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RESEARCH ARTICLE

Vocal differentiation parallels development of auditory saccular sensitivity in a highly soniferous fish

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ABSTRACT

Vocal differentiation is widely documented in birds and mammals but has been poorly investigated in other vertebrates, including fish, which represent the oldest extant vertebrate group. Neural circuitry controlling vocal behaviour is thought to have evolved from conserved brain areas that originated in fish, making this taxon key to understanding the evolution and development of the vertebrate vocal-auditory systems. This study examines ontogenetic changes in the vocal repertoire and whether vocal differentiation parallels auditory development in the Lusitanian toadfish *Halobatrachus didactylus* (Batrachoididae). This species exhibits a complex acoustic repertoire and is vocally active during early development. Vocalisations were recorded during social interactions for four size groups (fry: <2 cm; small juveniles: 2–4 cm; large juveniles: 5–7 cm; adults >25 cm, standard length). Auditory sensitivity of juveniles and adults was determined based on evoked potentials recorded from the inner ear saccule in response to pure tones of 75–945 Hz. We show an ontogenetic increment in the vocal repertoire from simple broadband-pulsed 'grunts' that later differentiate into four distinct vocalisations, including low-frequency amplitude-modulated 'boatwhistles'. Whereas fry emitted mostly single grunts, large juveniles exhibited vocalisations similar to the adult vocal repertoire. Saccular sensitivity revealed a three-fold enhancement at most frequencies tested from small to large juveniles; however, large juveniles were similar in sensitivity to adults. We provide the first clear evidence of ontogenetic vocal differentiation in fish, as previously described for higher vertebrates. Our results suggest a parallel development between the vocal motor pathway and the peripheral auditory system for acoustic social communication in fish.

KEY WORDS: Hearing, Vocal differentiation, Acoustic communication, Ontogeny, Batrachoididae

INTRODUCTION

A fundamental question for all vocal communication systems concerns the relationship between the developmental processes of vocal differentiation and auditory perception during ontogenetic development. Vocal differentiation, or the progressive increment in the number of call types with development, has been mostly documented in songbirds and mammals (e.g. Moss et al., 1997; Doupe and Kuhl, 1999; Hollén and Radford, 2009). An individual may undergo this process via vocal learning from adults during ontogeny (e.g. Brainard and Doupe, 2002). However, non-learner species also exhibit considerable ontogenetic changes in their vocalisations, which may result from the development of the vocal motor system in both peripheral vocal apparatus and central neural circuitry controlling vocal behaviour (e.g. Jürgens, 2002; Derégnaucourt et al., 2009). Equally important to consider is that acoustic signal perception originates through development of the auditory periphery, inner ear and sensory receptors, and also central neural pathways (e.g. Moore, 2002). Certain species are known to undergo a refinement process during a sensitive phase when young are first experiencing social acoustic signals in the auditory vocal environment. However, such interaction between vocal and auditory systems during development has only been clearly demonstrated in higher vertebrates, namely songbirds and mammals (e.g. Moore, 2002; Miller-Sims and Bottjer, 2012).

Soniferous fish are good candidates to examine vocal differentiation and auditory sensitivity during ontogeny because they have relatively simple central and peripheral vocal mechanisms. In addition, the neural circuitry that controls vocal behaviour in vertebrates seems to have evolved from conserved brain areas found in ancestral fish before they diverged into the major clades (Bass et al., 2008). Thus, studies that investigate the development of vocal-auditory systems in vocal fish are important to gain a comprehensive understanding of the mechanisms underlying social acoustic communication in all vertebrates.

Previous fish studies have reported ontogenetic changes in acoustic signal characteristics, such as repetition rate, amplitude, duration and dominant frequency. The refinement in some of these signal characteristics with age is probably due to ontogenetic changes in the size and/or resonance properties of the sound-generating apparatus (e.g. Myrberg et al., 1993; Amorim and Hawkins, 2005; Lechner et al., 2010). It remains unclear whether fish that produce more complex acoustic signals exhibit vocal differentiation, which could potentially result from ontogenetic modifications of the motor circuitry of the vocal pathways as in birds or mammals (e.g. Aronov et al., 2008).

Data on the development of hearing abilities in fishes is relatively limited compared with what is known for other taxa. The available studies across various taxonomic groups reveal common principles, namely ontogenetic increases in auditory sensitivity (e.g. birds, Dmitrieva and Gottlieb, 1994; anurans, Boatright-Horowitz and Simmons, 1995; mammals, Reimer, 1995; reptiles, Werner et al., 1998), an extension of the frequency hearing range (e.g. mammals, Rübsamen, 1992) and a shift in the most sensitive frequencies (e.g. anurans, Boatright-Horowitz and Simmons, 1995). Ontogenetic studies of fish hearing show diverse results, ranging from no differences (Popper, 1971; Zeddies and Fay, 2005), to expansion of

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the detectable frequency range (Higgs et al., 2002, 2003; Alderks and Sisneros, 2011), up to increases in auditory sensitivity with size/age (Kenyon, 1996; Wysocki and Ladich, 2001; Sisneros and Bass, 2005).

