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ONTOGENETIC DEVELOPMENT OF THE INNER EAR SACCULE IN A VOCAL TELEOST FISH: IMPLICATIONS FOR AUDITORY RECEPTION AND COMMUNICATION

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Resumo

Os peixes consistem no maior grupo de vertebrados e exibem uma diversidade excecional na estrutura e função dos seus sistemas sensoriais e comunicativos. A família Batrachoididae tornou-se importante no estudo do funcionamento dos sistemas auditivo-vocal no contexto da comunicação acústica. Um estudo recente relatou um paralelismo entre a diferenciação vocal e o aumento da sensibilidade auditiva em *Halobatrachus didactylus,* sugerindo uma potencial interação entre os circuitos auditivo-vocal durante o desenvolvimento. Os objectivos deste estudo foram: 1) verificar alterações ontogenéticas no principal órgão auditivo periférico (sáculo) que possam explicar o aumento da sensibilidade auditiva em *H. didactylus*; 2) comparar características morfológicas do sáculo entre Batracoidideos.

Espécimes em etapas ontogénicas distintas foram fixados e o seu sáculo removido, marcado com faloidina e analisado. A área do sáculo aumentou 10x, ocorrendo mudanças na sua forma. Os padrões principais de orientação das células ciliadas (CC) estabeleceram-se cedo no desenvolvimento pós-embrionário, com algumas variações subsequentes nas regiões rostral e caudal. A adição de CC aumentou rapidamente em relação ao crescimento epitelial, resultando numa redução de 1.6x na sua densidade. A densidade de células de suporte (CS) diminui significativemente. O aumento da área do epitélio deveu-se principalmente ao aumento da área da superfície apical de CS em conjunto com a adição de CC.

Comparações interespecíficas revelaram um padrão duplo comum com algumas diferenças nas regiões rostral e caudal que poderão explicar diferenças na sensibilidade auditiva.

Enquanto tais alterações ontogenéticas poderão facilitar a sensibilidade do sáculo, potenciando a comunicação acústica, outras características estruturais e moleculares deverão ser investigadas.

Keywords: epitélio do sáculo; células ciliadas; audição; ontogenia; *Halobatrachus didactylus*.

Abstract

Fishes, the largest group of vertebrates, display an exceptional diversity in structure and function of sensory and communication systems. The Batrachoididae has become an important family to study mechanisms of auditory-vocal functions for social communication. A recent study reported that vocal differentiation parallels developmental improvements in auditory sensitivity in *Halobatrachus didactylus*, suggesting a potential coupling between vocal-auditory circuitry. The goals of this study were: 1) to verify developmental changes in the main auditory endorgan (saccule) that may account for ontogenetic auditory improvements in *H. didactylus*; and 2) to compare saccular morphological features among Batrachoididae species.

Different size groups, from posthatched fry to adults, were PFA-fixed and their saccule removed for phalloidin-staining and structural analysis. Saccular epithelium area increased circa 10x with development, along with significant changes in shape (i.e. caudal region ratio). Most of hair cell (HC) orientation patterns seem to be established early in postembryonic development, with a few variations mostly in the rostral and caudal regions.

HC addition increased rapidly in relation to epithelium growth, resulting in 1.6x decrease in HC density. Supporting cell (SC) density decreased throughout development. SC apical surface increased significantly and together with HC addition explained the sensory epithelium growth. Interspecific comparisons within Batrachodidae revealed a common "dual pattern", but also differences in the rostral and caudal regions that may explain different auditory sensitivities.

While such developmental changes may facilitate in part saccular sensitivity and enhance social acoustic communication, other structural and molecular features of the saccule should be investigated.

Keywords: saccular epithelium; hair cell; hearing; ontogeny; *Halobatrachus didactylus*.

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Chapter 1. Aim of study

Over the years, scientists have struggled to understand how the sense of hearing and the vertebrate auditory system has evolved. Fish represent a basal lineage group within vertebrates, providing research platforms to study evolution and adaptation of the auditory system and its functions for social communication. Moreover, studies have confirmed the high variety and complexity of vocal communication systems in teleost fish, with sound providing a channel of communication that is crucial in a constantly changing environment, where visual acuity is sometimes limited.

The aims of this study were to investigate the morphological development of the inner ear saccular epithelium (the main auditory endorgan) and follow changes in the orientation patterns and density of hair cell bundles in the *Halobatrachus didactylus* throughout ontogeny. We also aimed to compare orientation patterns across species of the Batrachoididae family, such as *Opsanus tau*, *Opsanus* beta and *Porichthys notatus.*

Chapter 2. Ontogenetic development of the saccular epithelium in the Lusitanian toadfish: potential implications for auditory sensitivity and insights into the diversity of the Batrachoididae.

2.1 Abstract

The Batrachoididae family has become an important group to study mechanisms and evolution of auditory-vocal functions for social communication. A recent investigation reported ontogenetic changes in saccular auditory sensitivity in the Lusitanian toadfish *Halobatrachus didactylus*, coincident with an increase in the vocal repertoire. The major goals of this study were: 1) to verify ontogenetic changes in hair cell addition and other morphological features of the saccular epithelium during ontogeny; and 2) to compare saccular morphological features among different Batrachoididae species.

Sensory epithelia were removed from the inner ear saccules of previously PFA-fixed specimens of different sizes: posthatched fry (<1.4 cm, SL standard length) to adult stage (<23 cm, SL). Tissue was stained with phalloidin and analysed for epithelium area and shape, number of hair cells (HC), area of supporting cells (SC) and HC orientation patterns.

Saccular epithelium area increased circa 10x throughout development with a decrease and increase in the middle and caudal area ratio respectively. HC number increased about 8x with growth, but the HC density decreased 1.6x throughout the whole epithelium.

The SC density decreased with growth, but its area increased significantly. HC orientation patterns seem to be established in fry, although a few changes were observed in the most variable regions.

We propose that during early postembryonic development the number of HCs increased rapidly in relation to the overall epithelium growth, resulting in decreased HC density. The expansion of the sensory epithelium is mostly due to increased SC area. Interspecific comparisons among Batrachodidae revealed a common dual pattern with slight differences the extremities and the dorsal extension.

Even though increased number of HCs may in part facilitate saccular sensitivity, other morphological features of the inner ear and circulating steroid levels are most likely involved in the increased auditory sensitivity to enhance social acoustic communication during ontogeny.

2.2 Introduction

Fishes comprise the largest group of extant vertebrates displaying the greatest diversity in sensory structures for orientation and hearing in their highly diverse acoustic environments (Braun & Grande, 2008). However, comparative studies addressing relationship between form and function of the auditory system in this taxon are rather sparse compared to other vertebrate groups.

An ontogenetic perspective of the auditory system in fishes provides a readily testable framework for understanding structure-function relationships (Vasconcelos et al, 2015). Besides, studying lower vertebrate models such as fish is important to gain fundamental comparative insights into the evolution and ecology of the vertebrate auditory system, as early developmental events are most likely evolutionary conserved.

There are only a few studies describing ontogenetic changes in the post-embryonic development of the fish inner ear. These studies reported variable results, namely changes in size and shape of the sensory epithelia (Corwin, 1983; Lombarte & Fortuno, 1992; Lanford et al., 2000; Lombarte & Popper, 2004), number and density of hair cells (Popper & Hoxter, 1990; Lombarte & Popper, 1994; Higgs et al., 2001), number of nerves innervating the macula (Corwin, 1983), number of auditory ganglion cells, axon area and number (Barber et al., 1985), otolith size and hair cell orientation patterns (Lombarte & Popper, 1994; Edds-Walton & Popper, 1995).

Although the functional significance of most of the described morphological changes in the fish auditory peripheral system during development is not known, it is likely that changes in sensory morphology have functional implications and lead to sensitivity changes. Such structure-function relationships have been reported in other taxa, such as in mammals (Beutner & Moser, 2001; Jun et al., 2006; Tong et al., 2015), birds (Bartheld, 1994; Rubel & Fritzsch, 2002) and reptiles (Walsh et al., 2009).

The only fish species investigated so far that allowed establishing a developmental association between morphological changes and auditory sensitivity were the ray *Raja clavata* (Rajidae) and the zebrafish *Danio rerio* (Cyprinidae). Corwin (1983) did electrophysiological recording from neurons in the ear and found a 500-fold increase in auditory nerve sensitivity in *R. clavata.* Since hair cell numbers increased with age but no trend was found regarding an increase in the number of axons in the ramus neglectus it was suggested that the increase in

sensitivity might have been associated to the increased convergence ratio of sensory hair cells to auditory afferent neurons (Corwin, 1983).

With the use of AEP and saccular potentials in zebrafish, studies have delivered opposing results, with no changes in hearing sensitivity (Higgs et al., 2001) and a positive correlation between number and density of sensory hair cells respectively (Lu & DeSmidt, 2013).

Representatives of the Batrachoididae family have become important models to study mechanisms and evolution of auditory-vocal functions for social communication. These teleost fishes rely heavily on acoustic communication to mediate social interactions including territorial defence and mate attraction during the breeding season (Amorim et al., 2006; Vasconcelos, 2011; Mclver et al., 2014). For this reason, their auditory system has been focus of attention in several studies to understand how auditory plasticity can enhance social acoustic communication (Sisneros & Bass, 2003, 2005; Vasconcelos & Ladich, 2008; Sisneros, 2009; Alderks & Sisneros, 2011; Coffin et al., 2012). Coffin et al. (2012) also showed that seasonal changes in the saccular auditory sensitivity were correlated with changes in the hair cell density in the midshipman fish *Porichthys notatus*. A threshold shift of about 8–15 dB was observed between non-reproductive and reproductive females within 75 to 385 Hz.

A recent study reported a similar saccular sensitivity change of about 10 dB during ontogenetic development in another batrachoidid, the Lusitanian toadfish *Halobatrachus didactylus.* Auditory thresholds decreased significantly from small (2.4 – 8.7 cm SL) to large juveniles (5.0 – 8.7 cm) and then remained similar to the adult stage (Vasconcelos et al., 2015). Such developmental change in the peripheral auditory sensitivity was coincident with an increase in the vocal repertoire, suggesting a potential coupling between vocal-auditory systems (Vasconcelos et al., 2015). However, whether developmental enhancement in peripheral auditory sensitivity can be explained by morphological changes in the saccular epithelium and number of hair cells receptors has never been investigated.

In this study we used the Lusitanian toadfish to test the hypothesis that changes in auditory sensitivity might be concurrent with developmental modifications of the saccular sensory epithelium during ontogenetic development.

Our major goals were: 1) to investigate: the development of the inner ear saccular epithelium, including potential changes in the saccular epithelium area and shape, number of hair cells and supporting cells, and hair cell orientation patterns; and 2) to compare saccular morphological features among different Batrachoididae species.

2.3 Methods

2.3.1 Fish Collection and Maintenance

In the present study the following size groups of Lusitanian toadfish were considered: “posthatch fry”; “vocal fry”; “juveniles” and “adults”. In order to collect specimens from the three initial stages, we followed a method for egg collection previously described in other studies (Vasconcelos et al., 2011).

Prior to the onset of the breeding season, 60 artificial concrete nests (internal dimensions: 50-cm long, 30-cm wide and 20-cm height) were placed 1.5 m apart along an intertidal area of the Tagus River estuary in Portugal (38º42’N; 8º58’W). These nests, which had a hemicylinder shape and were closed at one end, were provided with a plastic sheet attached to the ceiling, where females usually deposit their eggs. Reproductive toadfish males readily occupied these nests and initiated vocal activity to attract females. During low tides (about 15 days later), when the nests were exposed to air and could be accessed, the plastic sheets containing eggs were removed and immediately placed in coolers containing fresh seawater. A total of 11 plastic sheets containing eggs were collected and transported to the laboratory at the University of Lisbon (Portugal), where they were placed in several stock saltwater tanks (20L) equipped with aeration and filtering systems.

Two plastic sheets containing healthy eggs were selected and suspended vertically underwater apart from each other in a separate 20L tank, allowing proper water flow and aeration of all embryos. Each plastic sheet contained more than one clutch, easily distinguished based on differences in developmental stages. The eggs were monitored daily and any evidence of potential fungal infection was immediately removed, similarly to parental care provided in many fish (Reebs & Colgan, 1991).

When the embryos started hatching becoming free-swimming, 20 individuals were randomly chosen and euthanized for further processing (group “posthatched fry”: 1.3-1.4 cm standard length - SL, 0.09-0.10g total body weight - TW, circa 51 dpf - days post fertilization (Félix et al., 2015).

Another group of 20 randomly chosen fry collected a similar stage were placed in an observation tank. These individuals were observed and recorded during the following 15 days (see next section for details), which was enough time to develop social interactions and vocal behaviour. Once fry started to vocalize and individuals could be clearly identified as sound producers, 15 specimens were collected and, similarly to the previous experimental group, euthanized for further processing (group “vocal fry”: 1.70-2.0 cm SL, 0.19-0.30g TW, 75 dpf).

