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Morphology and microchemistry of the otoliths of the inner ear of anuran larvae

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ABSTRACT

To navigate in space most vertebrates need precise positional cues provided by a variety of sensors, including structures in the inner ear, which are exquisitely sensitive to gravity and linear acceleration. Although these structures have been described in many vertebrates, no information is available for anuran larvae. The purpose of our study was to describe, for the first time, the size, complexity and microchemistry of the saccular otoliths of the larva of 13 anuran species from central Argentina, using electron microscopy and X-ray spectroscopy (N = 65). We concluded that a) these structures differ in area, perimeter, otolith relative size and fractal dimension, but are similar in terms of their microchemistry when compared by spatial guilds, b) that nektonic species have larger otoliths than nektonic-benthic and benthic species and c) that benthic species have larger otolith relative size than nektonic-benthic and nektonic species.

1. Introduction

Through adaptation vertebrates have incorporated different sensory receptors that provide a variety of cues that are advantageous for survival. For navigation, most vertebrates evolved structures in the inner ear that are exquisitely sensitive to gravity and linear acceleration. A peculiar adaptation is the presence of minute bio-minerals floating inside specific structures of the inner ear. These minerals (so-called otoconia or otoliths, depending on their size and quantity) mechanically stimulate the ciliary cell bundles initiating in this way the process of transduction from physical to neural energy (Bear et al., 2007).

It is well known that the inner ear of Anura includes three otolithic organs (saccule, utricle and lagena) containing otoconia, that are involved in the detection of linear acceleration and gravity, two dedicated acoustic end organs (basilar papilla and amphibian papilla) and three semicircular canals which detect angular acceleration or rate of body turning and tilting (Lannoo, 1999). Remarkable differences in shape and crystallographic properties of otoconia and otoliths of several Subphyla of chordates have been firstly described by Carlström (1963) and then enhanced mainly in teleost fish (Popper et al., 2005), rodents (Salamat et al., 1980), birds (Dickman et al., 2004; Li et al., 2006) caudates (Oukda et al., 1999a and Oukda et al., 1999b) and anurans (Pote and Ross, 1991; Kido and Takahashi, 1997). In addition, some authors (Lychakov, 2004) mentioned the existence of irregular and polyhedral otoconia clusters (teleost-like otoliths) in amphibians under different names like microstatolith (Carlström, 1963), compositional otolith (Fermin et al., 1998) and otolith (Oukda et al., 1999a) although these otoconia conglomerates have never been properly described. This study is the first description of the size, complexity and microchemistry of the otoliths (irregular and polyhedral conglomerate of individual otoconia) in the saccule of the inner ear of 13 neotropical anuran species at the larval stage interpreted from an ecological perspective. Differences in otolith morphometry regarding spatial guilds could be adaptive and partially explain differences in sensitivity to acoustic waves and particle motion.

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2. Materials and methods

2.1. Species selected

Larvae of thirteen anuran species were collected using sweep nets during the summer of 2013 from both temporary and permanent ponds. Twelve of these native anuran species (Peltzer and Lajmanovich, 2007) were collected from the central region of Argentina (Santa Fe and Entre Ríos provinces) and one exotic anuran species (*Lithobates catesbeianus*) was collected from the province of Cordoba in Argentina were this species was introduced. After their collection, larvae were transported in a non-transparent bucket filled with water collected from the same pond.

Once in the laboratory, specimens from different larval stages ranging from Gosner (Gosner, 1960) stages 30–42 ($n = 5$ individuals per species) were anesthetized using a solution of 0.1% ethyl p-aminobenzoate, measured (Total Length- TL), and decapitated with a scalpel. The extraction of the otoliths from the saccule was facilitated by using hypodermic needles under a stereoscope (Arcano® Ztx 1:4, Beijing, China) and without the use of fixatives. Subsequently, they were gently rinsed with distilled water and mounted on a double-sided sticky tape to be dried at room temperature (25 °C ± 2 °C).