Although some effort has been made to understand how fish vocal behaviour and auditory sensitivity develop during ontogeny, the question of whether there is concurrent ontogenetic development of the vocal motor and auditory systems for social communication remains unresolved. A few existing studies on this topic show that sound detection develops prior to the fish's ability to generate perceivable sounds (Wysocki and Ladich, 2001; Vasconcelos and Ladich, 2008). However, acoustic communication might be absent during early developmental stages because of poor hearing sensitivity, as in the gourami (*Trichopsis vittata*) (Wysocki and Ladich, 2001). Yet, at least in some species, it may occur during a wide range of developmental stages, as in the catfish (*Synodontis schoutedeni*) (Lechner et al., 2010). Nevertheless, these studies only focused on simple-pulsed sounds produced in distress/agonistic contexts and they do not report any evidence of vocal differentiation that would facilitate acoustic communication in these species.

In this study, we used the highly soniferous Lusitanian toadfish *Halobatrachus didactylus* (Bloch and Schneider 1801) (Batrachoididae, subfamily Halophryninae) to investigate the relationship between vocal differentiation and auditory sensitivity. This marine teleost relies on acoustic communication to mediate social interactions early in development. The Lusitanian toadfish exhibits an unusually large vocal repertoire that consists of about five different vocalisations used in various social contexts, including long tonal sounds (Amorim et al., 2008; Vasconcelos et al., 2010). Vasconcelos and Ladich (2008) using the auditory evoked potential (AEP) recording technique showed that the Lusitanian toadfish exhibits an ontogenetic increase in auditory sensitivity at the lowest (100 Hz) and highest tested frequencies (800–1000 Hz). These same authors recorded distress grunt sounds from different developmental stages and verified that the ability to communicate acoustically seems to occur when juveniles are able to generate perceivable grunts of higher amplitude and lower dominant frequency. However, whether such ontogenetic auditory improvements occur at the level of the auditory endorgans or more centrally in the auditory pathway has never been investigated. Also, previous work only analysed changes in the acoustic features of grunt calls produced in a distress context and nothing is known about development of the vocal repertoire. We propose that this model system offers an unmatched opportunity to investigate how the vocal motor and auditory systems develop during ontogeny enhancing social acoustic communication.

The aim of this study was twofold: (1) to verify how the vocal repertoire develops during ontogeny; and (2) to compare changes in the vocal behaviour with the auditory sensitivity at different developmental stages in the Lusitanian toadfish *H. didactylus*. Vocalisations were recorded during social interactions in different size groups, whereas auditory sensitivity was measured from equivalent juvenile and adult size groups based on auditory evoked responses from populations of hair cells in the saccule (main auditory endorgan in most teleosts).

RESULTS

Development of the vocal repertoire

Behavioural observations revealed that both fry and juveniles readily occupied available shelters, becoming territorial within 24 h. Individuals exhibited both visual and acoustic signalling during competition for space and food. Territorial visual displays included not only mouth opening and spreading of pectoral fins and opercula, but also more aggressive behaviour such as attacks, with occasional bites towards potential intruders.

Acoustic signalling was detected in all size groups analysed, including fry. Single grunts and grunt trains were produced during agonistic interactions while competing for food or space. Long grunt trains and double croaks were generally produced when individuals were inside the shelters and were not accompanied by any obvious social interaction. In contrast, the relatively rare boatwhistles recorded among large juveniles were detected only during active nest defence when the territorial individual was facing a potential intruder.

The vocal repertoire increased in the number of call types with increasing fish size, from simple broadband-pulsed sounds in fry and small juveniles to four different vocalisations in large juveniles and adults that included more complex amplitude modulated harmonic calls. Fig. 1 depicts the four representative call types produced by large juveniles, a grunt call produced by fry and a long grunt train generated by an adult.

Group size had a significant effect on the calling rate of all sound types: single grunts (H=26.63, d.f.=3, P<0.001), grunt trains (H=9.60, d.f.=3, P=0.029), long grunt trains (H=22.69, d.f.=3, P<0.001), double croaks (H=18.39, d.f.=3, P<0.001) and boatwhistles (H=35.63, d.f.=3, P<0.001) (see Fig. 2A–E). Single grunt production rate ranged from 1.65±0.63 calls h^{-1} per fish (mean number of sounds per session divided by the total number of individuals present) at the fry stage to none for the adults. Grunt trains also decreased in production rate with growth, ranging from 1.05±0.27 (fry) to 0.33±0.13 calls h^{-1} per fish (adults). In contrast, long grunt train production rate increased with increasing fish size, from 0.01±0.01 (fry) to 0.65±0.30 calls h^{-1} per fish (adults). Double croaks were only produced by large juveniles (0.05±0.30 calls h^{-1} per fish) and adults (0.23±0.06 calls h^{-1} per fish). Similarly, boatwhistles were only detected among large juveniles (0.02±0.02 calls h^{-1} per fish) and adults (10.38±3.10 calls h^{-1} per fish). Overall, the significant decrease in single grunt and grunt train production rates throughout development was accompanied by a significant increase in the calling rate of long grunt trains, double croaks and boatwhistles (Fig. 2F). While fry and small juveniles emitted exclusively various grunt-type sounds, large juveniles already exhibited the full adult vocal repertoire composed of grunt trains, long grunt trains, double croaks and boatwhistles.

