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Identification of nurseries areas of juvenile *Prochilodus lineatus* (Valenciennes, 1836) (Characiformes: Prochilodontidae) by scale and otolith morphometry and microchemistry

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The streaked prochilod *Prochilodus lineatus* (Valenciennes) is a commercially freshwater species from South America, distributed in the Plata basin. In the present work the morphometry (circularity, rectangularity, form factor, OL/OW and ellipticity indices) and chemistry (Sr:Ca, Ba:Ca, Zn:Ca) of *lapilli* otolith, and geometric morphometry of scales of streaked prochilod juveniles, in two sites in the Plata basin (Uruguay River and Estrella Wetland), were compared to determine if they are area-specific and to identify possible breeding areas. Otolith Ba:Ca ratios was 0.017±0.003 mmol/mol for Uruguay River while for Estrella Wetland individuals was bellow the detection limits. Zn:Ca ratios tended to be higher for the latter (0.03±0.002 mmol/mol). Significantly high circularity and low rectangularity values were obtained for Estrella Wetland otoliths ($p < 0.05$), while no significant differences for form factor, OL/OW and ellipticity were observed between sampling sites. Considering all scale geometric morphometry variables, discriminant analysis showed a good percentage of classification of individuals (90.5% for Estrella Wetland and 85.7 % for Uruguay River). These results indicate that the otolith microchemisty and morphometry (circularity and rectangularity indices) and scale morphometry are good markers of habitat and represent a potential tool for identification of streaked prochilod nursery areas.

El sábalo *Prochilodus lineatus* (Valenciennes) es una especie de importancia comercial de agua dulce de Sudamérica, distribuida en la cuenca del Plata. En este trabajo se comparó la morfometría (índices de circularidad, rectangularidad, factor de forma, OL/OW y elipticidad) y microquímica (Sr:Ca, Ba:Ca y Zn:Ca) del otolito *lapilli*, y la morfometría geométrica de las escamas de sábalos juveniles capturados en dos sitios de la cuenca del Plata (Río Uruguay y bañado la Estrella), para determinar si existen diferencias área-especificas e identificar posibles sitios de cría. La relación Ba:Ca del otolito fue 0.017±0.003 mmol/mol para el río Uruguay mientras que estuvo por debajo del límite de detección para los individuos del bañado la Estrella. La relación Zn:Ca tendió a ser más alta en el bañado la Estrella (0.03±0.002 mmol/mol). Se obtuvieron valores significativamente altos de circularidad y bajos de rectangularidad para el bañado la Estrella ($p < 0.05$), mientras que no se hallaron diferencias para el factor de forma, OL/OW y elipticidad. Considerando la morfometría geométrica de las escamas, el análisis discriminante mostró buenos porcentajes de clasificación (90.5% para el bañado la Estrella y 85.7 % para el Río Uruguay). Estos resultados indican que la microquímica y morfometría del otolito (índices de rectangularidad y circularidad) y la morfometría de las escamas son buenos marcadores de hábitat y representan una herramienta potencial para la identificación de sitios de cría del sábalo.

Keywords: Connectivity, Geocrochemistry, Geometric morphometrics, Plata Basin, Streaked prochilod.

Introduction

In the second part of the XXth century, world fishery production increased constantly at an average annual rate of 3.2% (FAO, 2014). In the last 15 years, the annual world fish extraction has remained constant at around 90 million tons per year (FAO, 2014). However, since the 50s, 25% of world fisheries have collapsed (Mullon *et al.*, 2005). A great number of marine and freshwater commercially important species are cross-border resources. They make vast movements, covering different jurisdictions, national, as well as international. One of the most commercially

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important freshwater species in South America is the streaked prochilod *Prochilodus lineatus* (Valenciennes, 1836), known as "sábalo or corimbatá" in southern South America. This species, distributed in the Plata basin (Paraná, Paraguay, Uruguay, Río de La Plata, Iguazú, Bermejo and Pilcomayo rivers, among others), is exploited by Argentina, Brazil, Bolivia, Paraguay and Uruguay (Espinach Ros & Fuentes, 2000). The mentioned basin has a natural flooding pulse regime (Neiff & 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 dispersion of eggs mechanism (Sverlij *et al.*, 1993; Espinach Ros & Sánchez, 2007). The streaked prochilod migrations for food or reproduction are more than 1000 km long (Bayley, 1973) though there is a lack of statistics on catching of this species for consumption, it is of common knowledge that some countries, like Argentina, have exported 36.000 t/year of streaked prochilod only captured in the lower region of the basin (Espinach Ros & Sánchez, 2007; Espinach Ros *et al.,* 2008).