An additional group of 6 juveniles was collected from the stock tanks about 5 months later after being brought to the laboratory ("juveniles: 2.3-2.9 cm SL, 0.33-0.79 g TW, 230 dpf), euthanized and preserved for later analysis.

Finally, 6 toadfish male adults were obtained by trawling in the Tagus river estuary ("adults": 20-23 cm SL, 205-334g TW) and transported to the laboratory for anaesthesia and perfusion fixation – see next section. Fish were further dissected after fixation perfusion to confirm morphotype (Modesto & Canário, 2002).

All fish developmental stages maintained in the laboratory were kept at 21 ± 2 ºC and under a day: night cycle of 12h: 12h. All fry and juveniles were fed with artemia flakes and small pieces of shellfish.

All specimens used in this study were weighted (total body weight, TW) and their length measured (standard length, SL; and total length, TL).

All experimental procedures conducted in Portugal comply with the local animal welfare laws, guidelines and policies. All specimens transported to the laboratory adapted rapidly to captivity and behaved normally in the stock tanks, suggesting that these animals were not exposed to overly stressful conditions.

2.3.2 Behavioural Observations

To collect fry at the developmental stage of vocal onset, posthatched fry were monitored for visual and acoustic behaviour in a tank (50 cm x 30 cm x 25 cm, 37.5L). The observation contained an appropriate filtering system, and was further equipped with sand substrate and three artificial nests evenly spaced to promote territorial competition and acoustic interactions. To significantly minimize vibrations and improve quality of sound recordings, the tank was placed on top of two marble layers that were interspersed with shock absorbing foam rubber.

Two hydrophones (High Tech 94 SSQ, Gulfport, MS, USA; frequency range: 30·Hz–6·kHz, ±1·dB; voltage sensitivity: –165·dB re. 1·V/µPa) were positioned close to each lateral nest. The hydrophones were connected to an A/D converter device (Edirol UA-25, Roland, Tokyo, Japan; 16bit, 8kHz) connected to a laptop running Cool Edit Pro 2.1 for windows software (Syntrillium Software Corporation, Phoenix, USA). The fish acoustic signals were detected in real time by simultaneously listening to the audio recording and checking sound features. This procedure enabled the identification of sound producers that were subsequently collected.

Behavioural observations of 50 min duration were performed twice a day, every day, during a period of 15 days.

2.3.3 Tissue Collection and Immunocytochemistry

Fry and juveniles were euthanized with an overdose (1.5 g/L) of MS-222 (Acros Organics, Geel, Belgium) in a saltwater bath. The heads were removed and immersed in 4% PFA (paraformaldehyde, 96% extra pure; Acros Organics, Geel, Belgium) overnight, and later stored in 0.1M PBS with 0.05% Sodium azide (Acros Organics, Geel, Belgium).

Adults were deeply anesthetized in MS-222 saltwater bath (1.5g/L), perfused through the heart with Flush solution (1% Lidocaine, 1% heparin, 0,9% NaCl) in 0.1M PB solution until the gills turned white. Specimens were subsequently perfused with 4% PFA in 0.1M PB solution. The heads were removed and stored in 0.1M PBS.

All PFA-fixed samples were sent to the laboratory in Macau, where the immunohistochemistry protocol and tissue analysis were conducted. The samples were shipped under stable cool conditions.

We followed the method described by Coffin et al. (2012) to remove the saccular epithelia. The fish inner ears were accessed from the dorsal part of the head, and the whole structure dissected and removed under the microscope (Zeiss Stemi 2000 CS; Fisher Bioblock Scientific, Pittsburgh, USA). The saccular epithelia were trimmed and separated from the otolith and the nerve bundles attached to the epithelia removed. The epithelia were then rinsed, blocked in 5% goat serum with 0.1% Triton-X (Sigma, Taufkirchen, Germany) in 0.1M PBS, and incubated overnight in anti-PH3 antibody (Ser28; Imgenex, diluted 1:200 in blocking solution; Littleton, USA). The tissue was subsequently rinsed thoroughly with 0.1M PBS and incubated in a secondary antibody (Alexa Fluor 568 goat-anti-mouse, diluted 1:500; Invitrogen, TermoFisher Scientific, Waltham, USA) diluted in 0.1M PBS with 0.1% Triton X. The tissue was finally counter stained with phalloidin (Alexa Fluor 488 phalloidin, diluted 1:100; Invitrogen, TermoFisher Scientific, Waltham, USA) diluted in 0.1M PBS, which labels actin and successfully allows the visualization of HCs and SC. All epithelia were mounted whole with Fluoromount-G (Southern Biotech, Birmingham, USA) and cover slipped.

2.3.4 Fluorescent imaging and morphological analysis

Samples were observed under a microscope Leica STP 6000 (DM6000B, Leica Microsystems CMS GmbH; Buffalo Grove, USA) equipped for epifluorescence and connected to a DM6000 camera. The saccular epithelia total area was determined at 200x (for fry and juveniles) and 100x (adults) magnification. Specific regions within the epithelia (rostral, middle and caudal) were also determined at the same magnification and based on changes in shape and orientation patterns of hair cells – see Fig 1. This analysis was performed with Stereo Investigator software (10.53.1/MBF Bioscience MicroBrightfield Inc.; Williston, USA). Moreover, the length and width of each of the epithelial regions was also measured.

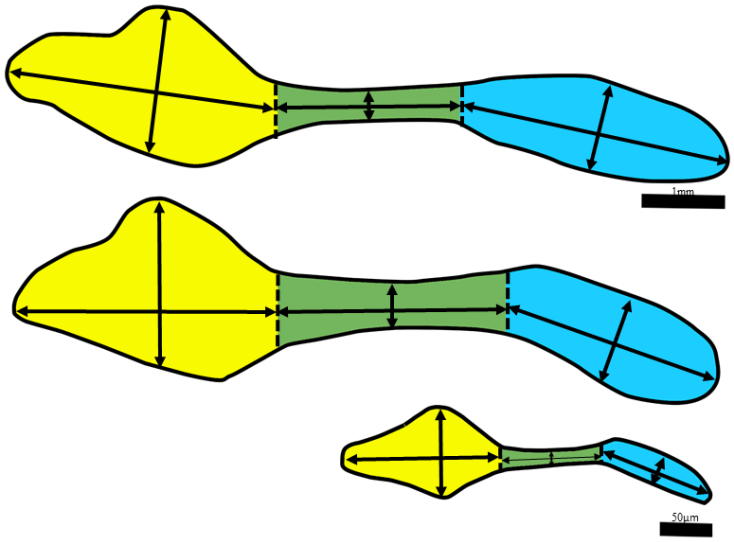


Figure 1. Sensory saccular epithelium from adult (20-23cm SL) to posthatched fry (1.3-1.4 cm SL) divided in three main areas separated by the dashed line: Rostral, Middle and Caudal (From left to right). The arrows represent the length and width of each area. The Rostral width was determined from a 90º angle starting from the dorsal extension, and the Caudal and Middle area's width, determined from the middle with also a 90º angle.

The epithelium shape, number of hair cell (HC) bundles and HC orientation patterns were determined at 400x using either Stereo Investigator software or Leica Application Suite (3.10 build 8587). In order to determine a representative epithelium shape for each size group, an averaged outline was created by superimposing the various shapes using Adobe Photoshop (Adobe Systems Incorporated; San Jose, USA).

As a mean to obtain an overall description and representative quantification of the HC number/density throughout the saccular epithelia, six different non-overlapping square regions (900 μm^2, 4900 μm^2 and 10000 μm^2 for fry, juveniles and adults, respectively) were defined

based on Coffin et al. (2012). All HC bundles that were within or overlapping the outline of the square regions were considered in the HC counts. The square regions were easily identifiable in all samples analysed and enabled a comparable measure of HC density across the different size groups – Fig 2.

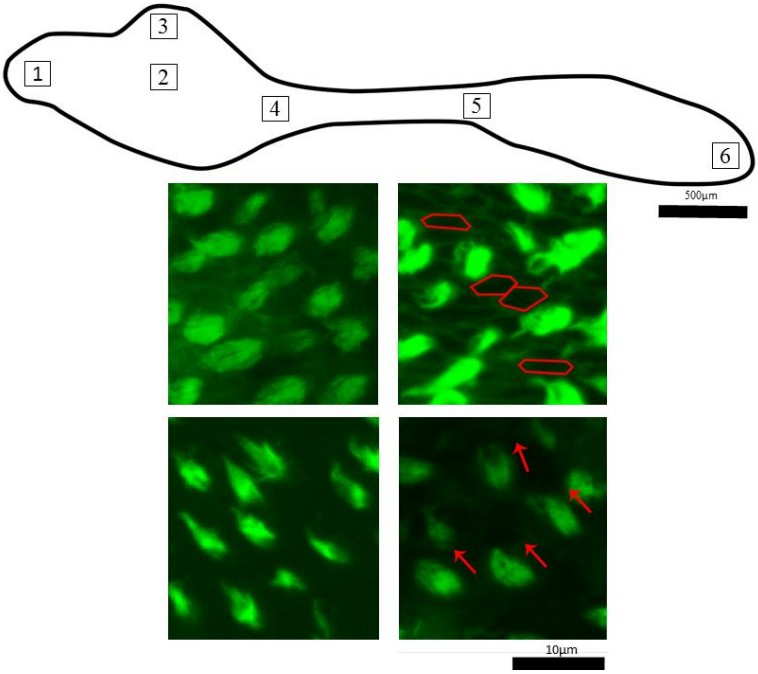


Figure 2. Determination of hair cell density, orientation and supporting cell area. (A) Sensory saccular epithelium of an adult (20-23 cm SL) with the selected areas for hair cell counts. (B) Saccular epithelium regions with higher density of hair cells for posthatched fry (top, 1.3-1.4 cm SL) and lower density for adults (bottom, 20-23 cm SL). (C) Supporting cell area measurements in adults (bottom). (D) Hair cell orientation determined by the direction of the kinocilium in relation to the stereocilia.

The SCs were generally more difficult to visualize and were only quantified in one region within the caudal epithelium near area 6 (see Fig. 2) and within smaller counting squares: 900 μm^2, 2500 μm^2 and 4500 μm^2 for fry, juveniles and adults, respectively. The SC area was also determined using Leica Application Suite software at 400x (see Fig 2). For comparison purposes with SC density, HCs was also counted in the same caudal region. It is important to note that we only had interest in analysing the apical projection of the supporting cells, since they were visible in the same layer as the hair cells.

The HC orientation patterns were defined based on the direction of polarization from the shortest stereocilia to the kinocilium, i.e. direction of depolarization (Popper, 1981). Although phalloidin does not label the kinocilium, its position appears as a dark black hole at the level of the cuticular plate (Lu & Popper, 1998; Coffin et al., 2012) - see Fig. 2.

The orientation patterns were defined based on general trends observed throughout the epithelium. The cilliary bundles may often flop over during preparation, which makes the determination of kinocillium and cell orientation difficult. The orientation patterns presented in this study were based on observation of HCs that could be clearly identified regarding the position of the kinocillium. In order to create a representative HC orientation map for each size group, the patterns from various samples were compared and an overall representation map determined based on consistent patterns using Adobe Photoshop.

2.3.4 Statistical Analyses

The total area of the saccular epithelia was determined and compared across different size groups with Kruskall-Wallis tests, followed by pairwise comparison post hoc tests to verify group specific differences. The ratios of area, length and width of each region (rostral, middle, caudal) within the saccular epithelium were compared across different size groups with One-way Anova, followed by pairwise comparison post hoc tests to verify ontogenetic changes in the saccular shape.

HC density was compared between different locations within the sensory epithelium with Kruskall-Wallis followed by pairwise comparison post hoc tests to verify location specific differences. An averaged value for HC density was then calculated per epithelium. The averaged HC density was compared across different size groups with Kruskall-Wallis and pairwise comparison post hoc tests.

SC density and area were determined and compared across different size groups with One-way Anova followed by pairwise comparison post hoc tests to verify group specific differences.

The relation of fish length (SL) with saccular epithelia area and HC density was determined with Pearson correlation tests, either considering all test groups or only smaller specimens.

Parametric tests were only used when data were normally distributed and variances were homogeneous. All data analysis was performed using IBM SPSS v22 (IBM, USA).

2.4 Results

2.4.1 Development of Size and Shape of the Saccular Epithelium

The saccular sensory epithelium increased circa 10x in area, from posthatched fry ($\overline{x}$ = 48637,25 μm²; sd= 11588,48) to adults ($\overline{x}$ = 2273711 μm²; sd= 413364). A correlation between area and fish length was found when only considering early developmental stages (r=0.944; p<0.01, n=34) – see Fig. 3A. Moreover, a comparison between different size groups also revealed significant differences regarding epithelia area (KW: H= -9.00-25.50; d.f.=3, p<0.01) – see Fig. 3B.