Development of the saccular auditory sensitivity

In order to compare the dynamic range of the response magnitudes or relative gain of the evoked saccular potentials between different size groups, the iso-level response profiles obtained for each individual under 130 dB re. 1 µPa sound stimulation were normalised and expressed relative to a value of 0 dB at the best frequency (BF, defined as the frequency that evoked the lowest saccular potential response threshold). Each data point of the iso-level profile (mean value in voltage) was converted into dB relative to the value measured at the BF. The data was then averaged to construct a relative gain plot for each group (Fig. 3A). Normalised iso-level curves obtained from the three different size groups (small and large juveniles and adults) were not significantly different (repeated-measures ANOVA, $F_{2,36}$=0.27, P>0.05). However, the range of relative gain from 75 to 945 Hz of small juveniles (range 28 dB) was about 10 dB lower than that of large juveniles and adults (38 and 37 dB, respectively).

The three size groups had best auditory saccular sensitivity at the lowest frequencies tested, between 75 and 165 Hz, and showed a

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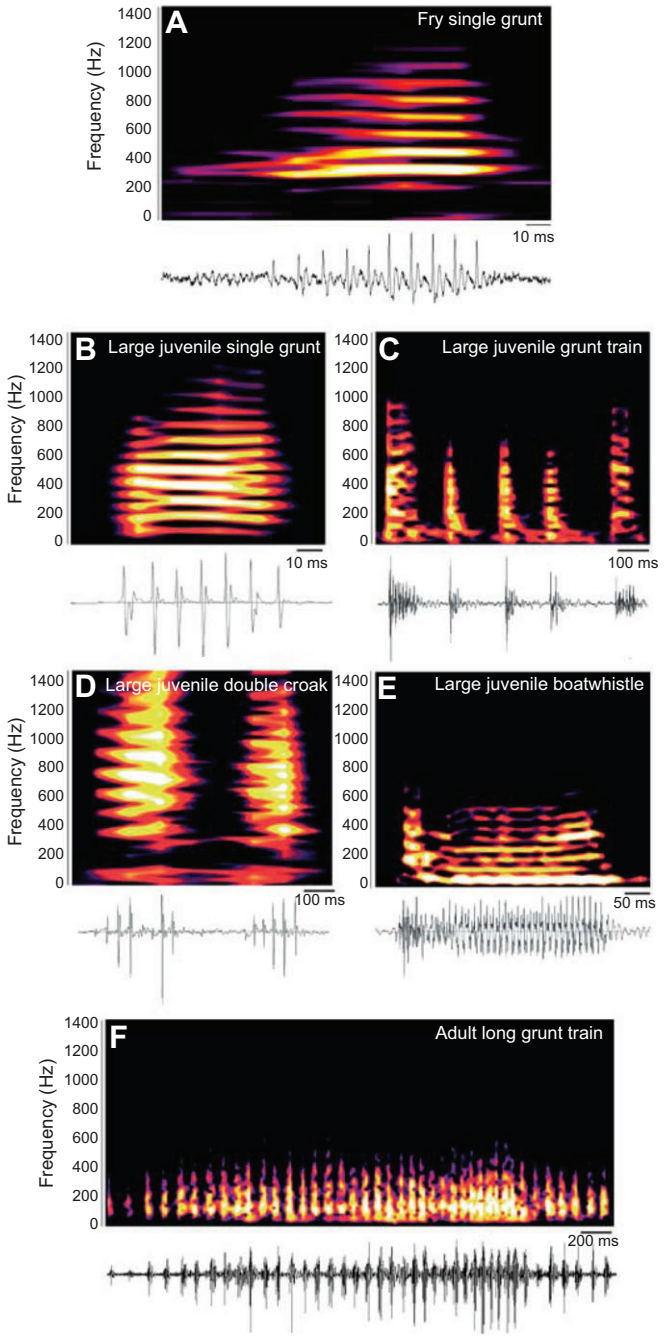

Fig. 1. Spectrograms and oscillograms of representative vocalisations produced by Lusitanian toadfish during social interactions. (A) Single grunt call produced by the earliest developmental stage (fry) considered in this study. Sound has been filtered <100 Hz to increase signal-to-noise ratio (SNR). (B–E) Sounds emitted by large juveniles, which already exhibited the full vocal repertoire. (F) Long grunt train produced by an adult (juvenile recordings of this sound type displayed poor SNR). Sampling frequency 8 kHz; hamming window, 30 Hz filter bandwidth.

gradual decrease in sensitivity with frequency up to 945 Hz (Fig. 3B). Mean auditory thresholds increased from 110 dB re. 1 µPa at 85 Hz (in adults) up to 151 dB at 785 Hz (small juveniles) and 945 Hz (adults). The threshold at the BF varied significantly with size: 100–133 dB (small juveniles), 97–136 (large juveniles) and 88–124 dB (adults) (one-way ANOVA, $F_{2,47}$=6.31, P<0.01).