In the last decades, studies on the species have intensified, but most research efforts were made in the middle and low section of the Paraná River in Argentina (Fig. 1) (Sverlij *et al*., 1993; Espinach Ros & Sánchez, 2007; Espinach Ros, 2008). It is suggested that this region represents the primary breeding area and the most important catching region of the Plata basin. However, in spite of the socioeconomical importance of the resources of the region, others sub-basins like Uruguay River (Argentina, Brazil and Uruguay border) and Pilcomayo River (Argentina, Bolivia and Paraguay border) (Fig. 1) are scarcely studied and managed (Bayley, 1973; Baigún *et al*., 2012; Comision Administradora del Río Uruguay (CARU), 2010, 2014). In recent years, presence of eggs and larvae of this species has been found in Uruguay River, downwards of the Salto Grande hydroelectric dam (Argentina-Uruguay), suggesting the area as a breeding region (CARU, 2010, 2014). On the other hand, in the Pilcomayo River floodplain, 1,700 km away, there are records of less than a year old juveniles, suggesting another breeding area nearby (Bayley, 1973).

Identification of nursery areas is a very important tool to generate strategies that ensure 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). Due to the strong relation between chemistry and morphometry of fish otolith with different environmental features, lately these tools have been widely used to identify breeding areas of different commercially important species (Rooker *et al*., 2001; Gillanders *et al*., 2003; Tournois *et al*., 2013; Avigliano *et al*.; 2015d; Bailey *et al*., 2015; Bouchard *et al*., 2015). Even though physiological factors can affect incorporation of trace elements in the otolith, temperature and salinity are among the most relevant environmental factors regarding the incorporation of elements in this structure (Campana, 1999; Secor & Rooker, 2000; Elsdon & Gillanders, 2002; Martin *et al*., 2004; Sturrock *et al*., 2012; Bouchard *et al*., 2015). The predominant source of most elements to otoliths is the surrounding water (Kerr & Campana, 2013). Likewise, otolith morphometry is also related to environmental factors as salinity, temperature, depth, among others (Lombarte, 1992; Lombarte *et al*., 2010; Avigliano *et al*., 2014; Reichenbacher & Reichard, 2014).

Given the aforementioned, the aim of this work was to analyze the importance of some microchemical and morphometrical tools to identify possible breeding areas of streaked prochilod *Prochilodus lineatus* juvenile individuals. For this, morphometry (circularity, rectangularity, form factor, OL/OW and ellipticity indices) and chemistry (Sr:Ca, Ba:Ca, Zn:Ca) of *lapilli* otolith, and geometric morphometry of scales, in two sites in the Plata basin (Uruguay River and Estrella Wetland), were compared to determine if they are area-specific and to identify possible breeding areas.

Material and Methods

Study area and sample collection. The Plata basin, with area 3,170,000 km^2, is among the largest in the world. The most important rivers are the Paraná (4,000 km long), Paraguay (2,600 km), Uruguay (1,800 km long) and Pilcomayo Rivers (1,500 km) (Fig. 1) (Guerrero *et al*., 1997). These rivers go through 5 South-American countries (Argentina, Bolivia, Brazil, Uruguay and Paraguay). Particularly, the Pilcomayo River rises in Central Bolivia at about 3,200 m above sea level (19° Lat. South), flowing approximately south-eastwards through the Cordillera Central of the Eastern Andes and then forms the border between Argentina and Paraguay (Organización de los Estados Americanos (OEA), 1971). In this section, overflowing forms semi-permanent wetlands, like the Estrella Wetland, that functioning as nurseries for the streaked prochilod (OEA, 1971). Uruguay River headwaters are the mountains of the Atlantic Forest of southeast Brazil, becoming then the international boundary between Argentina and Brazil, and in the southeast the boundary between Argentina and Uruguay (Fig. 1). The Plata basin discharges into the Plata River 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 simultaneously between April 2013 and April 2014 using multifilament three-layer nets in the Uruguay River, upstream of the hydroelectric dam of Salto Grande (Salto Grande, Corrientes province, international boundary of Argentina-Brazil) and in the Estrella Wetland, that is an alluvial fun of Pilcomayo

River at Formosa province, Argentina (Fig. 1). Fish were transported to the laboratory at 4 ºC where they were measured (standard length = SL) and the *lapilli* otoliths were extracted. Also, axial region scales were extracted, washed with distilled water, dried and stored in paper envelopes.