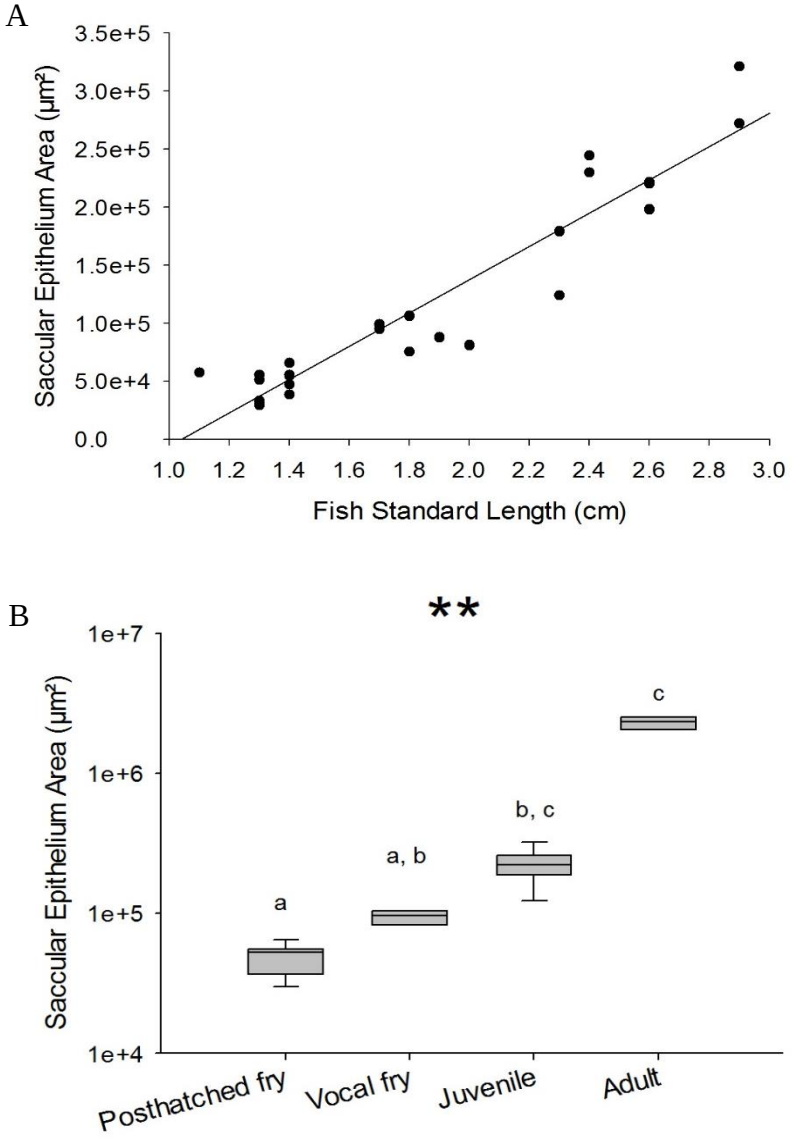
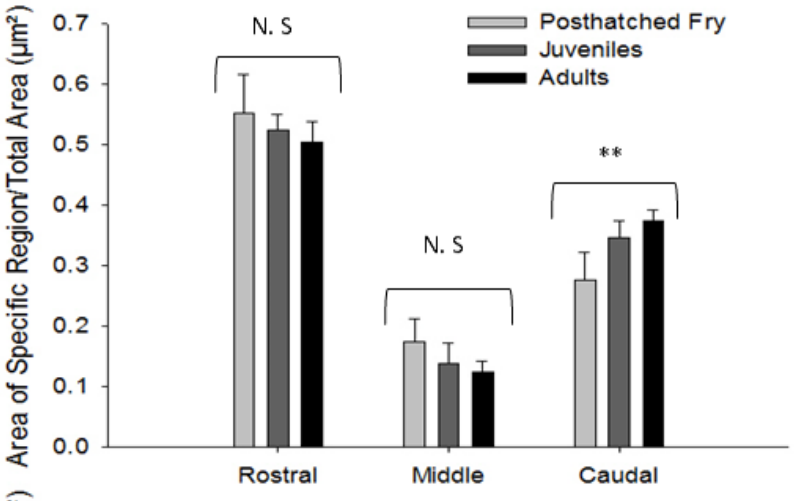


Figure 3. Ontogenetic changes in the area of the inner ear saccular epithelium in the Lusitanian toadfish *H. didactylus.* (A) Correlation between epithelium area and fish within early developmental stages. Pearson correlation, ** P<0.01. Regression equation: Saccular epithelium area=149518+143639xfish standard length. (B) Comparison of the epithelium area between different size groups. Differences are based in Kruskal-Wallis tests, followed by pairwise comparison post-hoc tests to verify group-specific differences. ** P<0.01. Data are medians ±10th, 25th, 75th and 90th percentiles. Different letters depict significant pairwise differences given by Kruskal-Wallis post-hoc tests. Groups: posthatched fry (1.3-1.4 cm SL); vocal fry (1.7-2.0cm SL); juveniles (2.3-2.6 cm SL); adults (20-23).

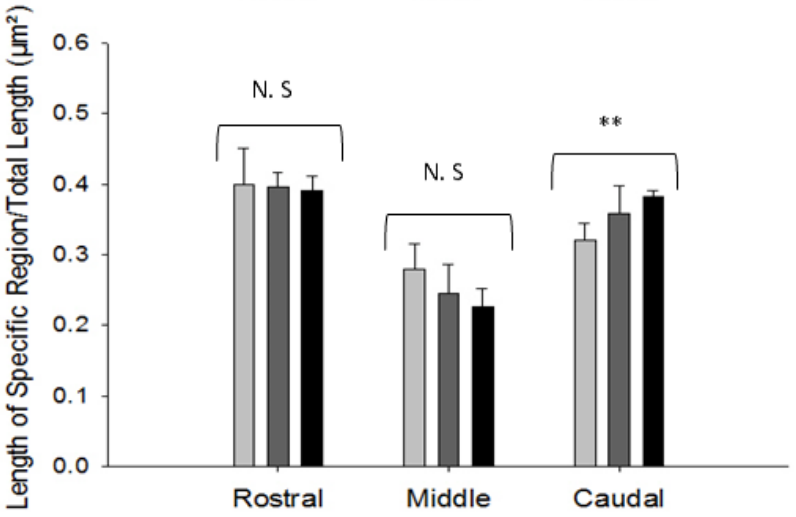
The saccular epithelium maintained a similar general shape throughout ontogenetic development. However, a few differences were observed. Early fry showed thinner rostral and caudal tips along with a thinner and markedly longer middle region. As the fish grew, the middle region became shorter and wider and Area 3 becomes more salient. The caudal region quantitatively showed significant changes, since the area of the saccule that it occupies increased with growth - See Fig. 1.

The saccular epithelium shape changed during ontogeny, namely the caudal region of the epithelium became larger during development. The area showed significant differences for the caudal region between Posthatched fry and Juveniles, and Posthatched fry and Adults ($F_{(2,12)}$= 9.53; $p<0.01$) – See Fig. 4A. With length the same tendency was registered, with significant differences for the Caudal region ($F_{(2,13)}$= 4.88; $p>0.01$) – See Fig. 4B. No significant differences were found for width ($F_{(2,13)}$= 0.35; $p<0.01$) – See Fig. 4C.

A


Posthatched Fry
Juveniles
Adults
Area of Specific Region/Total Area (μm²)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
N. S
N. S
**
Rostral
Middle
Caudal

B


Length of Specific Region/Total Length (μm²)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
N. S
N. S
**
Rostral
Middle
Caudal

C

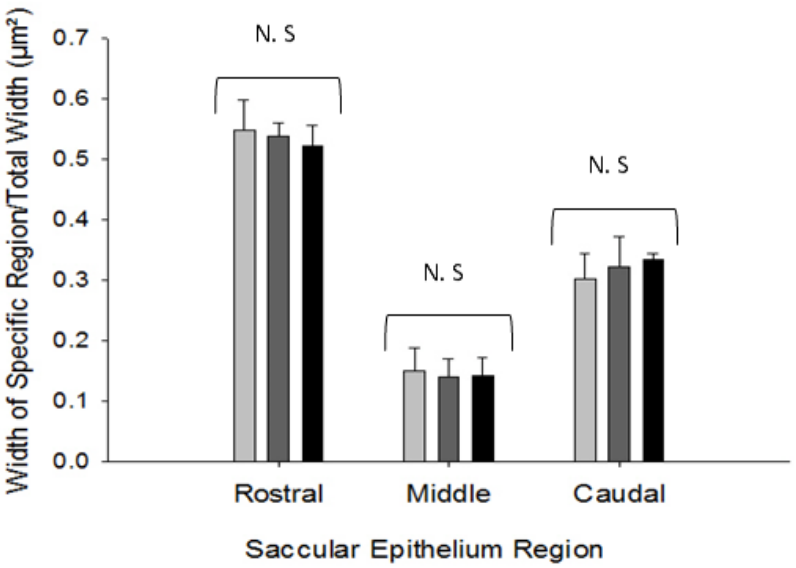

Width of Specific Region/Total Width (μm²)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
N. S
N. S
N. S
Rostral
Middle
Caudal
Saccular Epithelium Region

Figure 4. Growth variation of different saccular epithelium regions (rostral, middle, caudal) in relation to different size parameters, compared across different size groups of the Lusitanian toadfish *H. didactylus*. (A) Area over whole epithelium area for different regions. (B) Length over whole epithelium length for different regions. (C) Width over whole epithelium width for different regions. Differences are based in One-way Anova tests, followed by LSD comparison post-hoc test to verify group-specific differences. ** P<0.01; N.S no significant differences. Bars represent means ± standard deviation. Groups: posthatched fry (1.3-1.4 cm SL); vocal fry (1.7-2.0cm SL); juveniles (2.3-2.6 cm SL); adults (20-23 SL).

2.4.2 Variation in Orientation Patterns of Hair Cell Bundles during Development

The orientation patterns of the hair cell bundles in the saccular epithelium varied throughout development – see Fig. 5. The common patterns across different size groups were a patch of hair cells oriented towards area 3 (dorsal extension in the rostral region), the middle thinner region with a dorsal-ventral opposing pattern that extends to the caudal region and caudal oriented hair cells in the extremity of the caudal region.

In the adults, the orientation patterns were more complex, with the hair cell bundles changing from the rostral to the caudal region, including all possible orientations along the epithelium. In the rostral region (anterior wider area – Fig. 1), there were four main patterns: rostral, caudal, dorsal and ventrally oriented hair cell bundles. The rostral region was the most variable regarding bundle orientation patterns in inter-individual variation. Specifically, in adults, when moving caudally from the rostral tip, the orientation showed particular patterns. For example, in the dorsal extension there seems to be a curved pattern descending to the middle of the rostral tip, and a clear dorsal orientation following the outline of the lower bound in this area. In juveniles and posthatched fry we did not observe caudally oriented hair cells in the rostral area.

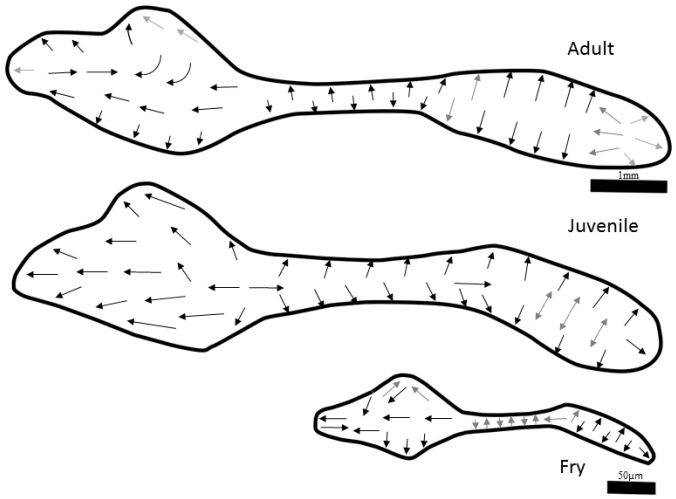


Figure 5. Representative patterns of hair cell bundles across different size groups of the Lusitanian toadfish *H. didactylus*. Only consistent hair cell patterns were considered. Grey arrows indicate orientation patterns that were less consistent. Epithelium shape is an average of individual outlines. Epithelium size was obtained from averages of different size groups epithelia: posthatched fry (1.3-1.4 cm SL); juveniles (2.3-2.6 cm SL); adults (20-23 SL).