Auditory threshold curves obtained from the three different size groups revealed significant overall differences within the frequency range 75–425 Hz, (repeated-measures ANOVA, $F_{2,51}$=5.80, P<0.01; Fig. 3B). In addition, results indicated a significant interaction between size and frequency ($F_{34,867}$=2.11, P<0.001), meaning that the effect of size on auditory sensitivity is frequency dependent. LSD *post hoc* tests showed significant group-specific differences between small and large juveniles at all test frequencies within 105 and 425 (except at 385 Hz). Auditory thresholds of large juveniles did not differ from that of adults at any frequency.

DISCUSSION

A major goal of this study was to investigate how the relatively large and complex vocal repertoire previously identified in the Lusitanian toadfish adults (Amorim et al., 2008) develops throughout ontogeny. Additionally, the focus of this study was to determine whether vocal differentiation parallels the development of the auditory sensitivity in this species. We showed that the number of stereotyped vocalisations increases in large juveniles above 5 cm standard length (SL) and that such vocal differentiation is coincident with an increase in saccular auditory sensitivity.

Ontogenetic vocal differentiation

The Lusitanian toadfish exhibited a gradual increase in the vocal repertoire, or number of stereotyped vocalisations, with increasing size throughout development. The vocal repertoire changed from simple broadband-pulsed grunts, mostly produced in fry and small juveniles, to four different call types, including amplitude-modulated and harmonic sounds (e.g. double croaks and boatwhistles) in large juveniles and adults. Interestingly, large juveniles already exhibited the full adult vocal repertoire, although differences in calling rates were found compared with the adults.

Previous studies in other fish species reported developmental changes in acoustic features of vocalisations such as amplitude, dominant frequency and temporal patterns (e.g. gurnards, Amorim and Hawkins, 2005; catfishes, Lechner et al., 2010; gouramis, Henglmüller and Ladich, 1999; Wysocki and Ladich, 2001). However, these studies never reported developmental changes in the vocal repertoire or vocal differentiation, probably because the vocal repertoire was more restricted and the sounds produced were not as elaborate as, for instance, those produced by batrachoidids (Amorim et al., 2008).

Vasconcelos and Ladich (2008) reported developmental changes in the acoustic features of grunt calls produced in a distress context by Lusitanian toadfish ranging from 3 to 32 cm SL. Dominant frequency, sound duration and number of pulses decreased, whereas pulse period and sound level increased with increasing fish size. These developmental changes were most likely associated with the growth of the sound-producing apparatus, i.e. swimbladder and intrinsic sonic muscles, which increase in size both ontogenetically and seasonally (Modesto and Canário, 2003a). Nevertheless, the development of the vocal motor pathways in the hindbrain may also have contributed for the vocal changes observed (Fine et al., 1984; Fine, 1989; Knapp et al., 1999).

In the present study, we investigated how the full vocal repertoire produced within a social context changes with growth in the Lusitanian toadfish, from fry to the adult stage. The vocal differentiation observed in this fish species most likely results from developmental changes in the premotor and motor vocal pathways located in the hindbrain. Knapp et al. (1999) reported that the vocal motor system, including sexual differentiation features, develops early in the larvae stage, well before any evidence of sexual maturation and vocal activity in *Porichthys notatus*