All the study protocols and field collections were approved by the bioethics' committee of the School of Biochemistry and Biological Sciences, National University of the Litoral (Santa Fe, Argentina) following the *Guidelines for use of live amphibians and reptiles in field and laboratory research* compiled by the American Society of Ichthyologists and Herpetologists, the Herpetologist's League and the Society for the Study of Amphibians and Reptiles (ASIH, 2004). The field collection did not involve endangered or protected species and was carried out in full compliance with the law "*Protection and Conservation of Wild Fauna*" (Argentina National Law N° 22.421) and local regulations.

2.2. Morphological description

Five otoliths per each of the selected species were examined using a SIGMA field emission scanning electron microscope (Carl Zeiss Microscopy GmbH, Germany) after coating with a ~20 nm carbon layer. The micrographs were acquired using the In-Lens Secondary Electron detector at a working distance between 2 and 10 mm with an acceleration potential of 10 kV. The resulting micrographs were saved as greyscale images (with 8 bits of intensity resolution) with a spatial resolution of 0.213 μm per pixel.

Otoliths are three-dimensional objects, thus the two-dimensional (2D) description used here can be only adequate as long as the otoliths lack exaggerated anisotropy (i.e., no disproportioned elongation in any particular coordinate). Nevertheless, otoliths with exaggerated anisotropy were excluded from this study. Considering that, a 2D silhouette was extracted from each otolith's digitized image (see examples in Fig. 1), by manually delineating its outline (Tomas and Geffen, 2003) using Corel Photo Paint X3 (Corel Corporation, Dublin, Ireland). All further calculations were done using this silhouette (Jelinek and Fernandez, 1998). Area and perimeter of each otolith were measured using the software ImageJ (Wayne Rasband, National Institute of Health, USA). Also, the otolith relative size index (OR) was calculated in accordance to the equation $OR = 0.01\ OA\ TL^{-2}$ (Lombarte and Cruz, 2007), where OA = Otolith area (μm^2) and TL = tadpole total length (mm) to reduce the effect of tadpole size.

The fractal dimension (FD) of each otolith was calculated by using the box-counting method (Mandelbrot, 1983; Krauss et al., 1994) included in the program FracLac 2.5 (Karperien, 2011), a plug-in of the ImageJ software. This algorithm is based on partitioning the image into square boxes of size $L \times L$ and subsequently counts the number of boxes ($n(L)$) containing any portion of the shape. By varying the box size L, the fractal dimension is calculated as the absolute value of the slope of the line computed from the linear regression of the ($\log(L), \log(n(L))$) curve. The mean FD for each specimen was computed from the data obtained from 12 random rotations of the silhouette of each otolith in order to avoid potential problems, such as anisotropy (Jelinek and Fernandez, 1998). In qualitative terms, higher FD values corresponds to more complex, more irregular and more fractured shapes than lower FD values.

2.3. Semi-quantitative elements microanalysis

The composition of the inorganic phase of each otolith was determined by a semi-quantitative elements microanalysis. An area of ~0.2 mm^2 of a smooth surface of three out of the five otoliths per species were examined for microanalysis by scanning electron microscopy using a 10 mm^2 silicon drift detector coupled with an advanced energy dispersion spectra (EDX) spectrometer connected to a computer operated by the Aztec software (Oxford Instruments, UK). The elements were graphed in spectra based on $K\alpha$ lines collected in the range 0–10 keV for 60 s and the percentage of atoms of each element measured, following the work in Oukda et al. (1999b). EDX maps were collected for the elements Cl, Mg, Na, P, Si and Al in order to see their spatial distribution in the sample. The frequency reported for each element represents the average estimated over all otoliths analyzed.