2.4.3 Variation in Hair Cell Density during Development

Hair cell bundle density quantified across 6 non overlapping regions – see Fig. 2, decreased approximately 1,6x times from posthatched fry ($\bar{x}$ = 0,02093 μm²; sd= 0,002061) fry to adults ($\bar{x}$ = 0,01316 μm²; sd= 0,000893). The hair cell bundle density varied significantly across the epithelium for all different size groups (KW: H= 13.45-23.01; d.f.=3; p<0.01) – see Fig. 6. The vocal onset group was included to explore possible changes from non-vocal post hatching fry to vocal fry. No changes in density were found between these earlier stages of development. The epithelial region that revealed the highest developmental changes was the A2 (P=0.00).

A negative correlation between hair cell density and fish length was found among animals from early developmental stages (r=-0.73; p<0.01, n=34) - see Fig. 7A. The mean hair cell bundle density (of all 6 areas) decreased significantly between different size groups (KW: H=-26.67-2.33; d.f.=3, p<0.01) - see Fig. 7B. Posthatched fry and vocal onset fry were statistically different from juveniles (p<0.05) and adults (p=<0.01).

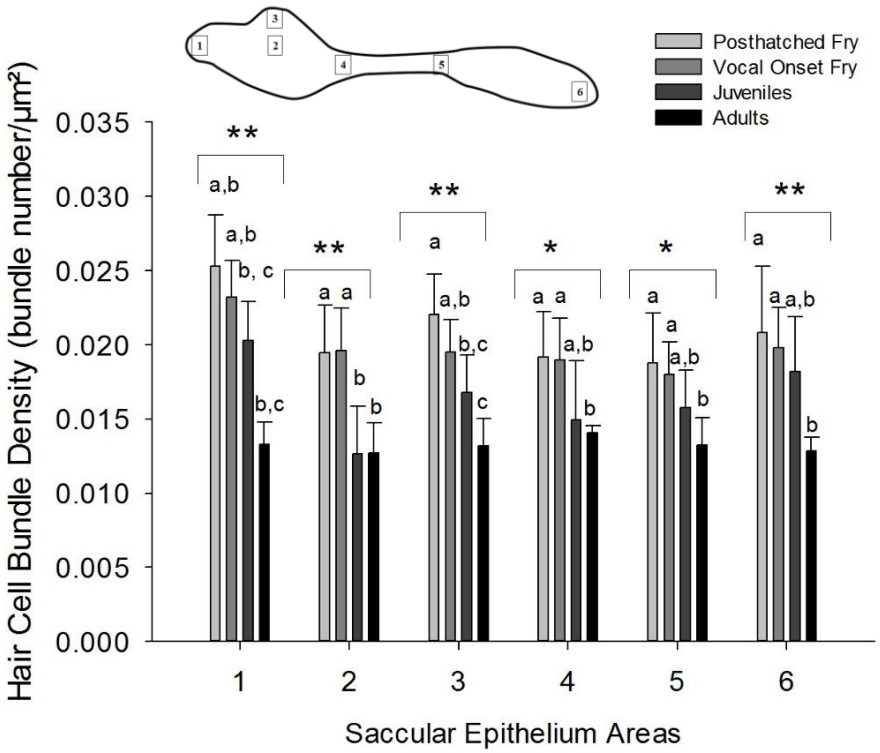


Figure 6. Variation in hair cell density for different epithelium regions across different size Lusitanian toadifish *H. didactylus.* Differences are based in Kruskal-Wallis tests, followed by pairwise comparison post-hoc tests to verify group-specific differences. * P<0,05; ** P<0.01. Bars represent means ± standard deviation and different letters depict significant pairwise differences given by Kruskal-Wallis post-hoc tests. Groups: posthatched Fry (1.3-1.4 cm SL); vocal fry (1.7-2.0cm SL); juveniles (2.3-2.6 cm SL); adults (20-23 SL).

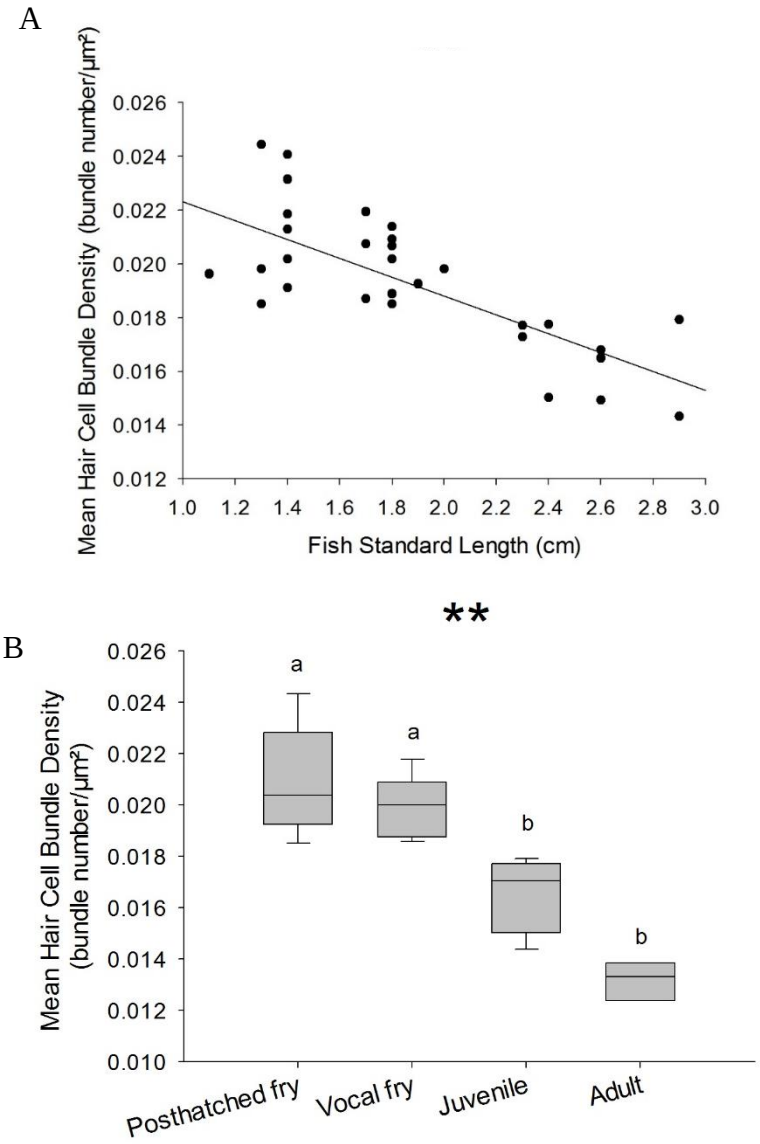


Figure 7. Differences in mean hair cell bundle density during ontogeny in the Lusitanian toadfish *H. didactylus*. (A) Correlation between the epithelium area and fish standard length (SL) from Post-hatched to Juveniles. ** P<0.01. Regression Line: Mean Hair Cell Bundle Density =0.026+0.004xfish standard length B) Comparison of the mean hair cell bundle density between different size groups. Differences are based in Kruskal-Wallis tests, followed by pairwise comparison post-hoc tests to verify group-specific differences. ** P<0.01. Data are medians ±10th, 25th, 75th and 90th percentiles. Different letters depict significant pairwise differences given by Kruskal-Wallis post-hoc tests Groups: posthatched fry (1.3-1.4 cm SL); vocal fry (1.7-2.0cm SL); juveniles (2.3-2.6 cm SL); adults (20-23 cm SL).

2.4.4 Variation in Supporting Cell Area and Density

We aimed to look at supporting cells and how their apical projections change during development to further understand how hair cell density changes are related to other ontogenetic morphologic changes in the sensory epithelium. Supporting cell density followed the same trend as hair cell density, with significant differences resulting in a decrease from posthatched fry to adults ($F_{(2,7)}$= 4.96; p<0,05) – see Fig. 8A. However, for supporting cell area there was a significant increase with development. A significant difference was found between groups ($F_{(2,82)}$= 13,72; p<0,01) with a clear increase from posthatched fry to juveniles and juveniles to adults – see Fig. 8B.

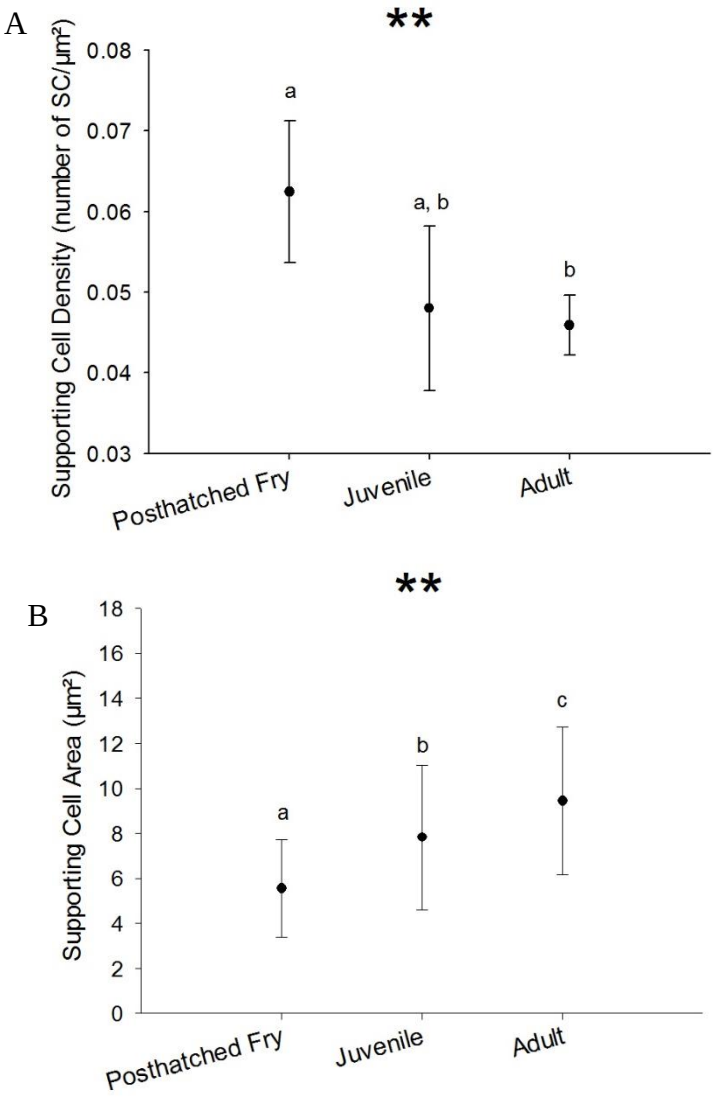


Figure 8. Differences in supporting cell density and area during the ontogeny in the Lusitanian Toadfish *H. didactylus*. (A) Comparison of supporting cell density between different size groups. (B) Comparison of supporting cell area between different size groups. Differences are based in One- way Anova tests, followed by LSD comparison post-hoc test to verify group-specific differences. * P<0,05; ** P<0.01. Bars represent means ± standard deviation. Different letters depict significant pairwise differences given LSD comparison post-hoc test. Groups: posthatched (1.3-1.4 cm SL) fry; juveniles (2.3-2.6 cm SL); adults (20-23 cm SL).

2.4.5 Comparative Approach: Batrachoididae

The saccular epithelium of the different Batrachoididae members analysed so far, revealed common features regarding shape and hair cell orientation patterns when compared with existing Batrachoididae data. Considering shape, *P. notatus* and *O. beta* seem to possess a bigger rostral region in relation to the whole saccular epithelium (Popper & Hoxter, 1981; Coffin et al., 2012), whereas in the *H. didactylus* and *O. tau* the rostral region seems to occupy an equivalent space in the tissue (Edds-Walton & Popper, 1995). The caudal region is more salient in the latter species whereas in *O. beta* and *P. notatus* the middle region seems to extend to the end of the saccule.

Regarding orientation patterns, the four species possess all possible orientations along the saccule length, a parallel opposition of caudal and rostral oriented hair cells in the rostral tip, a dorsal extension in the rostral tip (highly variable region) except in *O. beta*, and a dorsal-ventral opposing pattern in the middle thinner region. In all species the middle region opposing pattern seems to extend to the caudal region, shifting later in the tip to a caudal and/or rostral oriented pattern. In *H. didactylus* the dorsal extension in the rostral region and the caudal region are presented with grey arrows due to the difficulty in accessing a sufficient number of hair cells with clear orientation in those areas.