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

Age determination and selection of samples. The otoliths were washed with ultrapure water and dried. The left otolith of each pair was sectioned transversely through the core using a rotary saw equipped with a diamond blade (Dremel® 250 and 300) and they were burned directly onto a Bunsen burner (Fig. 2). The number of rings in the otolith section was counted using a stereomicroscope (Leica® EZ4-HD, Singapore) at 8X magnification. Age determination by counting the ring number in *lapillus* otoliths of *P. lineatus* was validated by Espinach Ros *et al.* (2008). To avoid possible year-class effects on trace element composition or scale and otolith morphometry analysis, only fish 0+ years old were selected for the study (Fig. 2). In total, 23 individuals from Estrella Wetland site (SL: 19.9±1.70; range: 14-22 cm) and 13 from Uruguay River site (SL: 16.4±2.20; range: 13-20 cm) were selected for the analysis.

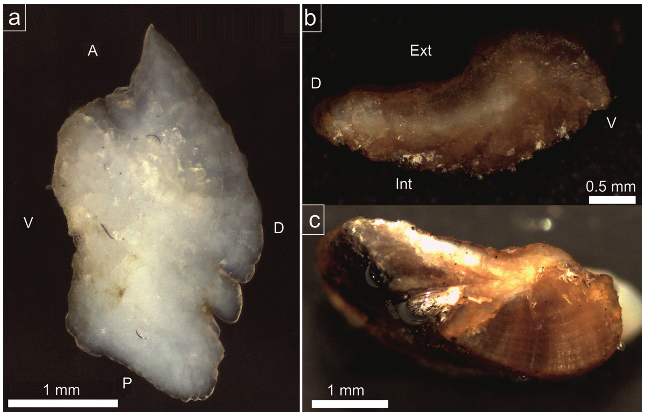

Fig. 2. *Lapilli* otolith of a streaked prochilod (*Prochilodus lineatus*). a) Right otolith of one of the sampled individuals, internal view (age 0+); b) right otolith section through the core (age 0+); c) example view of growth rings in an adult fish (age 8+). Abbreviations: A, anterior; D, dorsal; P, posterior; V, ventral; Ext, exterior; Int, interior.

Otolith microchemistry. The right otoliths were washed in water Mili-Q and, once dry, were transferred to a sterile centrifuge tube and weighed 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.*, 2015d). The concentration of Sr, Ba, Zn and Ca were determined (in triplicate) using 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) (U. S. Environmental Protection Agency (USEPA), 1994). The samples were introduced into the equipment with a PerkinElmer AS-90 Plus autosampler. External calibrations were performed in all cases using 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 using certified reference materials (FEBS-1, National Research Council, Canada) and an acceptable recovery percentage was obtained (99% for Zn, 100% for Ba, 105% for Sr and Ca). The detection limits (LOD) in µg/L based on three times the standard deviation of the blank signal was 8 for Ba and Sr, 12 for Zn and 15 for Ca.

The water used throughout the study was obtained from a Milli-Q water purification system (Millipore, São Paulo, Brazil) with a resistivity of 18.2 MOhm/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 material analyzed and the loss of material during the preparation process (Sinclair *et al.*, 1998; Bailey *et al.*, 2015).

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), rectangularity ($OA/[OL \times OW]$), ellipticity ($OL - OW/OL + OW$) and form factor ($[4\pi \times OA/OP^2]$). 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). Rectangularity gives information on the approximation to a rectangular or square shape, a value of 1 indicating a perfect rectangle or square (Tuset *et al.*, 2003b). Ellipticity reflects the similarity to an ellipse, 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, a value of 1 corresponding to a perfect circle (Tuset *et al.*, 2003b).

Scale geometric morphometry. Scales were cleaned with distilled water, dried and photographed using a stereoscopic microscope (Leica EZ4 HD). Only one scale per fish was used for the analysis (Ibañez *et al.*, 2007).

The selection of the landmarks was conducted according to Ibañez *et al.* (2007) and details of the framework of geometric morphometrics using landmarks can be found in Zelditch *et al.* (2004).

Landmarks 1 and 3 are the ventro and dorso lateral end of the anterior portion of the scale, respectively; landmark 2 is in the center of the anterior edge of the scale, landmarks 4 and 6 are at the boundary between the anterior area with *circuli* and the posterior area covered by cteni (spine-like ornamentations), respectively; landmark 5 is the focus of the scale; and landmark 7 is positioned at the tip of the posterior portion of the scale (Ibañez *et al.*, 2007) (Fig. 3).