2.4. Statistical analysis

We tested for potential differences among ecomorphological guilds (sensu Peltzer and Lajmanovich, 2007: Nektonic, Benthic, Benthic-Nektonic) on TL, Gosner stage (1960) and five otolith features: area, OR, perimeter, fractal dimension and number of chemical elements in otolith composition, using a non-parametric Kruskal–Wallis significance test. A guild is defined as a group of species that exploit the same class of environmental resources in a similar way regardless their taxonomic position (Simberloff and Dayan, 1991). Benthic species spend most of the time at the bottom of ponds and have globular or flattened bodies, dorsal eyes, and a short fin not extending onto the body; nektonic species live in the open water of ponds, often moving through vegetation and have laterally compressed or equidimensional bodies, lateral eyes, and their fins are often tall and sometimes extend onto their body as far as the head (Wells, 2007); benthic-nektonic present transitional traits between benthic and nektonic morphotypes and dwell near the bottom of ponds or in the water column (Peltzer and Lajmanovich, 2007).

The Spearman correlation coefficient was used to test for correlations between tadpole development (Gosner, 1960) and five otolith characteristics (area, perimeter, OR, fractal dimension and number of chemical elements). The Spearman correlation was also used to test for any relations between Ca and the diverse chemical elements detected in the composition of the otolith. The statistical analyses were performed using the statistical package IBM SPSS 22 (IBM, Armonk, NY, USA).

3. Results

3.1. Morphological description

The images presented in Fig. 1 are a typical otolith extracted from each of the species studied. Simple inspection of these pictures already reveals a large variation both in overall size and shape