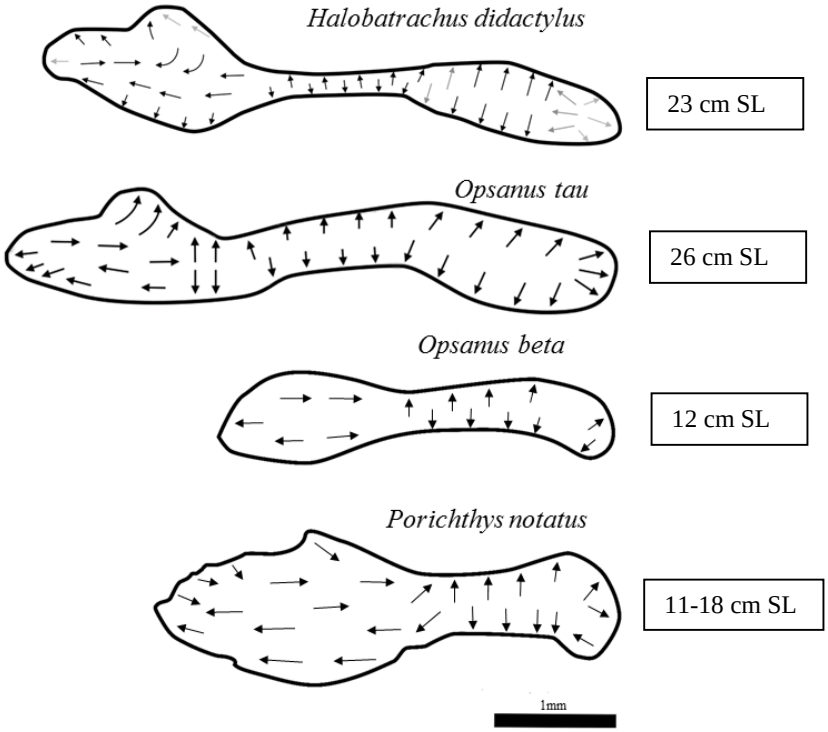


Figure 9. Representative hair cell orientation patterns across four Batrachoididae species: *Halobatrachus didactylus*, *Opsanus tau* (based on Edds-Walton & Popper, 1995), *Opsanus beta* (based on Popper, 1981), and *Porichthys notatus* (based on Coffin et al., 2012). Data are from adult males from each species. Grey arrows indicate orientation patterns that were less consistent.

2.4 Discussion

This study aimed to verify developmental changes in the main auditory endorgan (saccule) that may account for ontogenetic auditory improvements in *H. didactylus*. We verified that the main HC orientation patterns seem to be established early in the larval phase, and that throughout development epithelium area expands up to 10x. HCs increased rapidly in relation to the epithelium growth, resulting in decreased HC density, and the expansion of the sensory epithelium was mostly due to increased SC apical area.

Besides, interspecific comparisons among Batrachodidae species revealed a common dual pattern with some differences that may explain different auditory sensitivities.

To our knowledge, this is one of the few studies investigating the ontogenetic development of the structure and function of the saccule, i.e. how saccular epithelium develops and its relationship with auditory sensitivity. Besides, by focusing on a strongly vocal species that exhibits vocal differentiation, this study is the first attempt to investigate structural changes in the saccular sensory epithelium potentially associated with auditory enhancement for social acoustic communication during ontogenetic growth.

2.5.1 Development of size and shape of the saccular epithelium

The saccule is the largest of the three otolithic sensory epithelia in *H. didactylus* and considered the main auditory endorgan in fishes (Collin, 2003).

The trend of growth of the saccular sensory epithelia throughout development shown in this study is consistent with most studies on fish species that have reported that both the otoliths and sensory epithelia grow continuously throughout fish lifespan (Corwin, 1983; Popper & Hoxter, 1990, 1984; Lombarte & Popper, 1994; Webb et al., 2012). We reported a 10x increment in saccular epithelium area between posthatched fry (<1.4 cm, SL standard length) to adult stage (<23 cm, SL), whereas in *Merlucius merlucius* (Merluciidae) (10-75 cm SL) the epithelia area increased ± 50x (Lombarte & Popper, 1994), five times more than what we register for *H. didactylus*.

The implications of increasing saccular epithelium area with fish growth are not well understood. Previous studies pointed out that the growth of sensory epithelium and the parallel increase in the number of hair cells may help maintaining a stable hearing sensitivity as fish grows (Popper et al., 1988). If this general growth implies an increment in hair cell addition, it may also contribute for enhancement of saccular auditory sensitivity. However, since this is a common developmental morphological change and several fish species do not show obvious saccular sensitivity changes, e.g. the Batrachoidid midshipman fish *Porichthys notatus* (Alderks & Sisneros, 2011), it remains unclear the contribution of such morphological feature for fish auditory abilities. Further studies should focus on comparing saccular epithelium growing rates and saccular sensitivity changes from different fish species within similar size ranges.

Besides changes in total area, our data also indicates ontogenetic changes in saccular epithelium shape, namely the caudal region area increases in relation to the rest of the epithelium. In *M. merlucius* (Merluciidae) and *Opsanus tau* (Batrachoididae) the rostral and caudal areas also seem to differ in their growth rate, with the caudal area growing significantly

more (Lombarte & Popper, 1994; Edds-Walton & Popper, 1995). However, the significant differences in growth of both rostral and caudal areas in *M. merlucius* were just observed in adults (above 30 cm SL), whereas in *H. didactylus* such changes in shape (caudal area enlargement) were verified gradually from earlier stages (1.3 – 2.6 cm SL) to adulthood. In the Batrachoidid *O. tau*, Edds-Walton & Popper (1995) found similar enlargement in the caudal area of the saccular epithelium. However, this was not quantified and the observation was only made based on a single representative specimen per size group (14.5-24 cm SL).

The functional significance of an increased growth of the caudal region in relation to the rest of the sensory epithelium during ontogenetic development is not clear. An increment in this region can certainly affect the directional hearing sensitivity if the directional patterns of the hair cells are slightly different (Sisneros & Rogers, 2015). In the Batrachoidids, the contribution of the swimbladder for hearing is not known, but we can suggest that the increase in the caudal region of the inner ear saccule sensory epithelium could serve as an adaptation to shorten such distance, enhancing auditory sensitivity (Popper et al., 1988; Lombarte & Popper, 1994).

2.5.2 Variation in orientation patterns of hair cell bundles during development

Previous studies have shown that sensory hair cells in each epithelium are organized into different orientation patterns (Popper & Coombs, 1982), providing different patterns of activation according to the direction of acoustic stimulation (Saidel et al., 1990; Chang et al., 1992; Sisneros & Rogers, 2015) . Although the functional consequences of different hair cell orientation patterns is not fully understood, the ability of fish to accurately perceive sounds also relies on the ability to detect the direction of sound propagation, and the polarization of hair cells are then essential for processing of such acoustic information (Popper, 1976; Schuijf & Buwalda, 1980; Edds-Walton & Popper, 1995). The number and directionality of these cells in the saccule, varies greatly across species, with different orientation patterns being observed in species that possess differing auditory sensitivities and directional hearing (Coffin et al., 2012).

Popper (1981) placed saccular organization of teleost into 5 categories: standard, dual, opposing, vertical and alternating pattern (Popper, 1981). Four of the patterns exhibit horizontal and vertical orientation groups (standard, dual opposing and alternating), and the fitth patern type is characterized by vertical orientation groups only (Popper & Coombs, 1982). The standard consists of four quadrants, two in posterior end of the epithelium with vertically ciliary bundles dorsally or ventral oriented, and two in the rostral portion of the epithelium with

horizontal bundles either rostral or caudal oriented. The dual pattern is the standard pattern with added horizontal groups in the caudal tip of the epithelium. The alternating pattern shows the same posterior region found in the standard but its rostral region is divided in 3 sections of horizontal bundles with alternating rostral and caudal directions. The opposing pattern has two groups of vertical bundles on the posterior portion of the epithelium, oriented dorsally and ventrally, and two horizontal groups in the rostral region, that is bended down, making the two groups directly opposite to one another. The vertical pattern has only two groups of vertical bundles oriented dorsally and ventrally (Popper & Coombs, 1982).

Our data showed that *H. didactylus* exhibits a dual pattern, which can also be found in the other members of the Batrachoididae family studied so far (Coffin et al., 2012; Maruska & Mensinger, 2015). Such dual pattern is commonly found among various fish families, such as Gobidae (Popper, 1981), Cichilidae (Dehadrai, 1959; Mirbach et al., 2012; Schulz-Mirbach et al., 2013; Schulz-Mirbach et al., 2014) and Labridae (Retzius, 1881; Popper, 1981).

Few studies have approached ontogeny of hair cell patterns. In *Ictalurus nebulosus* (Ictaluridae) (size ranged analysed: 5-30 cm SL), *Clarius batrachus* (Claridae) (10-20 cm SL) and *M. merlucius* (10-75 cm SL) there are no differences registered in hair cell patterns with development (Jenkins, 1979a, 1979b; Lombarte & Popper, 1994). However, similar to our study, the Batrachoidid *O. tau* revealed some differences, namely a shift from a standard to a dual pattern, from 14 week posthatched to the adult stage (Jenkins, 1979a, 1979b; Lombarte & Popper, 1994; Edds-Walton & Popper, 1995). We propose that the first studies on this topic may not have shown orientation pattern differences due to either advanced age of specimens or short size range considered.

The notion that deflection of the hair cell bundle towards the longest cilium, along the axis to which the hair cell bundle is intrinsically tuned (by its geometry), leads to hair cell depolarization (Sisneros & Rogers, 2015), it is interesting due to the data we have showing a more complex hair cell orientation pattern in adults, especially in the rostral region of the saccule. The increasing differing axis of depolarization may allow larger toadfish to better perceive acoustic signal directional information.

Our results suggest that the ability of *H. didactylus* to perceive sound direction is enhanced throughout development as more complex hair cell orientation patterns appear and all possible directions are observed in adulthood. Future studies should investigate the directional hearing sensitivity of this species within different size/developmental groups, and compare it with the present data on saccular epithelium structure.

2.5.3. Variation in hair cell density

Changes in hair cell density are known to contribute for enhanced saccular sensitivity in fish, as shown in the Batrachoidid *P. notatus* (Coffin et al., 2012). Coffin et al (2012) reported a seasonal increase in hair cell density that paralleled an improvement in saccular sensitivity, enhancing social acoustic communication during the breeding season. Although this study was conducted in adults and changes were associated with seasonal patterns, such findings led us to hypothesise that a similar structural change in the saccular epithelium could be related with the ontogenetic increase in saccular sensitivity previously reported (Vasconcelos et al., 2015).

Our data showed that hair cell density decreased during development in *H. didactylus*. A similar finding has been previously reported for other non-related fish species such as *A. ocellatus* and *M. merlucius* (Popper & Hoxter, 1984; Lombarte & Popper, 1994;). In *M. merlucius* after fish reached 16 cm TL, the change in hair cell density slowed exponentially (Lombarte & Popper, 1994). In the zebrafish *D. rerio*, hair cell density remained stable with development (3-18 months old), with a linear increase in hair cell number registered in the first week of posthatched growth (Higgs et al., 2001).

We suggest that during early postembryonic development the number of hair cells increases rapidly in relation to the overall sensory epithelium growth/expansion. Later on during development, hair cell addition rate decreases, although the auditory sensory epithelia keeps growing and expanding. Altogether, this causes an ontogenetic decrease in hair cell density.

Our results showed that the ontogenetic enhancement of saccular auditory sensitivity is probably not due to changes in hair cell density. The considerable increment in saccular epithelial area during development and other structural and molecular features of the saccule are probably involved.

2.5.4. Supporting cell area and density

Supporting cells, unlike hair cells, span the entire depth of the epithelia from basal lamina to the lumen (Wan et al., 2013). They have various functions but mainly maintain the structural integrity of sensory organs during sound stimulation and head movements (Wan et al., 2013). Studies have shown that hair cells can regenerate from mitotic and proliferating supporting cells (Presson et al., 1995).

In fish, more specifically in the *Astronotus ocellatus* (Cichlidae) and *Carassius auratus* (Cyprinidae) inner ear saccule, supporting cells can enter mitotic S-phase and become hair cell precursors (Presson et al., 1995; Presson et al., 1996). Furthermore, it has been suggested that teleost may even have a population of supporting cells that undergo continual renewal and proliferation even without receiving signals from other supporting cells. How this may affect hair cell production in teleost is still unclear (Monroe et al., 2015).

Our results showed a gradual ontogenetic increase in the apical surface area of these cells. This study provides first evidence of such structural change in the saccular epithelium in fish. Future studies should focus on a comparison between the different auditory endorgans to evaluate whether such change is specific from the saccule. Besides, more information on the functional role of the supporting cells in fish is necessary in order to understand its potential impact on hearing sensitivity.

2.5.5 Comparative Approach: Batrachoididae

In this study we also compared saccular hair cell orientation patterns found in adults *H. didactylus* with other species from the Batrachoididae family.

The dual pattern seems to be common among the Batrachoididae, namely in the *O. tau, O. Beta and P. notatus* (Popper, 1981; Edds-Walton & Popper, 1995; Coffin et al., 2012), and was also found in our study species. More specifically, the hair cell orientation patterns of the middle and caudal regions seem to be similar in all Batrachoidids studied so far.