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 (Rohlf, 2001; Klingenberg, 2011).

Statistical analysis. Even though studied fish were of the same age, a Mann-Whitney U test was performed to analyze differences on the SL among sites. This allowed verifying that SL does not affect morphometrical or microchemical variables.

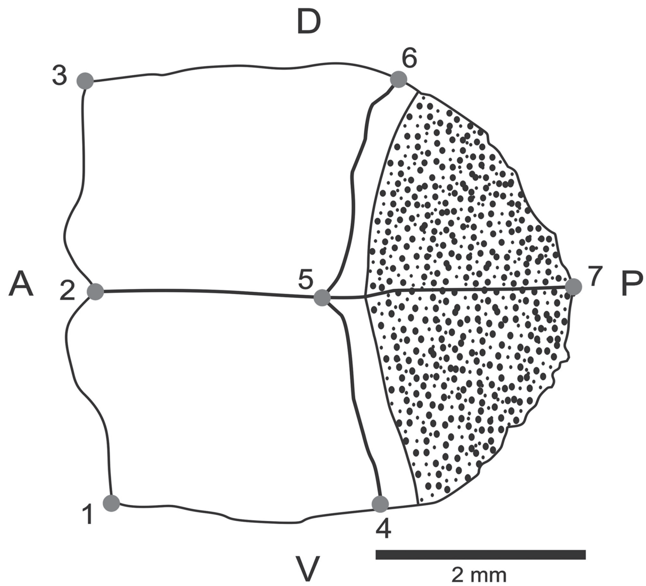

Fig. 3. Landmark definitions used in the fish scales. Abbreviations: A, anterior; D, dorsal; P, posterior; V, ventral.

T-test were used to explore the element:Ca ratios differences between locations. Previously, normality and homogeneity of variance were tested (Shapiro-Wilk test, $p>0.05$ and Shapiro-Wilk test, $p>0.05$, respectively). Moreover, we tested for relationships between elemental concentration and fish size (otolith weight) with analysis of covariance (ANCOVA, otolith weight as co-variate) (Campana *et al.*, 2000; Galley *et al.*, 2006; Burke *et al.*, 2008; Longmore *et al.*, 2010; Kerr & Campana, 2013; Avigliano *et al.*, 2015d, f). Element:Ca ratios were not significantly correlated with otolith mass.

In order to compare the shape indices from the selected sites, two sample tests were applied. After testing normality and homoscedasticity assumptions, circularity, form factor and rectangularity original data series were compared with a t-test while OL/OW and ellipticity were compared with a Mann-Whitney test. This analytical methodology is explained in Avigliano & Volpedo (2015). ANCOVA was then used to correct the effect of SL on the studied variables. It was only necessary to correct the form factor ($p < 0.01$, constant=-0.02).

The Hotelling T-square test was applied to determine whether differences existed among sites considering all the morphometric variables simultaneously. This test is appropriate for small samples and is robust to violations of the assumptions of normality and homogeneity of variance (Jørgensen & Rajeswaran, 2005; Avigliano & Volpedo, 2015; Avigliano *et al.*, 2015d). A canonical discriminant analysis (DA) was performed using the morphometric variables in order to obtain the cross classification matrix and determine the capacity of these variables to identify the site of origin of the fish (Longmore *et al.*, 2010; Silva *et al.*, 2011). Multicollinearity between variables was analyzed, thus preventing a false outcome in the DA analysis and the use of redundant variables in the study (Graham, 2003).

This geometric analysis was performed on the ten Cartesian coordinates or variables of 7 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 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 Discriminant Analysis (DA) to build a predictive model of group membership based on the observed characteristics in each case.

Results

Sampled fish sizes. No significant differences in the mean SL of fish among locations (W=230, $p > 0.05$) was obtained.

Otolith microchemistry. Means, deviations and ranges of otolith element:Ca ratios are shown in Table 1. Given that Ba:Ca and Zn:Ca ratios were below the detection limits for some groups, statistical tests were not applied for this variables.

While otolith Ba:Ca ratios for Uruguay River was 0.017±0.003 mmol/mol, it was below the detection limit of the used equipment for all Estrella Wetland individuals.

On the other hand, Zn:Ca ratios tended to be higher for Estrella Wetland (0.03±0.002 mmol/mol), while only 3 samples were above the detection limits in Uruguay River (0.01±0.002 mmol/mol).

