quadratic discriminant analysis of the otolith element:Ca ratios (a) morphometric indices (b) scale geometric morphometry (c) and all techniques combined (d).

Table 1
Cross-classification matrix of the discriminant analysis. The numbers represent the classification percentage.

	UpUR	DoUR	Paraná River	N
	Otolith microchemistry			
UpUR	**84.2**	10.5	5.3	19
DoUR	11.1	**77.8**	11.1	20
Paraná River	14.3	3.6	**82.1**	29
	Otolith morphometry			
UpUR	**63.2**	15.8	21.1	19
DoUR	9.5	**66.7**	23.8	20
Paraná River	7.1	14.3	**78.6**	29
	Scale geometric morphometry			
UpUR	**58.3**	20.8	20.8	19
DoUR	6.9	**82.8**	10.3	20
Paraná River	15.8	5.3	**78.9**	29
	All combined techniques			
UpUR	**89.5**	5.3	5.3	19
DoUR	5.0	**90.0**	5.0	20
Paraná River	7.1	0	**92.9**	29

specimen's centroid (Loy et al., 2000). The relative warps (RW) were used to construct a matrix (W matrix) and a PCA was performed (relative warp analysis, RWA) in order to describe major trends in shape variations (Márquez et al., 2010; Zelditch et al., 2012). RWs were submitted to the cross-validation QDFA to build a predictive model of group membership based on the observed characteristics in each case. In this case, a randomization test was also performed.Finally, a QDFA (followed by a randomization test) was performed to integrate the three techniques and to evaluate if classification percentages are comparable to those obtained by analyzing each technique individually.

3. Results

3.1. Otolith microchemistry

Otolith Ba:Ca, Sr:Ca and Zn:Ca ratios are shown in Fig. 3. High otolith Ba:Ca ratio was recorded for UpUR (0.07 ± 0.003 mmol/mol) ($p < 0.0001$) and there were no differences between DoUR and Paraná River (0.05 ± 0.002 mmol/mol and 0.05 ± 0.003 mmol/mol, respectively) ($p > 0.05$). The Sr:Ca ratio was 1.5 ± 0.09, 1.5 ± 0.08 and 1.6 ± 0.09 mmol/mol in UpUR, DoUR and Paraná River, respectively. No significant differences for otolith Sr:Ca ratio were observed between sampling sites ($p = 0.50$). Zn:Ca ratio differed significantly among sites ($p = 0.0005$), being lower in Paraná River site (0.06 ± 0.01 mmol/mol) than in UpUR (0.10 ± 0.01 mmol/mol) and DoUR (0.11 ± 0.01 mmol/mol).

The MANOVA and Hotelling T-square test showed significant differences for the element:Ca ratios among all sampling sites ($p < 0.0001$).

The QDFA plot showed a clear separation between *P. lineatus* from the three sampling sites (Fig. 4a). The QDFA cross-classification matrix (Table 1) revealed a high percentage of correctly classified individuals for Paraná River (82.1%), UpUR (84.2%) and DoUR (77.8%), while significantly different from random (prior probabilities for groups: 0.412 for Paraná River; 0.279 for UpUR and 0.309 for DoUR) (randomization test: $0.001 < p < 0.03$), still provides little discriminatory power.

3.2. Otolith morphometry

Otoliths from UpUR specimens showed high form factor and low ellipticity ($p < 0.05$) (Table 2). Significantly high circularity and low ellipticity values were also obtained for UpUR otoliths (Table 2), indicating high edge complexity and a circular shape. The lowest circularity and form-factor values were recorded for Paraná River otoliths, whereas ellipticity was significantly high ($p < 0.05$). No significant differences for rectangularity and roundness were observed between sampling sites ($p = 0.49$). Results from PERMANOVA analysis revealed significant differences for the morphometric variables among study sites ($p = 0.03$). The p values were 0.64 for the comparison between Paraná River and UpUR and $0.02 < p < 0.03$ for DoUR-Paraná River and UpUR-DoUR. The QDFA plot (Fig. 4b) showed a separation between sampling sites. The cross-classification matrix (Table 1) revealed a moderate-high percentage of correctly classified individuals (78.6, 63.2 and 66.7% for Paraná River, UpUR and DoUR, respectively), while significantly different from random ($0.01 < p < 0.05$).