The rostral region of the saccular epithelium seems to be more variable within the family, but is still possible to identify an antiparallel anterio-posterior orientation pattern in all species.

In terms of shape, O. tau and O. beta seem to share a more identical saccular epithelium shape compared to *H. didatylus*, with a longer middle region and larger caudal region. In contrast, *P. notatus* saccular epithelium shape exhibits a rather large rostral region and smaller middle and caudal areas.

Phylogenetically, *H. didactylus* represents a basal lineage among Batrachoididae (Rice & Bass, 2009), and it is probably more closely related to *O. tau* and *O. beta* than to *P. notatus*. Such statement is also supported by the life history and ecological information of these species (Rice & Bass, 2009).

Regarding saccular auditory sensitivity, previous investigations showed about 10 dB difference within the best frequency range between *H. didactylus* and *P. notatus* (Sisneros, 2007; Vasconcelos et al., 2015). If we consider that *H. didatylus* has a larger caudal region with

opposing dorsal-ventral hair cell orientation patterns and *P. notatus* a larger rostral region with anterior-posterior hair cell patterns, this could possibly explain the difference in saccular auditory sensitivity reported for these two species. Both species were recorded in the same lab, using the same criteria for auditory threshold determination, and were both stimulated acoustically mostly in the dorsal-ventral axis (see details in Sisneros, 2007; Vasconcelos et al., 2015). Based on the aforementioned, on the dynamics of hair cell stimulation (Sisneros & Rogers, 2015), and the results reported here, it is clear why such saccular sensitivity differences were described.

Future work should focus on determining saccular sensitivity in other Batrachoididae species, such as *O. tau* and *O. beta*. Besides, other morfological features of the inner ear should also be investigated, such as otolith shape and density, and compared across species. Finally, the potential contribution of the swimbladder to auditory sensitivity, specially at lower frequencies, should be analysed and compared interspecifically.

Chapter 3. Final Considerations

When following the developmental ontogenetic changes of the inner ear saccular epithelium from posthatched fry (<1.4 cm SL) to adults (<23 cm SL) in H. didactylus, our results showed a 10x increase of saccular epithelium area, due to the addition of HC and increasing SC area, and significant changes in its shape (i.e. caudal region ratio). HC orientation patterns in this species seem to be established early in postembryonic development, with a few variations mostly in the rostral and caudal regions. HC addition increased rapidly in relation to epithelium growth, resulting in a decrease in HC density in the later stages of development. Supporting cell (SC) density also decreased with development. Interspecific comparisons within Batrachodidae revealed a common "dual pattern", but also differences in the rostral and caudal regions that may explain different auditory sensitivities.

Complementary studies with this species should be done to investigate sensitivity according to directional hearing in developmental/size groups as we proposed, to further compare it to the present data on saccular epithelium structure. Not only that but potential contribution of the swimbladder to auditory sensitivity, specially at lower frequencies, should be analysed and compared interspecifically.

Other morphologic features of the inner ear, such as otolith shape and density, small hair cell bundles, hair cell bundle length/area, and physiological characteristics such as estrogen receptors and the effect of steroid hormones in ion channels that provide information on morphological changes in the hair cells and hair cell tuning respectively should be explored in the future.

More information on the functional role of the supporting cells in fish is necessary in order to understand its potential impact on hearing sensitivity along comparison between the different auditory endorgans to evaluate whether the changes on supporting cell area and density are specific from the saccule.

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Attachments

Introduction

1. The Sensory World of Fishes

Fishes represent the largest existing group of vertebrates, showing an exceptional diversity of structure and function of sensory systems. Throughout the evolutionary process, species have found ways to become more adapted and to obtain more information about their highly diverse aquatic habitats (Moyle & Cech, 2004) – see Fig. 10. Fishes rely on various sensory modalities, namely chemoreception (smell and taste), vision, electroreception, and mechanoreception (equilibrium/balance, hearing and lateral-line detection of water movements) (Moyle & Cech, 2004). Altogether, the information provided from the various senses is crucial for orientation, feeding, mating, social activities and ultimately survival.

Depending on the evolutionary history and ecological features of their habitats, fishes may rely differently on the various sensory systems. For example, species inhabiting murky waters where visual information is impaired may develop more their auditory and/or olfactory sensitivity such as in the Mexican blind cavefish (e.g. Jeffery, 2005; Plath et al., 2007). On the other hand, fishes that have been subject to rapid speciation in clear water habitats may rely more on visual information for social communication like numerous cichlids from Lake Malawi and Lake Victoria (e.g. Hofmann et al., 2009).

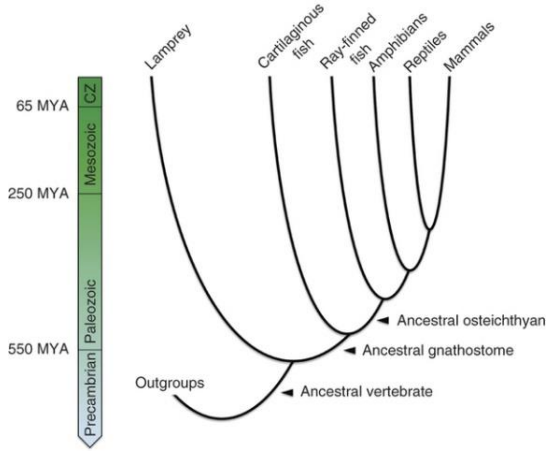

Figure 10. Timing of major radiation events within the vertebrate lineage. Cz, Cenozoic; MYA, million years ago (Smith et al., 2013).

Chemoreception is the most ancient sensory system, having evolved 500 million years ago. Fish detect water borne chemical stimuli through at least two different sensory channels,

olfaction and gustation (taste), which act at both distant and close ranges (Kleerekoper, 1969; Bardach & Villars, 1974; Hara, 1986; Noakes & Godin, 1988). Smell in teleosts is well developed, and odours such as amino acid, steroids and bile salts contribute for localization of food, detection of predators, mating and other interspecific communication (Bloom & Perlmutter, 1977; Hara, 1977, 1992, 1994; Hamdani & Doving, 2007). Olfaction is mediated by olfactory receptors located in the olfactory pits, which have incurrent and excurrent channels (nares) (Hara & Zielinski, 1989; Moyle & Cech, 2004). At hatching the olfactory apparatus is mostly complete (Hara & Zielinski, 1989).

Gustatory chemoreception, on the other hand, is especially important in the identification of both food and noxious substances. Taste buds are located mostly in the mouth and pharynx, but also in the skin, fins and barbels (Bardach & Villars, 1974; Smith, 1982; Noakes & Godin, 1988; Hansen et al., 2002). The primordial taste buds are the first structures of this system to appear during development (Hansen et al., 2002). The onset of feeding seems to be coincident with the appearance of these structures but the developmental stage at which they are fully developed varies considerably among species (Landacre, 1907; Noakes & Godin, 1988; Hansen et al., 2002).

Regarding vision or photoreception, fishes are generally well adapted to extract relevant visual information from their highly diverse habitats, e.g. from coral reefs to deep sea caves, and their eye structure and function usually reflect an adaptation to the constraints imposed by the different light environments (Li & Maaswinkel, 2006). Fish eyes are typically located on the side of the head and their structure is relatively similar to other vertebrates, with the visual receptors located in the eye retina and the lens providing the focusing of light. During development, the optic primordium appears normally at two days' post fertilization, followed by the differentiation of the temporal retina, ganglion cells, inner nuclear and outer nuclear layer. In teleosts, after hatching the increase in eye and lens size continues (Easter et al., 1977; Klein & Wang, 2002).

The electroreception is the ability to detect electric fields that in fish have been thought to play important roles in obstacle avoidance and prey capture (Zupanc & Bullock, 2005). Fish can either detect external electric field and do not possess electric organs (passive sense), or they may detect distortions in the water with a self-generated three dimensional electric field (Lissmann & Machin, 1958). The perception of such is mediated by electroreceptor organs spread throughout the body with typically higher density in the head (Coombs et al., 1988; Northcutt, 1989). The development of electroreceptors is dependent on the lateral line fibers

coming in contact with placodes in the ectoderm that form sensory ridges (Fortune & Chacron, 2005; Hofmann, 2005).

The acousticolateralis system of fish, which is remarkably diverse in this taxon (Braun & Grande, 2008), is important to sense sounds, vibrations and other water displacements, containing two main components: the inner ear and the lateral line system. Besides sound detection, the inner ear is also important for balance/equilibrium in the three-dimensional space (Moyle & Cech, 2004).

The lateral line is a primitive sensory system of vertebrates (Webb, 1989), found in both fish and aquatic amphibians (Coombs et al., 1988; Northcutt, 1989; Webb, 1989) and with various functional roles: feeding, swimming, navigation and communication. This close range system allows fish to sense water movements in relation to their body surface (Montgomery et al., 2014). The lateral line organs are the neuromasts containing mechanoreceptor hair cells that are positioned along the head trunk and tail (Allis, 1889). During embryonic development, neuromasts precursors are deposited by migrating primordium, which originate from otic placodes (Gompel et al., 2001; Stone, 2004). Later they integrate the epidermal layer and differentiate into neuromasts (Sapède et al., 2002). In the postembryonic development some neuromasts remain on the body surface and others become embedded in the body (canal neuromasts) (Webb & Shirey, 2003).

In aquatic environments, visibility is often impaired, and olfaction is restricted to close proximity or requires a receiver to be downstream in the water current, which makes hearing an important and crucial system for many fish species (Munk, 1974; Hawkins & Myrberg, 1983). Sound transmission in aquatic environments had a notable impact on the evolution of the auditory system in fishes. Water is an ideal medium (conductor) for acoustic propagation because of its higher density in comparison to air. Therefore, the speed of sound in water (circa 1,500 m/s) is closer to five times faster than in air (Au & Hastings, 2008). The following section describes in greater detail the development of the auditory system and hearing sense in fish.

2. The Fish Auditory System

Over decades, investigators have struggled to answer questions about how the sense of hearing and the vertebrate auditory system have evolved. Birds and mammals are known to have elaborate inner ear structures for sound detection and analysis (basilar papilla in birds and cochlea in mammals), and their sound analyses capabilities have been thoroughly explored (Fay

& Popper, 2000). However, this contrasts with the less information available on structure and function of the auditory system in lower vertebrates as fish.

The initial idea for the origin of the fish ear suggested that the structure evolved from the lateral line (acousticolateralis hypothesis), but modern anatomical and physiological methods suggested that the ear and the lateral line may have shared a common origin (Popper et al., 1992). However, recent data suggest that in the history of vertebrate's evolution, the ear evolved in an early stage, initially thought to function as a mechanism that would allow the measurement of motion and position of the head in relation to gravity (Popper et al., 2005). Various aspects of vertebrate hearing seem to have evolved very early in vertebrate history, pointing out that basic auditory functions found in mammals and birds are often changes first observed in fishes (Fay & Popper, 2000).

2.1 Auditory Neural Pathways: from the Inner Ear to the CNS

The inner ear of vertebrates has a general common structure with semicircular canals and, in most non-mammalian species, three otolithic endorgans: the saccule, lagena and utricle (Lu & Popper, 1998; Fay & Popper, 2000; Lanford et al., 2000; Popper & Lu, 2000; Popper et al., 2005; Webb et al., 2012). The modern inner ear of teleost fishes is a membranous sac located in the cranial cavity, lateral or below the hindbrain. The inner ear contains three otolithic endorgans, each one containing a calcium carbonate solidified single mass, which are connected by the semi-circular canals (Hawkins & Myrberg, 1983; Popper & Fay, 1999) – see Fig 4.

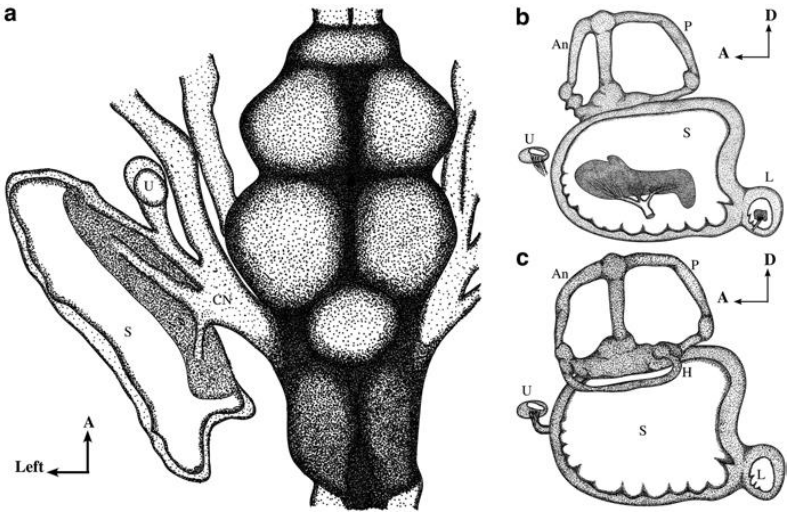

Figure 11. The inner ear in the adult plainfin midshipman. (a) depicts a dorsal view of the brain, auditory nerve (CN—VIIIth cranial nerve) and the inner ear (S—saccule, U—utricle). Notice the size of the saccule in relation to the brain. (b) and (c) show drawings or the right and left inner ears, respectively, in the plainfin midshipman. The three otolithic end organs (S—saccule, L—lagena, and U—utricle) as well as the three semi-circular canals (An—anterior, H—horizontal, P—posterior) are visible (Vasconcelos, Alderks, & Sisneros, 2015).