Sr:Ca ratio differed significantly among sites (T=-4.25, p=0.0002), being higher in the Uruguay River site (1.33±0.19 mmol/mol) than in the Estrella Wetland (1.08±015 mmol/mol).

Otolith morphometry. Significantly high circularity values were obtained for the Estrella Wetland otoliths (p = 0.002), indicating high edge complexity (Table 2). This study site presented otoliths with significantly low rectangularity (p = 0.04) indicating a morphology tending towards circularity, while Uruguay River otoliths tended towards rectangularity. No significant differences for form factor, OL/OW and ellipticity were observed between sampling sites.

The Hotelling's T-square test showed significant differences for the morphometric variables among the study sites ($F_{3, 37}$=0.60, p = 0.01). DA showed an acceptable percentage of classification of individuals (77.7% for Estrella Wetland and 83.3% for Uruguay River).

Scale geometric morphometry. Due to the existence of two sets of data, a discriminant canonical function was obtained. Data corresponding to the 10 RWs of the RWA were employed to perform the DA. The DA correctly classified 90.5% for fish caught in Estrella Wetland, whereas the cross-validated analysis correctly classified 85% of the fish. The DA proved to have greater accuracy in classifying the fish caught in Uruguay River (85.7%). In this case, the cross-validated analysis correctly classified was 60%.

Table 1. Mean ± standard deviation and range (minimum–maximum) in mmol/mol of the otolith ratios of juvenile *Prochilodus lineatus* for each study site. P: p-value from t-tests. N: sample size. N varied in those sampling sites where some element: Ca ratios were below the detection limit of the used equipment.

	Estrella Wetland	N	Uruguay River	N	P
Ba:Ca	-	0	0.017±0.003 (0.010-0.023)	13	-
Sr:Ca	1.08±0.15 (0.83-1.33)	28	1.33±0.18 (1.06-1.64)	13	0.0002
Zn:Ca	0.034±0.022 (0.005-0.083)	17	0.015±0.003 (0.013-0.018)	3	-

Table 2. Mean ± standard deviation and range (minimum–maximum) of the otolith morphometric indices of juvenile *Prochilodus lineatus*. OL, otolith length; OW, otolith width.

	Estrella Wetland	Uruguay River	Statistic	P
Circularity	18.4±1.02 (16.7-19.9)	17.31±1.08 (15.8-18.9)	T=3.38	0.002
Form factor	0.17±0.03 (0.10-0.24)	0.17±0.03 (0.12-0.24)	T=0.44	0.65
Rectangularity	0.69±0.07 (0.64-0.72)	0.71±0.02 (0.67-0.76)	T=-2.13	0.04
Ellipticity	0.20±0.02 (0.17-0.60)	0.20±0.03 (0.17-0.24)	W=214	0.88
OL/OW	0.67±0.05 (0.58-0.86)	0.66±0.04 (0.61-0.71)	W=206	0.88

The 1st 3 RWs explained 69.6% (37.4%, 20.3%, and 11.9%, respectively) of the total variance for the RWA analysis of the scale shape of the different sampling sites studied. Patterns of morphological variations described by the 1st 3 RWs are shown in Fig. 4. Shape changes along the 1st RW were expressed by the depression (negative RW1 scores) or expansion (positive RW1 scores) of the scale along the dorso-ventral axis (Fig. 4A). The shape of RW2 varied somewhat due to the displacement of landmarks 4-7, which formed 2 types of scale posterior region: for the 1st one (RW2+), a compression of the antero-posterior axis, while the 2nd one was characterized by an expansion of the scale posterior region.

Shape changes along RW3 were also expressed by depression (RW3+) or expansion (RW3-) of the anterior region (antero-posterior axis) (Fig. 4B).