3.3. Scale geometric morphometry

Due to the existence of three sets of data, two discriminant canonical functions were obtained (Fig. 4c). Data corresponding to the 18 RWs of the RWA were employed to perform the QDFA. The QDFA correctly classified 78.9% for fish caught in Paraná River, 58.3% for UpUR and 82.8% for DoUR and (Table 1). Since expected classification probabilities based on random assignment were 0.264, 0.333 and 0.403 for Paraná River, UpUR and DoUR, respectively, the discriminatory power of scale geometric morphometry was high (randomization test: $0.01 < p < 0.05$).

The 1st 3 RWs explained 58.7% (26.1, 18.1 and 14.5%, respectively) of the total variance for the RWA analysis of the scale shape

Table 2
Mean ± standard deviation and range (minimum–maximum) of the otolith morphometric indices of juvenile *Prochilodus lineatus*. Different letters indicate statistical significant differences between sampling sites ($p < 0.05$).

	UpUR (N = 19)					DoUR (N = 20)					Paraná River (N = 29)				
	mean ± SD			min	max	mean ± SD			min	max	mean ± SD			min	max
Circularity	17.3	±	0.74^{a}	16.10	18.7	18.2	±	1.43^{b}	15.1	21.1	17.2	±	1.46^{ab}	14.4	19.6
Ellipticity	0.20	±	0.02^{a}	0.17	0.24	0.20	±	0.03^{ab}	0.15	0.25	0.22	±	0.03^{b}	0.13	0.28
Form factor	0.21	±	0.03^{a}	0.18	0.28	0.16	±	0.05^{b}	0.10	0.26	0.15	±	0.03^{b}	0.11	0.24
Rectangularity	0.70	±	0.02^{a}	0.67	0.74	0.70	±	0.04^{a}	0.66	0.83	0.70	±	0.02^{a}	0.65	0.76
Roundness	0.60	±	0.03^{a}	0.54	0.56	0.59	±	0.05^{a}	0.51	0.74	0.57	±	0.04^{a}	0.50	0.70