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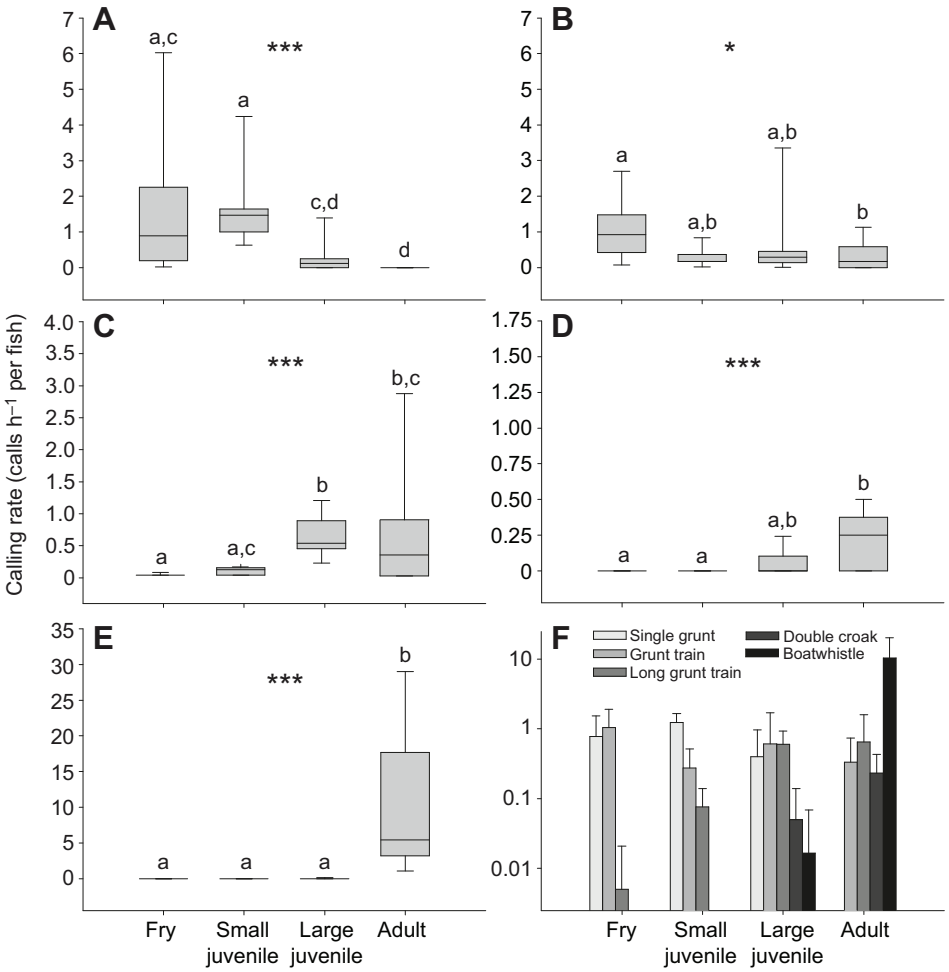

Fig. 2. Variation in the calling rates across different size groups for each sound type produced by Lusitanian toadfish. (A) Single grunt, (B) grunt train, (C) long grunt train, (D) double croak and (E) boatwhistle. Overall differences are based on Kruskal–Wallis tests, followed by pairwise comparison *post hoc* tests to verify group-specific differences. **P*<0.05; *** *P*<0.001. Data are medians ±10th, 25th, 75th and 90th percentiles. (F) Overall variation in the mean calling rate (±s.d.) of each sound type across different size groups. Groups: fry, <2.0 cm SL (*N*=12–20 fish, 10 sessions); small juveniles, 2.4–4.9 cm SL (*N*=17, 10); large juveniles, 5.0–8.69 cm SL (*N*=12, 10); adults, 25–35 cm (*N*=6, 10).

(Batrachoididae, subfamily Porichthinae). However, these authors also reported that motoneurons, which directly establish the firing rate of sonic muscles and vocal features, establish their connections with the sonic muscle prior to establishing connections with the premotor neurons in the hindbrain. More recently, Chagnaud and Bass (2014) showed that the central pattern generator (CPG) anatomy of two batrachoidids (*P. notatus* and *Opsanus tau*; subfamilies Batrachoidinae and Porichthinae, respectively) differs at the levels of both the pacemaker-motoneuron circuit and the afferent pre-pacemaker neurons and this may contribute to the species-specific patterning of frequency and amplitude-modulated vocalisations. Together, this suggests that the maturation of CPG and patterns of connectivity between hindbrain vocal nuclei may underline the vocal differentiation observed in the Lusitanian toadfish. Future studies should investigate this hypothesis and also whether steroid levels play a role in vocal patterning and plasticity, as reported for adult *P. notatus* (Bass, 2008). The ability to extend the vocal repertoire to include more elaborated calls through maturation of central vocal nuclei has been predominantly described in birds (Aronov et al., 2008) and mammals (Jürgens, 2002).

Behavioural observations of fry and juveniles suggested that the different vocalisations are produced during specific social contexts. Single grunts and grunt trains were typically emitted during agonistic interactions while competing for either food or territory, as observed by Vasconcelos and Ladich (2008) and Vasconcelos et al. (2010). Long grunt trains and double-croaks, which had so far been recorded only in adults in semi-natural conditions (Amorim et al., 2008), were also produced by juveniles inside their shelters but without any obvious social interaction or visual display, possibly to signal shelter occupancy. Finally, one of the most surprising results was that large juveniles of Lusitanian toadfish were capable of producing long harmonic boatwhistles. This signal was produced while the resident was facing an intruder, similar to agonistic boatwhistles previously recorded in territorial male adults (Vasconcelos et al., 2010). Such findings strongly suggest that similar to *P. notatus* (Knapp et al., 1999), the CPG must be fully developed at this developmental stage long before sexual maturity, which occurs around 30–35 cm total length for all morphotypes in this species (Pereira et al., 2011). Boatwhistles have been commonly described as mate advertisement calls among batrachoidids, used by nesting males to attract females for spawning (e.g. Vasconcelos et al., 2012). However, Vasconcelos et al. (2010) showed that Lusitanian toadfish also uses this signal in agonistic contexts during active nest defence. Although the sex of immature individuals used in the present study was not determined, it is likely that both males and females were present in the observation tanks. Our current findings further support the agonistic role of boatwhistles in this species, but future work should investigate potential sex-specific differences and the social role of the vocal repertoire in this species.