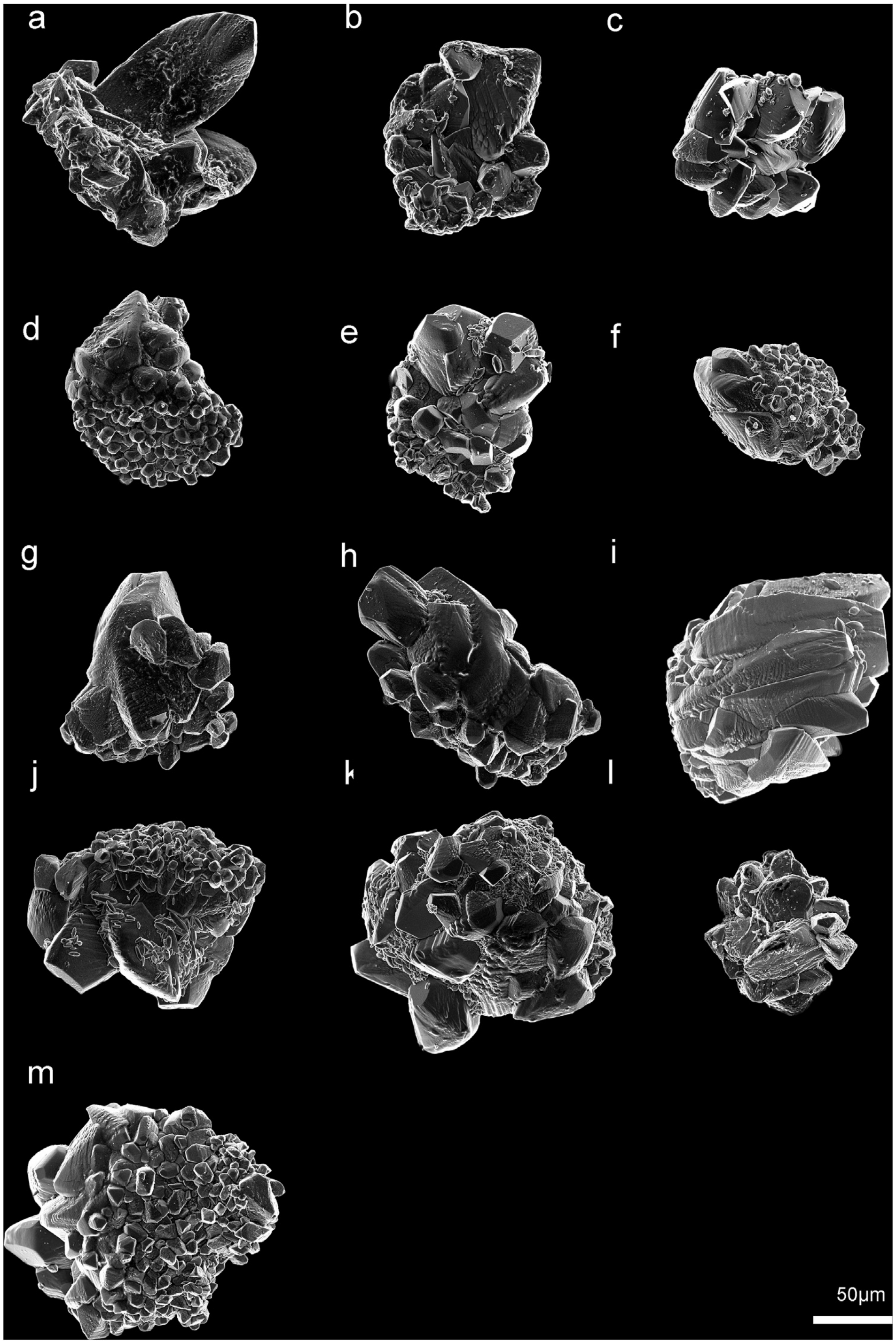

Fig. 1. Typical examples of otoliths extracted from the larvae of the thirteen anuran species studied. References: a) *Rhinella arenarum*; b) *Leptodactylus latrans*; c) *L. chaquensis*; d) *L. gracilis*; e) *L. mystacinus*; f) *L. latinasus*; g) *Pseudopaludicola falcipes*; h) *Odontophrynus americanus*; i) *Phyllomedusa azurea*; j) *Scinax nasicus*; k) *S. squalirostris*; l) *Elachistocleis bicolor*; m) *Lithobates catesbeianus*. Calibration Bar = 50 μm.

as well as in the amount of further details found when inspected at smaller spatial scales. For instance, consider the cases labeled *"d"* and *"l"* in Fig. 1. The specimen labeled *"l"* (from *Elachistocleis bicolor*) seems to be comprised of a few relatively large elements while the one depicted in *"d"* (from *Leptodactylus gracilis*) is comprised of a larger number of relatively smaller elements.

Table 1 summarizes otolith characteristics by species. Mean area varied between 7120 μm^2 (*E. bicolor*) and 18 230 μm^2 (*Phyllomedusa azurea*), mean perimeter between 376.5 μm (*E. bicolor*) and 613 μm (*L. catesbeianus*) and OR between 0.02 (*L. catesbeianus*) and 0.49 (*Pseudopaludicola falcipes*). No statistical differences in development stages were found between spatial guilds (Gosner, 1960) ($KW = 5.31$; $p > 0.05$), although significant differences in TL were found ($KW = 24.69$; $p < 0.0001$) with benthic species being significantly smaller than nektonic and B-N species (Fig. 2A). Statistical differences between spatial guilds were also found for

Table 1
Summary of otoliths characteristics for all the anuran species studied.