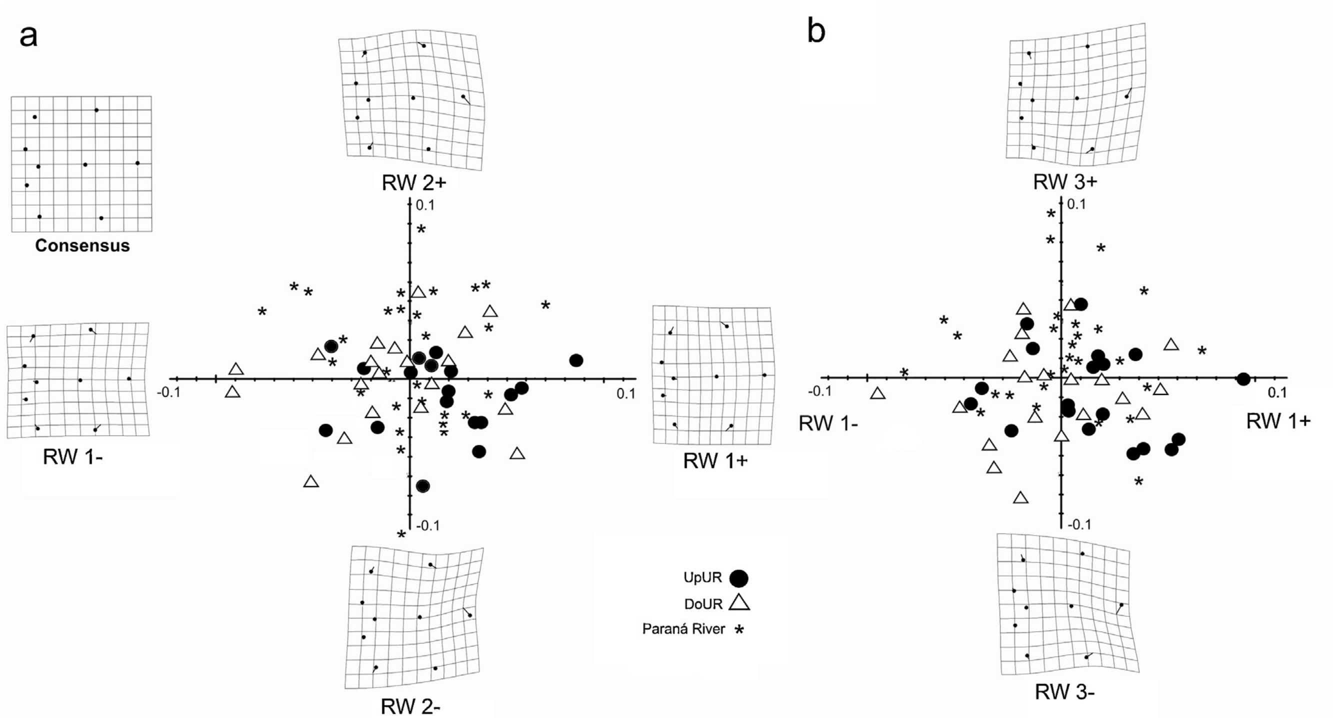

Fig. 5. Relative warp (RW) analysis based on landmark coordinates. Thin plate spline transformation grids for the extreme points of RW are shown; these were superimposed on the shapes predicted when the average landmark configuration of all scales was deformed into that of a hypothetical scale positioned at the extreme of the RW of interest. a) RW2 vs. RW1; b) RW3 vs. RW1.

of the different studied sampling sites. Patterns of morphological variations described by the 1st 3 RWs are shown in Fig. 5. Shape changes along the 1st RW were expressed by the depression (negative RW1 scores) or expansion (positive RW1 scores) of the scale along the antero-posterior axis (landmarks 1, 5, 6 and 8) (Fig. 5a). The shape of RW2 varied somewhat due to the displacement of landmarks 1, 5, 8 and 9 which formed 2 types of scale posterior region: for the 1st one (RW2+), an expansion of the scale posterior region, while the 2nd one (RW2−) was characterized by a compression. Shape changes along RW3 were also expressed by an expansion (RW3+) or depression (RW3−) of the posterior region (ventro-posterior region) (Fig. 5b).

3.4. Three combined techniques

The QDFA plot showed a clear separation between sampling sites (Fig. 4c). The cross-classification matrix integrating the three techniques also revealed a high percentage of correctly classified (92.9, 89.5 and 90.0% for Paraná River, UpUR and DoUR, respectively) (Table 1), while significantly different from random (prior probabilities for groups: 0.418 for Paraná River; 0.284 for UpUR and 0.299 for DoUR) ($0.0001 < p < 0.002$).

4. Discussion

Results indicate that the combined use of morphometric indices and microchemistry of the otolith with the geometric morphometric of scales showed high classification percentages and represent a good tool for identification of streaked prochilod nursery areas. Furthermore, the separate analysis of each methodology also showed moderate or high classification percentages, differing significantly from random chance. This means that it would be possible perform monitoring studies without the need to apply the three techniques simultaneously what reduces costs and effort, but if possible, it is advisable to use the combined techniques in order to obtain the highest power of classification. In addition, it also shows that there are several nursery areas, as it was suggested for the Paraná (Sverlij et al., 1993) and Uruguay rivers (CARU, 2014, 2010).

However, the classification rates were relatively moderate among some sites and techniques (e.g. otolith morphometry). This could be due to the existence of connectivity between study areas or to the moderate/low power of some variables to discriminate nursery areas. We ruled out temporal, climatic or hydrological variations that could affect the studied variables because fish were caught in a defined time window. There is a flow of individuals from UpUR to DoUR revealed by capture and recapture studies (Delfino and Baigun, 1985; Sverlij et al., 1993) which could have a negative effect on the classification rates among these sites. Also, Fuentes et al. (2016) reported that the larvae of fish that spawn in the upstream section are partly transported to the lower section. Several marking studies indicated that adult shads tend to move predominantly from the Uruguay to the Paraná rivers crossing between 400–700 km in about 6 months (CARU, 2010; Espinach Ros et al., 2008; Sverlij et al., 1993). In this sense, the movements of juveniles with aged 0 between the Paraná River and DoUR are unlikely because fish should travel more than 1500 km in a few months. So the moderate classification rates may not be due to an effect of connectivity, but rather to the discrimination power of the studied variables.

In our study area there is a decreasing temperature gradient in north-south direction. Furthermore, sampling sites have different climatic and topographic features, depths and hydrographic dynamics. All these factors undoubtedly affect the characteristics of the water and could print unique and distinctive signatures in the otoliths, which explains some of the multi-elemental differences found in this work.