Ontogenetic development of peripheral auditory sensitivity

Comparisons of auditory sensitivity measured from populations of hair cells within the saccule across different size Lusitanian toadfish revealed a threefold ontogenetic increase in sensitivity at most test

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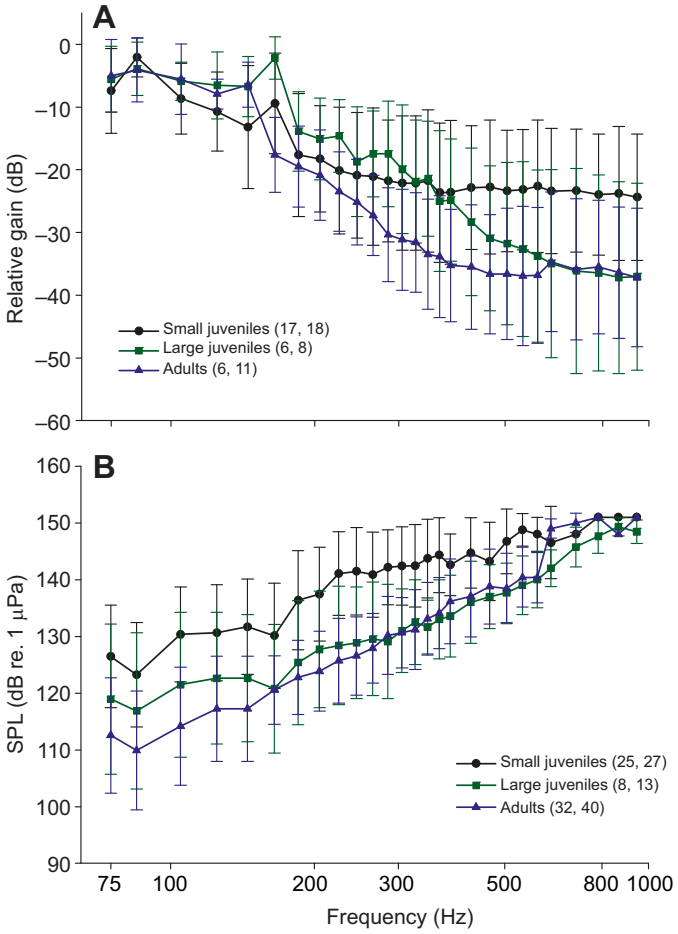

Fig. 3. Comparison of the mean relative gain and auditory threshold curves of the evoked potentials recorded from the saccule in Lusitanian toadfish of different size groups. (A) Comparison of the mean (±s.d.) relative gain curves based on responses to iso-level pure tones at 130 dB re. 1 µPa. The iso-level response data were normalised, i.e. a relative value of 0 dB was assigned to the peak response for each recording and the remaining data for other frequencies were expressed in relative dB (relative to best frequency). (B) Comparison of the mean (±s.d.) auditory threshold curves between different size groups. Number of animals and number of recordings per group, respectively, are indicated in parentheses. Group sizes: small juveniles, 2.4–4.9 cm; large juveniles, 5.0–8.69 cm; adults, 25–35 cm, standard length. SPL, sound pressure level.

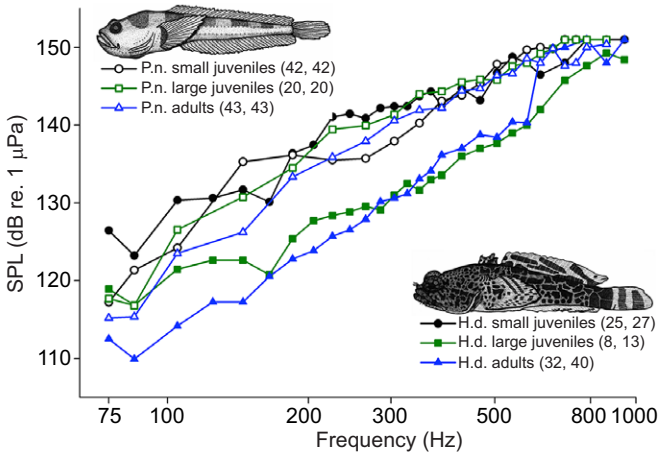

Fig. 4. Comparison of mean auditory threshold curves across different size groups in the Lusitanian toadfish and the midshipman fish. Two Batrachoidae species are compared: the Lusitanian toadfish (*Halobatrachus didactylus*; *H.d.*) and the midshipman fish (*Porichthys notatus*; *P.n.*). Note that within Lusitanian toadfish, small juveniles differed significantly from large juveniles and adults, which revealed higher auditory sensitivity and produced the full vocal repertoire. In contrast, in the midshipman fish, the auditory sensitivity of different size groups is similar and vocal behaviour has only been detected in adults (based on Alderks and Sisneros, 2011). Number of animals and number of recordings per group are indicated in parentheses. Illustrations represent a typical small juvenile of each species. SPL, sound pressure level.

frequencies. Saccular sensitivity increased by 10 dB between the smaller (2.4–4.9 cm SL) and the larger juveniles (5.0–8.7 cm), which already exhibited similar auditory thresholds to adults.