Each endorgan has a sensory epithelium (macula), which contains the transducers of acoustic (or vestibular) information, the hair cells. These cells are mechanosensory receptors found in the ears of all vertebrates and in the lateral line of fish and amphibians (Hawkins & Myrberg, 1983; Popper & Lu, 2000; Popper et al., 2005). The calcareous otolith attaches to the macula via a gelatinous otolithic membrane (Popper & Lu, 2000).

Hair cells have a tuft of cilia at their apical surface projecting into the lumen of the otolithic chamber (Popper et al., 2005). These cells contain large conductance calcium activated potassium channels (BKCa) responsible for regulating the cell excitability (Popper et al., 2005). When the cilia bend under acoustic stimulation, it causes the release of a neurotransmitter from the basal end of the cell, which will excite the afferent endings of the eighth cranial nerve that projects into central brain regions. This will alert the brain for a mechanical event that could be either sound or movement of the head (Popper & Lu, 2000; Popper et al., 2005). The variable morphological polarization of the ciliary bundles within the sensory epithelium allows the bending in different directions and, consequently, different neuronal firing actvity. Each hair cell contains one kinocilium (longest cilium) and smaller stereocilia. The direction of bending, i.e. towards the kinocilium or the smallest stereocilia,, determines whether the cells undergoes depolarization or hyperpolarization, respectively (Popper & Lu, 2000). Besides the hair cells, the sensory epithelia also contains supporting cells that are important for the maintenance of the structural integrity of sensory organs during sound stimulation and head movements (Wan et al., 2013), and may acts as hair cell precursors (Presson et al., 1995; Presson et al., 1996).

As mentioned above, each end organ has a macula (or sensory epithelium) and an otolith, which serve as inertial systems, as a stimulus causes motion of the fish body in relation to the otoliths. As the otolith is approximately three times denser than the fish body it will move at a different amplitude and phase than the sensory epithelium. The direct or indirect mechanical contact between the tips of the cilia and the otolith, will bend the cilia leading to the detection of a mechanical signal (Popper & Lu, 2000; Popper et al., 2005).

Although the specific function of each endorgan is not clear, recent investigations suggest that the saccule is the main auditory endorgan (Collin, 2003) and that the utricle and lagena may have mixed vestibular and auditory functions (Popper & Lu, 2000). The utricle and

lagena may be essential for directional processing, since their epithelium is positioned in a different spatial orientation (horizontal and vertical plan, respectively), and to extend the hearing dynamic range (Wysocki & Ladich, 2001; Maruska & Mensinger, 2015).

The central auditory pathways have a quite common organizational scheme across different taxa, even though the peripheral auditory organs are not homologous (McCormick, 2011).

The inputs from the auditory receptors in the saccule are collected by the VIII cranial nerve, an aggregate of nerve branches entering the brain at the level of the medulla oblongata (Edds-Walton & Fay, 2005; Kittelberger & Bass, 2013). The information is distributed to the first order nuclei in the medulla and to areas within the cerebellum. In teleosts, the descending (Do) and secondary (So) nuclei innervate auditory portions of the midbrains' torus semicircularis (Ts), a structure homologous to the mammalian inferior colliculus (Echteler, 1984; Kozlowski & Crawford, 1998; Bass et al., 2000; Lu & Bass, 2006) (Fig. 12). Fibres from the saccule provide the majority of the input to the Do nuclei that contains the largest first order acoustic population nuclei (McCormick, 2011). On the other hand, the fibres from the lagena and the utricle overlap saccular inputs at some levels but are largely laterally or ventrolateral adjacent to the Do (McCormick, 2011). Projection from the auditory Ts innervates the isthmal midbrain, rostral midbrain, anterior hypothalamus, dorsal thalamus, tectum and preglumerulosus complex of the diencephalon (Bass et al., 2000; Echteler, 1984; Kozlowski & Crawford, 1998; Lu & Bass, 2006). In the telencephalon the auditory areas are portions of area ventralis lateral to the supracommisural (Vs) and central (Vc) nuclei, receiving inputs from the dorsal thalamus and anterior hypothalamus, from the Ts and portions of the dorsal telencephalon, which receive auditory inputs from preglomerular complex (Bass et al., 2000; Goodson & Bass, 2002; Northcutt, 2008; Yamamoto & Ito, 2008) - see Fig 5 for an overview

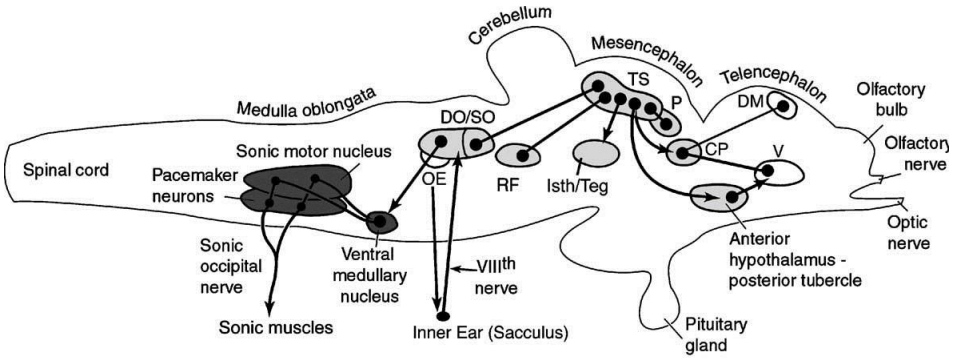

Figure 12. Side view of the brain portraying the organization of the central auditory. Solids dots represent somata, whereas lines represent axonal projection pathways. Two connected dots represent reciprocal connections. Shown here (lightly shaded are the relative positions of the nuclei identified as linked to the midbrain auditory region in the torus semicircularis(TS). The central posterior nucleus (CP) in the dorsal thalamus also projects to the dorsomedial (DM) and ventral (V) telencephalon, while the anterior hypothalamus also provides input to V; all these pathways are a likely source of auditory input to the telencephalon. Other abbreviations: DO, descending octaval nucleus; Isth/Teg, isthmal and midbrain tegmental regions; OE, octaval efferent nucleus; P, medial pretoral nucleus of the pretectum; RF, reticular formation; SO, secondary octaval nucleus (Bass & Mckibben, 2003).

2.2 Development of the Auditory Sensitivity

Most species studied so far, exhibited auditory sensitivity improvements with age/size. Some exceptions were registered, especially when different methods were applied. The only species that exhibited a decrease during development was the *Abudefduf saxatilis* (Pomacentridae) (Egner & Mann, 2005).

Table 1 provides a systematic overview of the ontogenetic changes in auditory sensitivity in fish, based on different techniques – subject is further explored in section 2.3 and 2.4.

Table 1. Systematic overview of developmental changes in auditory sensitivity in fish, based on various techniques. AEP auditory evoked potentials, ASR acoustic startled response, BC behavioral conditioning, SEP saccular evoked potentials, SUR single unit recordings, HR heart rate. Development changes are indicated as (+) increase, (-) decrease, or (=) no changes (adapted from Vasconcelos, Alderks, & Sisneros, 2015).

Order	Family	Species	Common Name	Technique	Auditory changes	Reference
Rajiformes	Rajidae	*Raja clavata*	Thornback ray	SUR	+	(Corwin, 1983)
Clupeiformes	Clupeidae	*Alosa sapidissima*	American shad	AEP	=	(Higgs et al., 2004)
	Clupeidae	*Clupea spp.*	Herring	ASR	+	(Blaxter & Batty, 1985)
Cypriniformes	Cyprinidae	*Danio rerio*	Zebrafish	AEP	=	(Higgs et al., 2001)
				AEP	=	(Higgs et al., 2003)
				ASR	+	(Zeddies & Fay, 2005)
				ASR	+	(Bhandiwad et al., 2013)
				HR	+	(Lu & DeSmidt, 2013)
Siluriformes	Mochokidae	*Synodontis schoutedeni*	Squeaker catfish	AEP	+	(Lechner et al., 2010)
	Claroteidae	*Lophiobagrus cyclurus*	African bullhead catfish	AEP	+	(Lechner et al., 2011)
Gadiformes	Gadidae	*Theragra chalcogramma*	Walleye pollock	AEP	=	(Mann et al., 2009)
Batrachoidifomes	Batrachoididae	*Halobatrachus didactylus*	Lusitanian toadfish	AEP	+	(Vasconcelos & Ladich, 2008)
		Porichthys notatus	Plainfin midshipman fish	SUR	+	(Sisneros & Bass, 2005)
				SEP	=	(Alderks & Sisneros, 2011)
				ASR	=	(Alderks & Sisneros, 2013)
Perciformes	Serranidae	*Epinephelus coioides*	Orange-spotted grouper	AEP	+	(Wright et al., 2011)
		Epinephelus fuscoguttatus	Brown-marbled grouper	AEP	=	(Wright et al., 2011)
	Carangidae	*Caranx ignobilis*	Giant trevally	AEP	+	(Wright et al., 2011)
	Pomacentridae	*Abudefduf saxatilis*	Sergeant major damsefish	AEP	-	(Egner & Mann, 2005)
		Pomacentrus nagasakiensis	Nagasaki damsefish	AEP	+	(Wright et al., 2005)
		Stegastes partitus	Bicolor damselfish	BC	+	(Kenyon, 1996)
		Amphiprion ephippium	Saddle anemonefish	HR	+	(Simpson et al., 2005)
		Amphiprion rubrocinctus	Red anemonefish	HR	+	(Simpson et al., 2005)
	Polyprionidae	*Polyprion oxygeneios*	Hapuka	AEP	+	(Caiger et al., 2013)
	Gobidae	*Neogobius melanostomus*	Round goby	AEP	=	(Belanger et al., 2010)
	Osphronemidae	*Trichopsis vittata*	Croaking gourami	AEP	+	(Wysocki & Ladich, 2001)
	Sciaenidae	*Sciaenops ocellatus*	Red drum	ASR	+	(Fuiman et al., 1999)
	Polynemidae	*Eleutheronema tetradactulum*	Indian salmon	AEP	+	(Wright et al., 2011)
	Percichthyidae	*Macquaria novemaculeata*	Australian bass	AEP	+	(Wright et al., 2011)
	Chaetodontidae	*Chaetodon ocellatus*	Spotfin butterflyfish	AEP	=	(Webb et al., 2012)

2.3 Structure-function relationships

The otoliths and the sensory epithelia grow in most fishes throughout their lifespan. The growth of the sensory epithelia is typically combined with the addition of auditory hair cells (Lombarte & Popper, 1994). Studying such ontogenetic morphological changes and their effect in terms of auditory sensitivity is important because each step forward in development will presumably influence physiology and behaviour, which are adaptive for survival and reproduction of the individual (Alderks & Sisneros, 2011).

Some of the changes occurring during development in the fish inner ear are common across different species. Growth and shape changes in the sensory epithelia, as well as the addition of hair cells were found in elasmobranchs such *Raja clavata* (Rajidae) (13.5-97 cm TL) (Corwin, 1983), and in teleosts such as *Astronotus ocellatus* (Cyprinidae) (2.0-19 cm SL) (Popper & Hoxter, 1984, 1990) and *Merluccius merluccius* (Merluccidae) (7- 75 cm TL) (Lombarte & Popper, 1994). Hair cell number increase was also registered in *Carassius auratus* (Cyprinidae) (3.5-8 cm SL) (Platt, 1977).

In *Danio rerio* (Cyprinidae) (2-7 dpf) (Lu & DeSmidt, 2013) authors found an increase in hair cell density, whereas in *A. ocellatus* (Popper & Hoxter, 1984) (2.0-19 cm SL) and *M. merluccius* (Lombarte & Popper, 1994) the opposite occurred.

Regarding orientation patterns results vary, in *M. merlucius* no changes were registered (Lombarte & Popper, 1994) with increasing age, whereas in *O. tau* (Batrachoididae) there seems to be a pattern shift (standard to dual) from 4 week post hatched to the adult (13.5-26 cm SL) (Sokolowski & Popper, 1987; Edds-Walton & Popper, 1995).