Water concentration of Sr is in general positively related to salinity (Avigliano and Volpedo, 2013; Bouchard et al., 2015; Campana, 1999; Sturrock et al., 2012); while environmental concentration of Ba is generally higher in freshwater than in salt or estuarine waters

(Bouchard et al., 2015; Campana, 1999; Tabouret et al., 2010). There are some exceptions, for example, Bouchard et al. (2015) found a positive ratio between Ba:Ca and salinity in juvenile *Boreogadus saida.* However, the incorporation of these elements in the otoliths is not always related to the salinity (Bouchard et al., 2015; Brown and Severin, 2009; Sturrock et al., 2012; Walther and Limburg, 2012). In this case, salinities are similar between sampling sites (0 UPS) (Avigliano and Volpedo, 2013; Avigliano and Schenone, 2016). In freshwater environments like this, Sr dissolved ambient values will be dominated by bedrock geological composition (Garcez et al., 2014; Pouilly et al., 2014; Walther and Limburg, 2012). This is consistent whit the homogeneity of the Sr results; it would be expected that these river basins have similar geologies (Garcez et al., 2014; Pouilly et al., 2014).

On the other hand, Zn incorporation into the otoliths is mainly influenced by fish diet and may be independent of water concentration (Ranaldi and Gagnon, 2008). For this reason, Zn was proposed as an indicator of habitat (Avigliano et al., 2015c; Ranaldi and Gagnon, 2008). Temperature has been shown to influence elemental incorporation in otoliths of larval and juvenile fish (Bath et al., 2000; Bouchard et al., 2015; Martin and Thorrold, 2005). All of these factors can make it difficult to know exactly how the environmental characteristics affect otolith microchemistry.

The patterns of concentration of Sr and Ba reported for the study area are consistent with those observed for otoliths in this paper. In the Paraná and Uruguay rivers, there have been reports with similar concentration of Sr in water (11–32 and 11–33 μg/L, respectively) (Avigliano and Volpedo, 2013; Avigliano and Schenone, 2015; Avigliano et al., 2014). On the other hand, Ba values are generally lower for the Uruguay River (6.5–24 μg/L) (Avigliano and Schenone, 2015; Avigliano et al., 2014) compared to the Paraná River 9–47 μg/L (Avigliano and Schenone, 2015). Rivers show great chemical heterogeneity due to upwelling, fluvial and anthropogenic inputs, but often the pollutants would contribute to such geographic variation in the concentration of Zn (Sturrock et al., 2012). In the Plata Basin, Zn levels in water are highly variable between the different tributaries of the sampled rivers (Avigliano and Schenone, 2015; Avigliano et al., 2015d). These variations in the concentration of Zn could be related to variations in the Zn:Ca ratio found in otoliths.

In conclusion, otolith microchemistry showed to be a good habitat indicator, especially Ba:Ca and Zn:Ca ratios. It would be interesting to evaluate simultaneous relations with multiparametrical analysis, so as to identify nursery areas more robustly. The otolith element:Ca ratios proved to be a good marker of habitat for other species from the Plata Basin as *Lycengraulis grossidens* (Mai et al., 2014), *Odontesthes bonariensis* (Avigliano and Volpedo, 2013; Avigliano et al., 2014), *Percophis brasiliensis* (Avigliano et al., 2015c), *Micropogonias furnieri* (Albuquerque et al., 2012, 2010) and *Genidens barbus* (Avigliano et al., 2016, 2015e, 2015f).

In relation to the otolith morphometry, circularity, ellipticity and form factor indices were efficient to differentiate breeding areas. Circularity, ellipticity and form factor indices showed to be a good tool as habitat indicators for other species of the same basin, like *O. bonariensis* (Avigliano et al., 2015g) silverside. Circularity was also a good habitat indicator for other species like *Plagioscion ternetzi* (Avigliano et al., 2015a), *Coryphaenoides rupestris* (Longmore et al., 2010) and *Lophius piscatorius* (Cañás et al., 2012). In *Argyrosomus japonicus* (Ferguson et al., 2011) and *Scomberesox saurus* (Agüera and Brophy, 2011) circularity and ellipticity also proved to be good indicators. According to our results, the rate of rectangularity was not efficient as an indicator for *Coryphaena hippurus* (Duarte-Neto et al., 2008), *Gadus morhua* (Petursdottir et al., 2006) and *Scomberesox saurus* (Agüera and Brophy, 2011). Contrary to our results, roundness proved to be a good indicator of habitat to distinguish populations of *A. japonicus* (Ferguson et al., 2011), *S. saurus* (Agüera and Brophy, 2011) and *Serranus cabrilla* (Tuset et al., 2003b). Finally, in some species such as *Mulloidichthys flavolineatus*, descriptors such as circularity, ellipticity, form factor and rectangularity have shown significant differences between juveniles caught in different parts of the Indian Ocean (Pothin et al., 2006). These discrepancies among different species show that the use of morphologic indices to separate breeding areas or populations is species and/or environmental dependent and should be correctly evaluated before their use for monitoring or population studies.