Vasconcelos and Ladich (2008), using the AEP recording technique, also reported reduced auditory sensitivity in small juveniles (2.8–3.8 cm SL, equivalent to the smallest size class used in this study) compared with large juveniles (5.4–6.6 cm SL) and adults. However, significant differences were only found at 100 Hz (~7 dB less) and at higher frequencies of 800 and 1000 Hz (~15 and 11 dB, respectively). Auditory thresholds recorded using the AEP technique were considerably lower, namely 22–40 dB less (within about 100–500 Hz), compared with those reported in this study using the auditory saccular potentials. The AEP recording technique measures the overall synchronous neural electrical activity induced by acoustic stimulation and includes the evoked responses from potentially one or more endorgans (i.e. saccule, lagena and utricle), the primary afferents of the eighth nerve and central auditory nuclei (Kenyon et al., 1998). Therefore, the summed AEP neural response is expected to be more complex and of different amplitude compared with the receptor-specific responses reported in this study. Saccular potential recordings reflect only the receptor potentials recorded from a limited population of hair cells within the saccular macula close to the recording electrode.

Our findings are in contrast to those reported from a study conducted in the batrachoidid *P. notatus*, which showed via saccular potential recordings that auditory threshold curves were similar throughout development (Alderks and Sisneros, 2011) (Fig. 4). The smallest juvenile midshipman fish tested (2–3 cm SL) had auditory sensitivity similar to that of large juveniles (7–8 cm SL) and adults (>9 cm SL), with auditory thresholds varying from ~115 dB re. 1 µPa (at 75–145 Hz) to 150 dB (at 945 Hz). Such species-specific differences within Batrachoididae may be due to their divergent life histories. An important behavioural difference between these two species is that *P. notatus* juveniles do not reveal aggressive territorial behaviour: they engage in far fewer social interactions than *H. didactylus*. The vocal activity of *P. notatus* seems to start much later in development, probably associated with sexual maturity and reproductive behaviours (our unpublished observations). Moreover, Vasconcelos et al. (2011) showed that there is a notable difference in seasonal and gender saccular sensitivity variation in these two species. While in *P. notatus* saccular sensitivity is increased during the breeding season when adult males start vocalising to attract females (Sisneros, 2009), in *H. didactylus* the sensitivity does not change seasonally and vocal activity is detected all year round (Vasconcelos et al., 2011).

Studies on the ontogeny of auditory sensitivity in fish have shown varying results. For instance, Popper (1971) and Zeddies and Fay (2005), using acoustically evoked behavioural responses, found no differences among different size goldfish *Carassius auratus* and zebrafish *Danio rerio*, respectively. Although in the latter, acoustic startle response thresholds were adjusted as the fish develop, in order to maintain appropriate reactions to relevant stimuli switching

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from particle motion, at larvae stage, to sound pressure sensitivity, in juveniles and adults. Yet, Higgs et al. (2003), using AEP recordings and sound pressure stimulation, reported an expansion of the maximum detectable frequency from 200 Hz to 4000 Hz with increasing zebrafish size, which coincided with the development of the Weberian ossicles and sensitivity to sound pressure.

Most studies conducted on fish, however, have revealed improvements in hearing abilities throughout development, similar to our data on the Lusitania toadfish and to other taxa (e.g. amphibians, Boatright-Horowitz and Simmons, 1995; reptiles, Werner et al., 1998; birds, Dmitrieva and Gottlieb, 1994; mammals, Reimer, 1995). For instance, Kenyon (1996), using behavioural conditioning techniques, reported ontogenetic increases in auditory sensitivity in the damselfish (*Stegastes partitus*) of 45 dB re. 1 µPa at their most sensitive frequency (300 Hz). Wysocki and Ladich (2001), using the AEP recording technique in different size croaking gourami (*Trichopsis vittata*), found an increase in sensitivity of about 14 dB re. 1 µPa at 0.8–3.0 kHz and a shift in the best frequency from 2.5 kHz to 1.5 kHz. Likewise, Lechner et al. (2010, 2011) reported a considerable increase in auditory sensitivity and shift in the best frequency with development in two catfish species. Lechner et al. (2010) reported an ontogenetic increase in *Synodontis schoutedeni* auditory sensitivity of 26 dB re. 1 µPa and a change in the best frequency range from 2–3 kHz down to 0.3–1 kHz. According to Lechner et al. (2011), auditory sensitivity in *Lophiobagrus cyclurues* increased up to 40 dB re. 1 µPa during ontogeny and the small juveniles were unable to detect frequencies higher than 2–3 kHz, whereas large juveniles showed best sensitivity to higher frequencies of 4–6 kHz. Recently, Caiger et al. (2013) described an ontogenetic enhancement of auditory abilities in the hapuka (*Polyprion oxygeneios*) based on AEP measurements, i.e. a 22× increase in auditory sensitivity and an expansion of the auditory bandwidth (from the maximum 800 Hz to 1000 Hz) from larvae to the juvenile stage.