Anura	Spatial guild	Gosner stage	Total length (mm)	Area (μm^2)	OR	Perimeter (μm)	Fractal dimension	Element composition
Bufonidae								
a. *Rhinella arenarum*	B	32–37	20.8–24.8	14,234 ± 359	0.30 ± 0.05	586.3 ± 24.0	1.73 ± 0.01	Ca,Na,Mg,Al,Si,P,S,Cl,K
Leptodactylidae								
b. *Leptodactylus latrans*	B-N	31–34	33.4–44.5	9739 ± 1755	0.06 ± 0.02	448.0 ± 51.2	1.70 ± 0.01	Ca,Na,Mg,Al,Si,P,S,Cl,K
c. *Leptodactylus chaquensis*	B-N	40–41	50.6–62.7	7773 ± 201	0.03 ± 0.01	412.9 ± 30.0	1.67 ± 0.01	Ca,Na,Mg,Al,Si,P,S,Cl,K
d. *Leptodactylus gracilis*	B-N	37–40	25.9–34.6	8309 ± 1310	0.09 ± 0.01	414.8 ± 41.9	1.68 ± 0.01	Ca,Na,Mg,Al,Si,P,S,Cl,K
e. *Leptodactylus mystacinus*	B-N	31–37	23.9–29.7	8585 ± 2747	0.12 ± 0.03	422.6 ± 87.2	1.68 ± 0.03	Ca,Na,Mg,Al,Si,P,S,Cl,K
f. *Leptodactylus latinasus*	B-N	35–42	20.0–30.8	9084 ± 4673	0.20 ± 0.15	414.4 ± 107.1	1.68 ± 0.06	Ca,Na,Mg,Al,Si,P,S,Cl,K,Sr
g. *Pseudopaludicola falcipes*	B	30–40	9.4–17.2	9401 ± 1609	0.49 ± 0.23	458.6 ± 42.5	1.69 ± 0.02	Ca,Na,Mg,Al,Si,P,S,Cl,K
Odontophynidae								
h. *Odontophrynus americanus*	B-N	34–37	18.3–25.3	11,008 ± 1805	0.26 ± 0.12	482.5 ± 49.9	1.71 ± 0.03	Ca,Na,Mg,Si,P,S,Cl,K
Hylidae								
i. *Phyllomedusa azurea*	N	38–38	34.8–36.2	18,230 ± 1643	0.15 ± 0.01	612.8 ± 34.2	1.84 ± 0.01	Ca,Na,Mg,Al,Si,P,S,Cl
j. *Scinax nasicus*	N	31–37	19.3–20.0	14,887 ± 1792	0.37 ± 0.04	511.9 ± 24.1	1.74 ± 0.02	Ca,Na,Mg,Al,Si,P,S,Cl,K
k. *Scinax squalirostris*	N	33–36	24.9–28.8	13,972 ± 120	0.20 ± 0.04	525.6 ± 30.2	1.78 ± 0.05	Ca,Na,Mg,Al,Si,P,S,Cl,K
Microhylidae								
l. *Elachistocleis bicolor*	B	31–34	16.5–21.5	7120 ± 510	0.21 ± 0.07	376.5 ± 22.6	1.67 ± 0.01	Ca,Na,Mg,Al,Si,P,S,Cl,K
Ranidae								
m. *Lithobates catesbeianus*	N	37–39	101.5–113.2	16,889 ± 2288	0.02 ± 0.01	613.0 ± 60.2	1.73 ± 0.01	Ca,Na,Mg,Al,Si,P,S,Cl,K

The first three columns correspond to parameters of the larvae and the remaining five to otolith morphology and chemistry. Numerical values represent either range (hyphenated values) or mean ± SD. Species in the first column are labelled with the same letters used in the examples shown in Fig. 1. The spatial guild in the second column follows the criteria described in Peltzer and Lajmanovich (2007). Abrev. N: Nektonic, B: Benthic, B-N: Benthic-Nektonic.