Even though some variables were not efficient by themselves to separate breeding areas, simultaneous use of all variables (QDFA) showed moderate or good classification percentages. The QDFA classification percentages are similar to those reported by other authors (Agüera and Brophy, 2011; Avigliano et al., 2015g; Tuset et al., 2003b) who have indicated that the otolith shape indices can be used as natural markers, not only to separate the species, but also to identify populations or nurseries.

Environmental (salinity, water temperature and depth) and genetic factors have been suggested to be responsible for some inter- and intra-specific differences in the otolith morphometry (Avigliano et al., 2014; Lombarte, 1992; Lombarte et al., 2010; Reichenbacher and Reichard, 2014). Therefore, the taxonomic value of otoliths is well established (Gierl et al., 2013; Reichenbacher and Reichard, 2014). Because of these characteristics, otolith morphometry has been widely used to identify fish stocks (Campana and Casselman, 1993; Cañás et al., 2012; Sea et al., 2008), to differentiate species (Harvey et al., 1996; Tuset et al., 2013, 2012; Zhuang et al., 2014; Zischkea et al., 2016), to describe ecomorphological patterns of species (Avigliano et al., 2015b; Gauldie, 1988; Jaramillo et al., 2014; Tuset et al., 2003a; Volpedo and Fuchs, 2010; Volpedo et al., 2008; Volpedo and Diana Echeverría, 2003), and as an environmental indicator (Avigliano et al., 2015b; Nelson et al., 1994). Among the most commonly used indexes are rectangularity, circularity and aspect ratio (Avigliano et al., 2015b; Bani et al., 2013; Cañás et al., 2012; Jaramillo et al., 2014; Lombarte et al., 2010; Longmore et al., 2010; Sadighzadeh et al., 2012; Tuset et al., 2008; Zischkea et al., 2016).

The analysis of scale morphometrics was robust to differentiate breeding areas. This technique, unlike the others we have studied, is more economical, requires less work and permits to liberate fish afterwards. Finally, its use could be recommended to study and monitor vulnerable species, allowing the capture of larger sample volumes and the freeing of specimens.

The usage of geometrical morphometry of scales has been used to identify species (Ibañez et al., 2007) and populations (Staszny et al., 2012). Moreover, some authors have used Fourier elliptic analysis on scales to identify populations (Poulet et al., 2005; Richards and Esteves, 1997). However, no morphologic aspect of scales has been previously used as habitat marker to identify breeding areas. There is only one precedent where it was shown that morphology of scales of streaked prochilod juveniles captured in Pilcomayo River could vary with different hydrologic conditions (Bayley, 1973).

In relation to the obtained results, we recommend the use of the different methodologies to identify and monitor streaked prochilod breeding areas in the national and international management programs of the region. For a proper long-term management of fisheries, it is necessary to evaluate if these areas are maintained in time or if they vary in different regions of the basin. With this information gathered, stable nursery areas could be protected during spawning and breeding phases. In relation to monitoring and use of otolith microchemistry, analyzing the geochemical origin of the different studied elements, as well as the incorporation of isotopical relations could generate other markers of origin to study even adult individuals (Ashford and Jones, 2007; Garcez et al., 2014; Hegg et al., 2015, 2013; Hobbs et al., 2010; Newman et al., 2010; Niklitschek et al., 2010; Schloesser et al., 2010; Walther and Thorrold, 2009).

Finally, data generated in relation to the chemical signature of the areas could be used in the near future to predict the origin of adult fish captured in other regions of the basin, by using specialized software (e.g. HISEA) (Avigliano and Volpedo, 2016). For this, otolith cores of adults of the same cohort (2013–2014) could be compared with the chemical signature obtained in this research.

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