



## Research Paper

# Ontogenetic development of the inner ear saccule and utricle in the Lusitanian toadfish: Potential implications for auditory sensitivity



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## ARTICLE INFO

## Article history:

Received 1 March 2017

Received in revised form

1 June 2017

Accepted 13 June 2017

Available online 15 June 2017

## Keywords:

Saccule

Utricle

Hair cell

Ontogeny

Batrachoididae

Auditory sensitivity

## ABSTRACT

Studies addressing structure-function relationships of the fish auditory system during development are sparse compared to other taxa. The Batrachoididae has become an important group to investigate mechanisms of auditory plasticity and evolution of auditory-vocal systems. A recent study reported ontogenetic improvements in the inner ear saccule sensitivity of the Lusitanian toadfish, *Halobatrachus didactylus*, but whether this results from changes in the sensory morphology remains unknown. We investigated how the macula and organization of auditory receptors in the saccule and utricle change during growth in this species. Inner ear sensory epithelia were removed from the end organs of previously PFA-fixed specimens, from non-vocal posthatch fry (<1.4 cm, standard length) to adults (>23 cm). Epithelia were phalloidin-stained and analysed for area, shape, number and orientation patterns of hair cells (HC), and number and size of saccular supporting cells (SC).

Saccular macula area expanded 41x in total, and significantly more (relative to body length) among vocal juveniles (2.3–2.9 cm). Saccular HC number increased 25x but HC density decreased, suggesting that HC addition is slower relative to epithelial growth. While SC density decreased, SC apical area increased, contributing to the epithelial expansion.

The utricle revealed increased HC density (striolar region) and less epithelial expansion (5x) with growth, contrasting with the saccule that may have a different developmental pattern due to its larger size and main auditory functions. Both macula shape and HC orientation patterns were already established in the posthatch fry and retained throughout growth in both end organs.

We suggest that previously reported ontogenetic improvements in saccular sensitivity might be associated with changes in HC number (not density), size and/or molecular mechanisms controlling HC sensitivity. This is one of the first studies investigating the ontogenetic development of the saccule and utricle in a vocal fish and how it potentially relates to auditory enhancement for acoustic communication.

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## 1. Introduction

Fishes comprise the largest group of extant vertebrates displaying the greatest diversity in sensory structures for hearing and orientation in their highly diverse acoustic environments (Braun and Grande, 2008; Ladich and Schulz-Mirbach, 2016). Studying organisms from an early diverging vertebrate lineage such as fish is important to gain fundamental comparative insights into the evolution and function of the vertebrate inner ear (Fay and Popper, 2000; Schulz-Mirbach and Ladich, 2016), as early developmental

events are often evolutionary conserved (Fritzsche and Beisel, 2003). An ontogenetic perspective of the auditory system in fishes provides a readily testable framework for understanding structure-function relationships (Vasconcelos et al., 2016). However, studies addressing ontogenetic development between form and function of the auditory system in this taxon are rather sparse (e.g. Corwin, 1983; Lu and DeSmidt, 2013).

There are only a few studies describing developmental changes in the fish inner ear end organs. These studies reported common developmental trends, such as enlargement and changes in shape of the sensory macula (Corwin, 1983; Lombarte and Fortuno, 1992; Lombarte and Popper, 1994, 2004; Popper and Hoxter, 1984), increased hair cells number and decreased density (Corwin, 1983; Higgs et al., 2001; Lombarte and Popper, 1994, 2004; Lu and

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DeSmidt, 2013; Popper and Hoxter, 1984; Sokolowski and Popper, 1987), increased number and size of nerves innervating the sensory macula (Barber et al., 1985; Corwin, 1983; Popper and Hoxter, 1984), increased otolith area or length (Lombarte and Fortuno, 1992; Lombarte and Popper, 2004; Mann et al., 2009) and early establishment of hair cell orientation patterns (Lombarte and Popper, 1994, 2004; Sokolowski and Popper, 1987).

Although the functional significance of most of these morphological changes in the fish auditory peripheral system is not known, it is likely that changes in sensory morphology have important functional implications leading to sensitivity changes (Schulz-Mirbach and Ladich, 2016; Vasconcelos et al., 2016). Such structure-function relationships have been mostly reported in more recently diverged vertebrates, such as in birds (e.g. Bartheld, 1994; Rubel and Fritzsche, 2002) and mammals (e.g. Beutner and Moser, 2001; Jun et al., 2006; Tong et al., 2015).

The association between morphological changes in the auditory end organs and hearing sensitivity during development was only investigated in two fish species belonging to different classes. The first study to report ontogenetic structure-function changes in the auditory system in fish was conducted by Corwin (1983) in the elasmobranch *Raja clavata*. Through multiunit recordings from the macula neglecta (nonotolith inner ear end organ), the author described a 500x auditory improvement in skates from 21 to 91 cm total length most likely caused by hair cell addition. Among teleosts, the only studies were conducted in zebrafish *Danio rerio* (Cyprinidae) and the results are not consistent. For instance, Higgs et al. (2001, 2003) based on overall auditory evoked potential recordings reported absence of improvements in hearing sensitivity and response latency despite continuous hair cell addition (25–50 mm; 10–45 mm body length), and described an expansion of the maximum detectable frequency probably due to development of Weberian ossicles. However, Wang et al. (2015) using the same technique reported auditory sensitivity improvements in zebrafish ranging from 12 to 37 mm, which followed an increase in hair cell number and density and changes in the organization of the hair cell stereocilia.