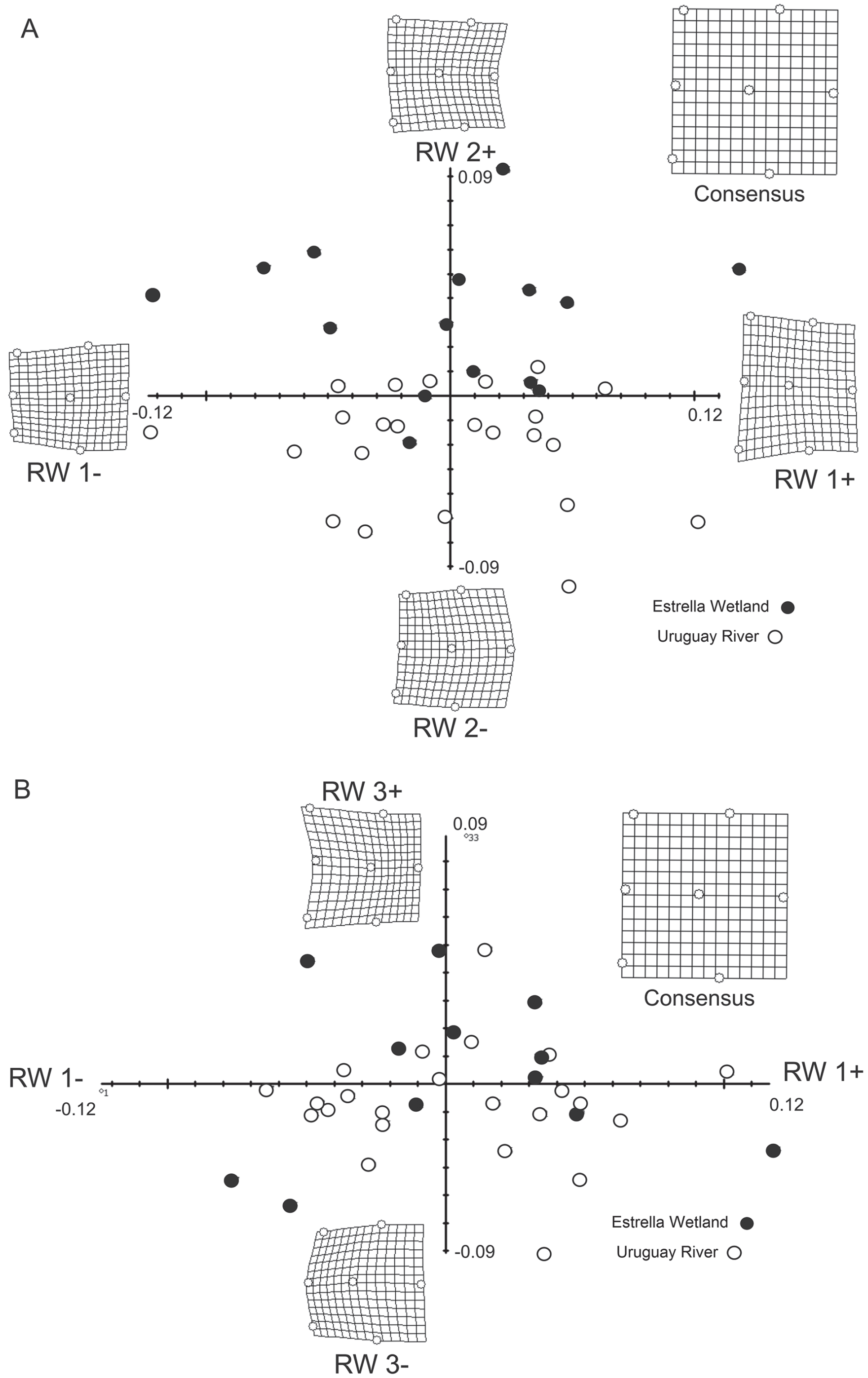

Fig. 4. Relative warp (RW) analysis based on landmark coordinates. Thin plate spline transformation grids for the extreme points of RW are shown; these were superimposed on the shapes predicted when the average landmark configuration of all scales was deformed into that of a hypothetical scale positioned at the extreme of the RW of interest. A) RW2 *vs*. RW1; B) RW3 *vs*. RW1.

Discussion

Integrated results of this work (scale and otolith morphometry and microchemistry) show that there are different breeding areas in the Plata basin, as it was suggested for the Pilcomayo (Bayley, 1973) and Uruguay Rivers (CARU, 2010). Besides, the simultaneous use of morphometric indices and microchemistry of the otolith, with the geometric morphometric of scales represent a potential tool for identification of streaked prochilod nursery areas.

Tendencies and differences found in element:Ca ratios could be due to diverse factors, like geology of the study area, chemical composition of the water and by less extent to temperature and diet (Campana, 1999; Ranaldi & Gagnon, 2008; Brown & Severin, 2009; Sturrock *et al.*, 2012). Given that research on trace element distribution on the study area are scarce and there is not a geochemical distribution map of the location, it becomes difficult to relate elements found in otolith to their geological origin. In the Uruguay River, there has been reports of Sr concentration in water of around 11-33 µg/L (Avigliano & Volpedo, 2013; Avigliano *et al.*, 2014; Avigliano & Schenone, 2015), while values observed in Estrella Wetland are higher, about 37-104 µg/L (pers. obs.). However, this pattern was opposite to the one observed in this study for otolith Sr:Ca ratios (Table 1), not explaining why Uruguay River values were higher.