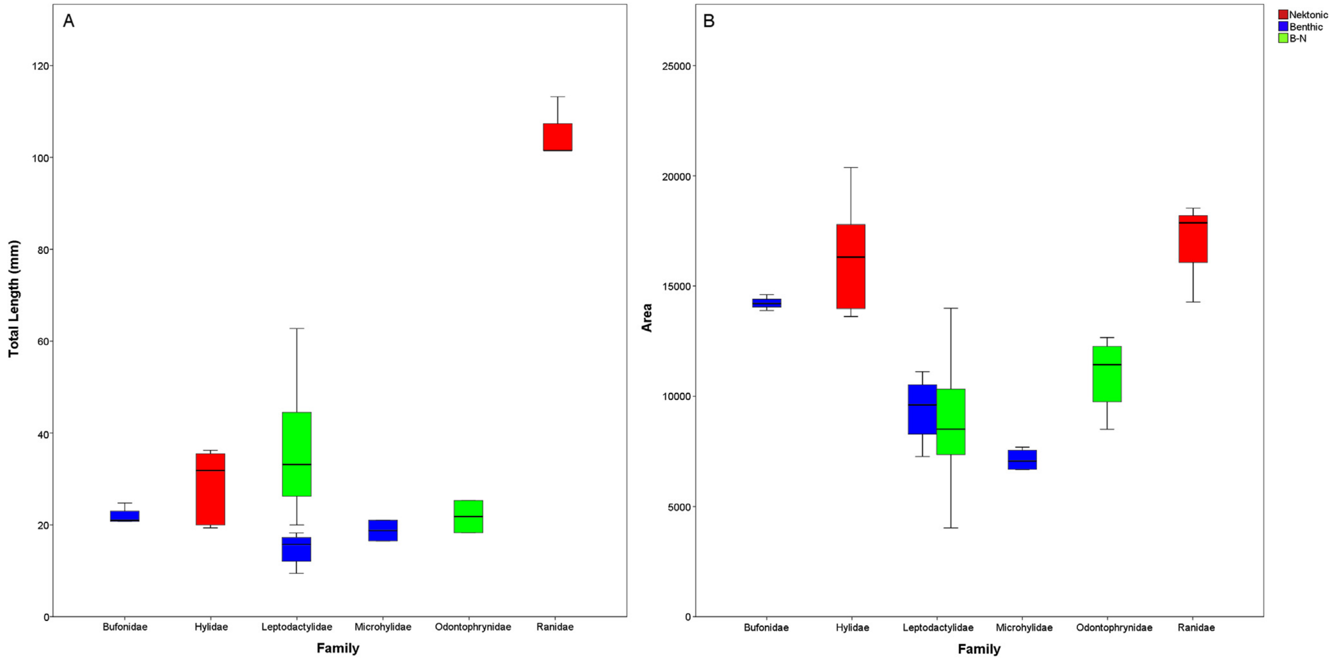

Fig. 2. Box and whisker diagram of total length (TL) (A) and area (B) related to spatial guild and taxonomic family. Leptodactylidae is the only analized family that include species of two different spatial guilds (benthic and B-N). Note that species with bigger tadpoles do not necessary have bigger otoliths.

otolith area ($KW = 24.72$; $p < 0.0001$) (Fig. 2B) and perimeter ($KW = 20.55$; $p < 0.0001$), with nektonic species having significantly higher values than benthic and B-N species in both cases. The benthic species was the group with significantly higher values ($KW = 12.51$; $p < 0.005$) as it presented a higher proportion of big OR than nektonic and B-N species (Fig. 3).

No Spearman correlation was found between Gosner stage and area ($r = -0.061$; $p > 0.05$), perimeter ($r = -0.032$; $p > 0.05$) and FD ($r = -0.163$; $p > 0.05$). Moreover, a negative Spearman correlation was found between Gosner stage and OR ($r = -0.474$; $p < 0.01$).

Differences among spatial guilds regarding FD were significant ($KW = 20.55$; $p < 0.0001$). FD varied between 1.67 (*E. bicolor* and *L. gracilis*) and 1.84 (*P. azurea*) with nektonic species presenting higher values—more complex shapes-than benthic and B-N species.

3.2. Semi-quantitative microanalysis composition

The chemical composition of the otoliths was characterized by a high percentage of Ca (~90%), as expected from previous results, and the presence of other eight chemical elements (Na, Mg, Al, Si, P, S, Cl, K) in different frequency and concentrations (Table 2). The number of chemical elements were similar among spatial guilds (*$KW = 2.96$; $p > 0.05$*). However, significant correlations ($p < 0.05$ and $p < 0.01$) were found between Ca and all the other elements in