Representatives of the Batrachoididae family have become important model organisms to study mechanisms and evolution of auditory-vocal functions for social communication. These teleosts rely on acoustic communication since early developmental stages to mediate social interactions (Vasconcelos et al., 2015) and for mate attraction during the breeding season (McIver et al., 2014; Vasconcelos et al., 2012). For this reason, their auditory system has been the focus of attention in several studies investigating the mechanisms underlying auditory plasticity for enhancing social acoustic communication (Coffin et al., 2012; Forlano et al., 2016; Sisneros et al., 2004). For example, Coffin et al. (2012) reported that seasonal changes in the saccular auditory sensitivity (of about 8–15 dB) are correlated with a 13% increase in the hair cell density in females midshipman fish *Porichthys notatus*.

A recent study reported a similar saccular sensitivity improvement 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). Whether such improvements in saccular auditory sensitivity can be explained by concurrent morphological changes in the sensory macula remains unknown.

In present study we investigated how the macula and organization of auditory receptors in the sacculus change during growth in the highly vocal Lusitanian toadfish. Morphological analysis of the sensory epithelia considered changes in the area and shape, density and orientation patterns of hair cells, density and size of supporting cells. An additional inner ear end organ, the utricle, with mixed

auditory-vestibular functions (Messinger, 2016), was also considered for comparison purposes.

## 2. Methods

### 2.1. Fish collection and maintenance

In the present study the following ontogenetic groups of Lusitanian toadfish were considered: 1) “posthatch (PH) fry” (1.3–1.4 cm standard length, SL; 0.09–0.10 g body weight, BW; circa 10–17 dpf - days post fertilization); 2); “vocal onset (VO) fry” (1.7–2.0 cm SL, 0.19–0.30 g BW; circa 34–41 dpf); “juveniles” (2.3–2.9 cm SL, 0.33–0.79 g BW, circa 165–172 dpf); and “adults” (20–23 cm SL, 205–334 g BW). The size groups selected were related to specific ontogenetic stages, which have been previously investigated regarding auditory sensitivity and vocal behaviour (Vasconcelos and Ladich, 2008; Vasconcelos et al., 2015).

To obtain specimens from the first three groups, we followed the procedure for collecting toadfish eggs that has been successfully adopted in previous studies (e.g. Vasconcelos et al., 2012). Prior to the onset of the breeding season, semicylinder concrete shelters were placed along an intertidal area of the Tagus River estuary in Portugal (38°42'N; 8°58'W). Toadfish adults readily occupied these shelters, which contained a plastic sheet attached to the ceiling to facilitate egg collection. During spring low tides (about 15 days later), after the shelters were used for breeding and became air-exposed, the plastic sheets containing eggs were removed, placed in coolers containing fresh seawater and transported to the laboratory at the University of Lisbon (Portugal). Two plastic sheets containing several clutches and healthy eggs were selected and suspended vertically underwater in a 20 L tank equipped with filtering system. When the embryos started hatching becoming free-swimming, 20 individuals were randomly chosen and euthanized for further processing (group “PH fry”).

Another group of 20 fry were randomly selected and transferred to a 20 L observation tank provided with sand substrate and 3 small plastic shelters to promote territorial social interactions and determine the vocal onset. We used the experimental setup described in Vasconcelos et al. (2015) for sound recordings of toadfish fry and, therefore, only a brief explanation will be given. Two hydrophones (High Tech 94 SSQ, Gulfport, MS, USA; frequency range: 30·Hz–6·kHz,  $\pm 1$ ·dB; voltage sensitivity:  $-165$ ·dB re. 1·V/ $\mu$ Pa) were positioned close to the nests and connected to an A/D converter device (Edirol UA-25, Roland, Tokyo, Japan; 16bit, 8 kHz) connected to a laptop running Adobe Audition 3.0 for windows software (Adobe Systems Inc., San José, CA, USA). The vocal onset was detected *in situ* by daily monitoring audio recordings of 60 min duration performed twice a day up to 24 days post hatching. Vocal specimens were collected and euthanized for further processing (group “VO fry”). This vocal group represented an important developmental stage to evaluate potential association between auditory-vocal systems (Vasconcelos et al., 2015).

Later on, a group of “juveniles” (N = 6) was collected from the stock tanks and euthanized for tissue analysis. The “adult” group (N = 6) was obtained outside the breeding season by trawling in the Tagus river estuary. Toadfish adults were transported to the laboratory for anaesthesia and perfusion-fixation. All collected adult specimens were males, as confirmed through gonadal inspection.

All fish developmental stages maintained in the laboratory were kept at  $21 \pm 2$  °C and under a day: night cycle of 12 h: 12 h. All fry and juveniles were fed with artemia flakes and small pieces of shellfish. All experimental procedures complied with the local animal welfare laws, guidelines and policies in Portugal and Macau (ref. AL017/DICV/SIS/2016).

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