Although very few data exists on the ontogenetic changes in the auditory nerve morphology, studies have shown that in *R. clavata* there were no changes in the number of nerves innervating the macula (Corwin, 1983), whereas in *Raja ocellata* (Rajidae) there was an increase in axon number and total axon area (Barber et al., 1985).

Most of the studies done so far, have focused on later stages of development, it would be interesting to explore such morphologic differences in earlier phases, and in species that are highly vocal, such as *H. didactylus*. With this approach we would be able to relate morphological changes to the onset of sound production and social communication, and to changes in auditory sensitivity.

3. Development of Auditory-Vocal Systems in Fish

One of obvious questions rising in audiology research is the relationship between the auditory system and the vocal system, i.e. how these two systems interact during ontogenetic development for social communication. When focusing on vocal differentiation studies most of the information is available in higher vertebrate groups, such as mammals and songbirds, and there is a common trend of increment in the number of call types with growth (Moss et al., 1997; Doupe & Kuhl, 1999; Hollén & Radford, 2009). Vocalizations often start on the first day after hatching or birth and sound characteristics change with growth often increasing in production rate until vocal maturation (Wurdinger, 1968; Ripley & Lobel, 2004). The process is different when considering vocal and non-vocal learners species, as the latter also exhibit vocal differentiation but probably as a result of the concurrent development of the vocal motor system in both the peripheral vocal apparatus and central neural circuitry controlling vocal behavior (Derégnaucourt et al., 2009; Jürgens, 2002)**.**

Looking at fish, 40 families are known to vocalize during agonistic interactions (Amorim & Hawkins, 2005), but the ontogeny of sound from hatching to maturation has only been investigated in the *Trichopsis vitatta (*Osphronemidae) (Wysocki & Ladich, 2001) and in *Tramitichromis intermedius* (Cichlidae) (Ripley & Lobel, 2004).

Only a few studies have explored the ontogeny of vocal behaviour and mainly found common trends such as an increase in sound duration, pulse period, pulse number and sound pressure, and a decrease in sound dominant frequency level. (Myrberg et al., 1993; Henglmuller & Ladich, 1999; Amorim & Hawkins, 2005; Colleye et al., 2009; Lechner et al., 2010).

The few existing studies concerning the relationship between the vocal motor and auditory systems during development in fish revealed that sound detection develops prior to the fish's ability to generate perceivable sounds (Vasconcelos & Ladich, 2008; Wysocki & Ladich, 2001) In the croaking gourami (*T. vitatta*) acoustic communication might be absent during early developmental stages because of poor hearing sensitivity (Wysocki & Ladich, 2001). On the other hand, acoustic communication may occur during a wide range of developmental stages, as in the catfish (*Synodontis schoutedeni*) (Lechner et al., 2010).

A recent study with the Lusitanian toadfish *H. didactylus* (Batrachoidiae) showed that the developmental stage when large juveniles started producing the full vocal repertoire was coincident with a significant enhancement in auditory saccular sensitivity (Vasconcelos et al., 2015) – see fig 13. This data provides a clear evidence that the development of the vocal motor

control systems parallels the development of the peripheral auditory system in a vocal fish species (Vasconcelos et al., 2015).

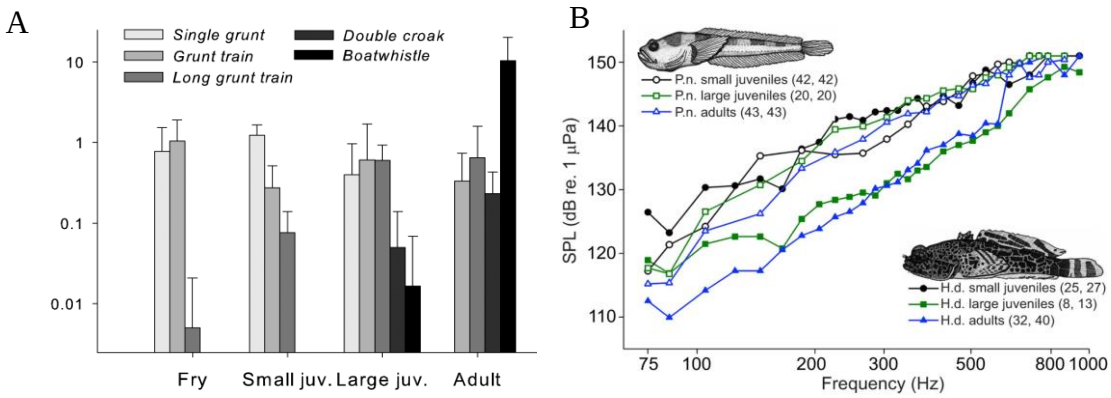

Figure 13. (A) Variation in the mean calling rate (±s.d) of each sound type across different size groups in *Halobtrachus didactylus*. (B) Comparison of mean auditory threshold curves across different size groups in the *H. didactylus* and the *Porichthys notatus*. Illustrations represent a typical small juvenile of each species. SPL, sound pressure level. Groups: fry, <2.0cm SL (N=12-20 fisg, 10 sessions); small juveniles, 2.4-4.9cm SL (N=17, 10); large juveniles, 5.0-8.69cm SL (N=12,10); adults, 25-35cm (N=6, 10) (Vasconcelos et al., 2015).

Although there is already information about how vocal-motor control system and peripheral auditory systems interact during development, science has not been able to uncover what are the underlying anatomical changes that influence how both systems develop.

4. The model system

4.1 Why fish?

Fish represent the largest group of vertebrates and at least 100 families rely on acoustic signals during various social interactions including mating and territorial defence (Amorim & Hawkins, 2005; Radford et al., 2014). This taxon is not only highly diverse in species life histories and interesting to investigate adaptation mechanisms to different habitats, but also contains species with simpler social behaviours (e.g. vocalizations) that can be easily quantified.

In addition, vocal neural pathways controlling for vocal behaviour are organized similarly in all vertebrates and evolved from and ancestrally shared brain region originated in ancestral fish before the radiation of the major clades (Bass, 2008). Thus, studies that investigate the development of vocal-auditory systems in fish are important to gain a

comprehensive understanding of the mechanisms and evolution underlying social acoustic communication in all vertebrates.

Finally, the lower costs in terms of space and maintenance of the majority of fish species and their relatively easy breeding (e.g. zebrafish), makes this taxon even more appealing to use as research models (Lu & DeSmidt, 2013). Even though many fish species may note exhibit their full behavioural repertoire in captivity, certain species are especially suited for lab research and also especially tolerant to experimental manipulations (e.g. Batrachoididae, Amorim et al., 2010).

4.2 The Lusitanian toadfish (Halobatrachus didactylus)

In the last decades the toadfishes and the plainfin midshipman fish from the Batrachoididae family have emerged as important model species to study mechanisms and evolution of auditory-vocal functions for social communication in fish (Bass, 2008). Although teleosts are known for their diversity when it comes to acoustic communication, the Batrachoididae are specially unique in terms of vocal complexity and repertoire size (Amorim et al., 2006; Vasconcelos & Ladich, 2008; Vasconcelos, 2011).

The Lusitanian toadfish *H. didactylus* has become a surprising interesting model system to investigate the role of communication signals in various social contexts. Its place as a basal lineage in the Batrachoididade family makes this species an unmatched opportunity to explore the underlying mechanisms that motored the evolution of acoustic signalling and communication (Amorim et al., 2006; Vasconcelos et al., 2011; Vasconcelos et al., 2015).

H. didactylus occurs in subtropical regions, along the Northeasthern Atlantic and in the Mediterranean Sea – see Fig. 14. This sedentary benthic species inhabits shallow coastal waters (up to 50m depth) living in sand and mud substrates (Roux, 1986). Inhabiting these shallow waters often precludes or limits visual communication and so this species seems to heavily rely on acoustic signalling to interact with conspecifics throughout life in various social contexts such as to attract mates or to defend nests (Amorim et al., 2006; Vasconcelos & Ladich, 2008; Lechner et al., 2010; Vasconcelos, 2011).

Figure 14. Adult Lusitanian toadfish (*Halobratchus didactylus*) in a nest in the Tagus river estuary. Rock has been lifted to see the specimen.

The reproductive season of *H. didactylus* goes from May to July in Portugal, depending on the temperature, during which territorial males build nests in aggregations under rocks or in crevices. During the mating season these nesting males produce long distance advertisement calls, the boatwhistle, from their nests to form conspicuous choruses and attract females to spawn (Vasconcelos et al., 2011).

Like other batrachoidids, the Lusitanian toadfish has sexual polymorphism with two male morphotypes: the "type I" are territorial males that build and guard nests, and the "type II"(sneaker) are smaller males with higher gonadsomatic index but smaller sonic muscles, and seek for opportunistic fertilizations (Modesto & Canário, 2002; Pereira et al., 2011).

Studies have shown that this species exhibits a rich vocal repertoire composed of at least five different vocalizations, something rare among fish (Amorim et al., 2008). At least three sounds, the grunt call, croak and double croak, are associated with agonistic contexts (dos Santos et al., 2000), and the boatwhistle, a complex amplitude-modulated call seem to have an important role in mate attraction (Vasconcelos et al., 2011) but that is also produced during agonistic interactions (Vasconcelos et al., 2010) – see Fig. 15.

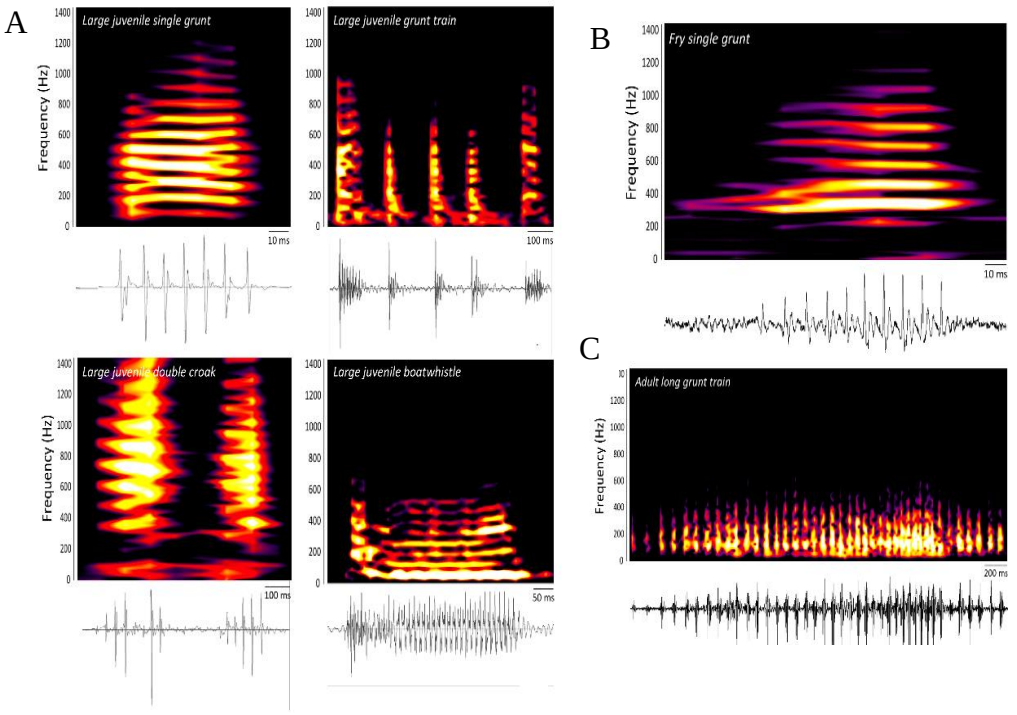

Figure 15. Spectrograms and oscillograms of representative vocalizations produced by the Lusitanian toadfish during social interactions. (A) Sounds emitted by large juveniles, which already exhibited the vocal repertoire. (E) Single grunt call produced by the earliest developmental stage (fry) – Sound has been filtered <100 Hz to increase signal-to-noise ratio (SNR). (F) Long grunt trains produced by an adult. Sampling frequency 8 kHz; hamming window, 30 Hz filter bandwidth (Vasconcelos et al., 2015).

Vasconcelos et al. (2015) recently reported that juveniles (5-10.6 cm SL) of *H. didactylus* already possess the full vocal repertoire of an adult, surprisingly showing that they are already capable of producing boatwhistles, a capacity until then only describe in adults (Vasconcelos et al., 2010). Vasconcelos et al. (2015) also recorded vocal activity from fry (1.7-2.0 cm SL) in agonistic contexts, reenforcing the importance of vocal communication for this species since early stages of development (Vasconcelos et al., 2015).

The toadfish *H. didactylus* offers the opportunity to study development of vocalization and social behaviour from a post-hatching phase and correlate behavioural changes to development of the inner ear apparatus and vice-versa. This structure-function exploration is necessary to pinpoint crucial development stages and to answer questions about the adaptive mechanism behind such high vocal diversity and hearing plasticity.

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