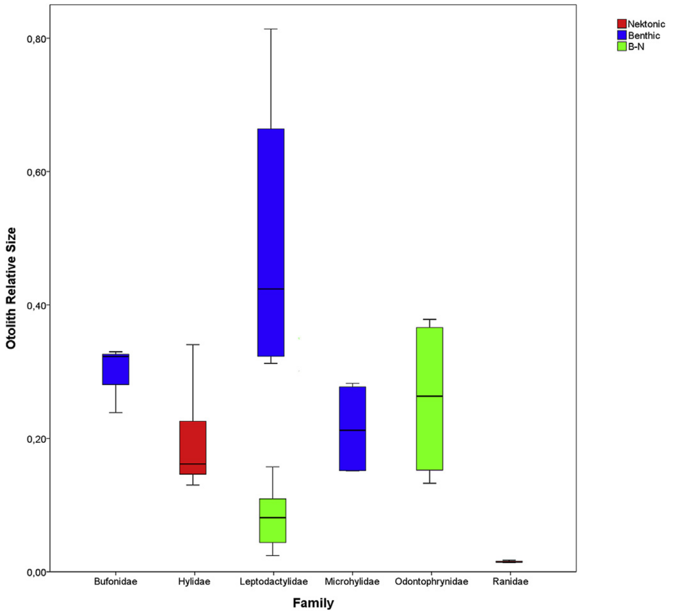

Fig. 3. Box plot of otolith relative size (OR), taxonomic family and spatial guild. Leptodactylidae is the only studied family that includes species of two different spatial guilds (benthic and B-N).

the otoliths' composition and between Gosner stage and the number of elements composing the otolith (Spearman's r = 0.416; $p < 0.05$). Moreover, the spatial distribution of these elements was homogenous, as shown by the typical example presented in Fig. 4.

4. Discussion

Our findings provide the first description of saccular otoliths in the inner ear of tadpoles of 13 anuran species from central Argentina. Saccular otoliths were previously reported in the larvae of the other two orders of amphibians: caecilians (Carlström, 1963) and caudates (Carlström, 1963; Oukda et al., 1999a). Otolith size differed among spatial guilds. The results showed that nektonic species have bigger otoliths than benthic and B-N species (Fig. 2B). On this subject Lychakov and Rebane (2000) proposed that higher otolith mass provides higher acoustic sensitivity at low frequencies —which could enhance the auditory bandwidth of nektonic species-. In this context, it is important to highlight the saccule's acute sensitivity to substrate-borne vibrations (Lewis and Narins, 1985) and probably to water vibrations (Walkowiak and Münz, 1985). In these context, other authors (e.g. Wells, 2007) suggested that the saccule —and therefore its otolith-is probably used by most anurans to detect the approach of predators. It is evident that predators like birds hunt their tadpole prey from different angles than fish and aquatic invertebrates so, tadpoles from different spatial guilds may have developed different strategies to detect them including a wider hearing range. On the other hand, nektonic species have fins capable of producing high acceleration and moving them quickly through dense vegetation. Lychakov and Rebane (2000) suggested that fish that swim between obstacles require highly sensitive otolith organs in order to be able to perform precise, well-calculated movements around those obstacles as well as otolith organs with rapid action capable of recording the strong acceleration, sharp turns and halts during the maneuvering around obstacles, all of which are skills provided by large otoliths. In sum, in the case of the tadpoles considered in this study, otoconia crystals with different masses could simultaneously provide high sensitivity for recording weak accelerations -small otoconia- and for operating under high accelerations -bigger otoliths-.

Table 2
Frequency and concentration of the different elements found in the inner ears of otoliths and the Spearman correlations (R) between Ca and the other constitutive elements.

Chemical elements	OEF%[a]	a% Mean ± SD	R
Ca	100	90.31 ± 8.90	
P	97.8	1.59 ± 1.53	−0.77**
S	95.6	1.18 ± 1.05	−0.84**
Mg	95.6	0.90 ± 0.46	−0.58**
Na	88.9	2.82 ± 3.56	−0.83**
Si	84.4	0.99 ± 0.90	−0.37*
Cl	80	1.71 ± 2.08	−0.71**
K	68.9	1.76 ± 1.46	−0.82**
Al	46.7	0.49 ± 0.46	-0.36^{NS}
Sr	2.2	0.15 ± 0.04	ND

*Significant correlation $P < 0.05$.
**Significant correlation $P < 0.01$.
NS = not significant correlation $P > 0.05$.
ND = not determined.
[a] (OEF%) defined as the fraction between the number of occurrences of element x and the total number of otoliths examined by 100.