On the other hand, element incorporation to the otolith does not depend only on geology of the areas. For example, in general, the Sr concentration in otoliths of freshwater and estuarine fish species correlates positively with the salinity, while Ba correlates negatively (Elsdon & Gillanders, 2005; Sturrock *et al.*, 2012; Avigliano *et al.*, 2014; Bouchard *et al.*, 2015; Stanley *et al.*, 2015). In this research, the observed pattern in otolith Sr:Ca ratios did not coincide with the positive relation between salinity/conductivity and Sr:Ca otolith ratio reported for other species (Brown & Severin, 2009); given that Estrella Wetland normally presents higher conductivity values than Uruguay River ones (130-820 µS/cm and <70 µS/cm, respectively) (Instituto Nacional de Tecnología Industrial (INTI), 2010; Comisión trinacional para el desarrollo de la cuenca del rio Pilcomayo (CCP), 2014; per. obs.). Nevertheless, relationship between salinity and Sr concentration in water among study areas was consistent to that reported by other authors (Martin *et al.*, 2004; Elsdon & Gillanders, 2005; Brown & Severin, 2009; Sturrock *et al.*, 2012; Avigliano *et al.*, 2014; Bouchard *et al.*, 2015; Stanley *et al.*, 2015). In this sense, Sr levels in superficial water of study areas and their salinities don't seem to explain the observed differences in otolith Sr:Ca ratios, thus, other factors might be influencing. Experimental essays showed that Sr precipitation in aragonite is negatively affected by temperature (Kinsman & Holland, 1969), suggesting that this process may also occur in otoliths (Campana, 1999). This could explain the low Sr:Ca ratios obtained for the Estrella Wetland, since its water temperature is usually around 16-35°C (CCP2014; per. obs.) while Uruguay River temperatures vary between 14-28°C (INTI, 2010). On the other hand, Campana (1999) discussed temperature, major life history transitions, somatic growth rate, and the rate of crystal formation as factors, other than water chemistry, that may influence otolith Sr:Ca variation. Campana (1999) proposed that high rates of system-wide protein synthesis extended to endolymph proteins, which are thought to dictate otolith growth. Also, it has been reported that maximum ages and growth rate for Pilcomayo River and Estrella Wetland are lower than those of Uruguay River (Sverlij *et al.*, 1993, pers. obs). High growth rates are thought to reduce Sr incorporation into otoliths, whereas low growth rates are thought to enhance Sr incorporation (Brown & Severin, 2009).

Related to Ba levels, superficial water values has been reported around 6.5-13 µg/L for Uruguay River (Avigliano *et al.*, 2014; Avigliano & Schenone, 2015), while for Estrella Wetland values are around 26-46 µg/L (pers. obs.). Just as Sr:Ca ratios, otolith Ba:Ca ratios were higher in Uruguay River fish, not been consistent to observed water levels. However, as it was previously mentioned by other authors (Tabouret *et al.*, 2010; Avigliano *et al.*, 2014; Bouchard *et al.*, 2015), higher Ba:Ca ratios were associated to sites with less conductivity. In this case, otolith Ba incorporation appears to be independent of water temperature (Martin & Thorrold, 2005; Martin & Wuenschel, 2006).

Otolith Zn incorporation appears to be related to diet (not water) (Ranaldi & Gagnon, 2008). For this reason, Zn:Ca ratio has also been used as habitat indicator for species associated to La Plata River basin such as *Percophis brasiliensis* (Avigliano *et al.*, 2015b). In this case, relatively elevated Zn:Ca ratios found in Estrella Wetland could be associated to geological features of the sampling areas, given that in Pilcomayo River Zn deposits has been found (Gemmell *et al.*, 1992). Actually, tributaries of Estrella Wetland are contaminated with Zn due to mining. For example, in its primary tributary, Pilcomayo River, values of up to 6,000 µg/L of Zn (Fang *et al.*, 2015) were registered, while in Uruguay River no values above 50 µg/L (INTI, 2010; Avigliano & Schenone, 2015) were detected.