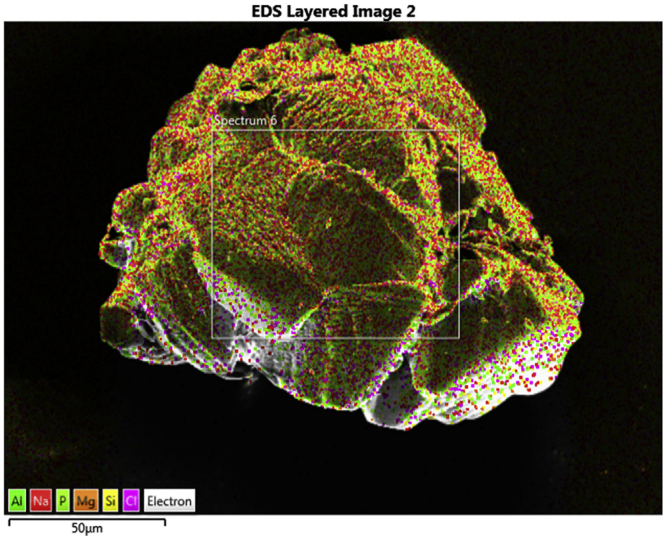

Fig. 4. Example of an X-ray intensity map of the chemical element contents demonstrating the typical sparse distribution of Al, Na, P, Mg, Si and Cl. (Otolith from *Scinax nasicus* larva).

Relative otolith size also differed among spatial guilds. In this case, benthic species exhibited a bigger OR index than nektonic and B-N species —even within the family Leptodactylidae which includes species from two different spatial guilds-because the benthic group included species with smaller TL (Fig. 2A). Surprisingly, other authors (Dewitt et al., 1990; Ekau, 1991; Lombarte y Cruz, 2007; Lombarte et al., 2010) observed the same phenomena (benthic species with bigger OR) in fishes. On this subject, Paxton (2000) linked relatively larger otoliths in fish to evolutionary responses that compensate for the absence of or great reduction of sunlight, which are precisely the type of habitats frequented by the anuran larvae studied here (Lajmanovich, 2000).

The fractal dimension of the otoliths varied between FD = 1.67 and FD = 1.84. The significant differences among spatial guilds in terms of FD showed that nektonic species have more irregular and more fractured otoliths than benthic and B-N species. The processes underlying otoconia formation, shaping and maintenance are not yet fully understood (Lundberg et al., 2015). Regarding differences in otolith shape (in this case, more or less fractured shapes), Popper et al. (2005) suggested that shape variations could impose different motion dynamics in response to vestibular and auditory stimuli. In addition, Fermin et al. (1998) suggested that otoconia shape could also affect otoconia density and the intensity of the ion interchange between otoconia and endolymph.

Microchemical analysis of the otoliths revealed a ~90% Ca purity, slightly lower than the calcitic and aragonitic otoconia of newts at ~94% Ca (Oukda et al., 1999b). If any, the minor differences between otolith and otoconia purity could be due to the small number of samples analyzed or due to real interspecific differences. Furthermore, the presence of chemical elements different from $CaCO_3$ does not seem to depend on spatial guild as demonstrated by this work or by different taxonomic levels, as six out of nine trace elements found in our work (Cl, K, Mg, Na, P, S) were similar to previous findings for calcitic otoconia of guinea pig rodents (Takumida and Zhang, 1997) and for calcitic and aragonitic otoconia of newts (Oukda et al., 1999a). On the contrary, two (Al and Si) have not been previously reported in other amphibian otoconia while Sr was found in otoconia of the endolymphatic sac of newts (Oukda et al., 1999b) but not in the saccule or the utricle. In our study, Sr was found in two individuals of the same species (*Leptodactylus latinasus*). Thus, although it is a limited sample, the results show no statistical differences in the otolith's composition among spatial guilds.

5. Conclusions

This study described the otoliths in the inner ear of larvae of 13 anuran species from Argentina for the first time. It is concluded that, a) across spatial guilds, these structures differ in area, perimeter, OR and fractal dimension, but are similar in terms of its microchemistry, b) that nektonic species have larger otoliths than nektonic-benthic and benthic species and c) that benthic species have larger otolith relative size than nektonic-benthic and nektonic species.

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