In conclusion, Sr:Ca ratio showed to be a good habitat indicator. Ba:Ca and Zn:Ca ratios could also be used as indicators, but it is necessary the use of more sensitive techniques (ICP-MS). It would be interesting to evaluate simultaneous relations with multiparametrical analysis, so as to identify nursery areas more robustly. Differences of Sr:Ca ratios and tendencies observed for Ba:Ca and Zn:Ca ratios may indicate the occurrence of two different breeding sites.

The otolith Sr:Ca and Ba:Ca ratios proved to be a good marker of habitat for other euryhaline species from La Plata basin, *Lycengraulis grossidens* (Mai *et al.*, 2014), *Odontesthes bonariensis* (Avigliano & Volpedo, 2013; Avigliano *et al.*, 2014) and *Micropogonias furnieri* (Albuquerque *et al.*, 2012) and anadromus fish, *Genidens barbus* (Avigliano *et al.*, 2015c, d). However, these relationships depend on the species and should be analyzed before being used in nurseries and fish stock identification studies.

In relation to the otolith morphometry, only circularity and rectangularity indices were efficient to differentiate breeding areas. These indices showed to be a good tool as habitat indicators for other species of the same basin, like pejerrey *O. bonariensis* (Avigliano *et al*., 2015e) and river croaker *Plagioscion ternetzi* (Avigliano *et al*., 2015a). The DA classification percentages are similar to those reported by other authors (Tuset *et al*., 2003a; Avigliano *et al*., 2015e), 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. Circularity was also a good habitat indicator for other species like *Coryphaenoides rupestris* (Longmore *et al*., 2010) and *Lophius piscatorius* (Cañás *et al*., 2012). On the other hand, form factor did not showed to be a good indicator for the streaked prochilod, as well as for other species like *C. rupestris* (Longmore *et al*., 2010). However, form factor index along with ellipticity index, led to the identification of populations of *O. bonariensis* (Avigliano *et al*., 2015e). These discrepancies among different species show that the use of morphologic indices to separate breeding areas or populations is species and/or environmentally dependent, and should be correctly evaluated before their use for monitoring or population studies.

Even though some variables were alone not efficient to separate breeding areas, simultaneous use of all variables (DA) showed good classification percentages. For this, the use of morphometrical indices cannot be discarded for studies on nursery areas of streaked prochilod.

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 (Lombarte, 1992; Lombarte *et al*., 2010; Avigliano *et al*., 2014; Reichenbacher & Reichard, 2014). Therefore, the taxonomic value of otoliths is well established (Gierl *et al*., 2013; Reichenbacher & Reichard, 2014). Because of these characteristics, otolith morphometry has been widely used to identify fish stocks (Campana & Casselman, 1993; Cañás *et al*., 2012), to differentiate species (Tuset *et al*., 2012, 2013; Zhuang *et al*., 2014), to describe ecomorphological patterns of species (Tuset *et al*., 2003a; Jaramillo *et al*., 2014; Avigliano *et al*., 2016), and as an environmental indicator (Nelson *et al*., 1994; Avigliano *et al*., 2016). Among the most commonly used indexes are rectangularity, circularity and aspect ratio (Longmore *et al*., 2010; Cañás *et al*., 2012; Jaramillo *et al*., 2014; Avigliano *et al*., 2016).

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

The use 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 (Richards & Esteves, 1997; Poulet *et al*., 2005). However, no morphologic aspect of scales has been previously used as habitat marker to identify breeding areas. There is only one precedent were it was shown that morphology of scales of streaked prochilod juveniles captured close to Estrella Wetland could vary with different hydrologic conditions (Bayley, 1973).

In relation to the obtained results, we recommend to integrate 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. This information could be obtained by an annual repetition of this study after reproductive periods. With this gathered information, stable nursery areas could be protected during spawning and breeding phases. However, if some nurseries are not stable in time, is advisable an active monitoring to ensure their identification every year and to act accordingly to guarantee the reproduction and breeding of streaked prochilod juveniles.

In relation to monitoring and use of otolith microchemistry, analyzing the geochemical origin of different studied elements, as well as the incorporation of isotopical relations could generate other markers of origin to study even adult individuals (Ashford & Jones, 2007; Walther & Thorrold, 2009; Niklitschek *et al*., 2010; Hegg *et al*., 2013, 2015; Garcez *et al*., 2015).

On the other hand, these methodologies could be used to evaluate the existence of nursery areas in others areas, for example, Uruguay River high basin and in Paraná River, allowing a proper conservation and management of the areas according to fisheries needs. Also, the use of some or all applied methodologies could be helpful for the study of connectivity between nursery areas.

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, using specialized software (*e.g.* HISEA). 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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