Although relatively few studies have examined the structure–function relationships in the developing fish ear, increases in auditory sensitivity during ontogeny in fish have been typically explained as a result of general inner ear development with continued addition of hair cells (Popper and Hoxter, 1984; Lombarte and Popper, 1994; Lu and DeSmidt, 2013), number of auditory nerve ganglion cells, innervation patterns of the eighth nerve (Corwin, 1983; Popper and Hoxter, 1984) and/or increase in swimbladder size and development of the morphological connections (Weberian ossicles) to the ear (Lechner et al., 2011). Another possibility could be the otolith enlargement with fish growth, providing increased inertial mass for the accelerometer-like auditory endorgan (Gauldie, 1988).

Future work should identify potential morphological differences in the inner ear throughout ontogeny in the Lusitanian toadfish, with particular focus on early developmental stages (e.g. hair cell addition and density; otolith size and density) that may contribute to the changes observed in the peripheral auditory sensitivity. This species does not present accessory morphological hearing structures and the swimbladder shape may actually shield the nearby ears during sound production (Barimo and Fine, 1998). Nevertheless, the potential effect of the swimbladder on auditory sensitivity especially at lower frequencies has yet to be investigated.

Parallel development of vocal–auditory systems

In this study we show that the developmental stage, when large juveniles start producing the full adult vocal repertoire, is coincident with a significant enhancement in auditory saccular sensitivity. Our data provide clear evidence that the development of the vocal motor control system parallels the development of the peripheral auditory system in a vocal fish species. We suggest that this parallel development may serve an important and conserved function in the development of social acoustic communication among vertebrates.

Juveniles of vocal vertebrate species often gradually transform primitive unstructured vocalisations into complex, stereotyped calls that constitute the adult repertoire (e.g. birds, Tchernichovski et al., 2001; Derégnaucourt et al., 2009). Among birds and mammals, previous research showed that auditory feedback is crucial during the vocal differentiation phase and that the acoustic signals perceived from the auditory–vocal environment play an important role in the refinement of the neural connections within the vocal circuitry (e.g. Miller-Sims and Bottjer, 2012). The onset of coupling between the vocal motor and auditory system throughout the evolution of vertebrates remains unknown. Future studies should investigate the potential coupling of auditory–vocal systems in fish by testing whether early acoustic experience can shape vocal behaviour. Such studies have the potential to ultimately shed light onto the mechanisms and evolution of vocal behaviour common to all vertebrates (Bass et al., 2008).

MATERIALS AND METHODS

Animals

Toadfish were collected in Portugal, mostly from the Tagus River estuary but also from the Mira River estuary (only the small and large juveniles used for auditory measurements were collected from this location). Collections were carried either by trawling or directly from an intertidal nesting area from February to June. Although we are aware of size differences among populations from Tagus and Mira (Costa, 2004), this study mostly focuses on comparisons of either auditory sensitivity or sound production among animals from the same population.

Based on previous behavioural observations and auditory information (Vasconcelos and Ladich, 2008), fish were classified into four different size groups: fry, 1.7–2.00 cm standard length (SL), 0.19–0.30 g body mass; small juveniles, 2.4–4.9 cm SL, 0.60–3.10 g; large juveniles, 5.0–10.60 cm SL, 3.17–15.02 g; and adults, 25–35 cm SL, 279–651 g. Fish within these size ranges, with the exception of the smallest group (fry: N=12–20) that was only studied in terms of vocal behaviour, were either used for saccular sensitivity measurements (small juveniles: N=25; large juveniles: N=8; adults: N=32, including 27 type I or nest-guarding males and 5 females) or vocal recordings (small juveniles: N=17, large juveniles: N=12, adults: N=6). Note that type I males nest under rocks in the breeding season from where they vocalize in choruses to attract gravid females and care for the offspring, in contrast to the smaller and less vocal type II or sneaking males, which attempt opportunistic fertilisation (Modesto and Canário, 2003b). The juvenile groups used in this study represent early development stages and were probably less than one year old (Pereira et al., 2011). The Lusitanian toadfish reaches sexual maturity at 30–35 cm total length (around 4–6 years old) in females and both male morphotypes (Pereira et al., 2011).

All fish, with the exception of the adults recorded in the field, were transported to the laboratory at the University of Lisbon (Lisbon, Portugal) where they were kept in stock aquaria (juveniles in 50–60 litre tanks; adults in 80 litre tanks) and maintained at 21±2°C under a 12 h light:12 h dark cycle. Vocal–acoustic recordings of fry and juveniles were carried out in the same laboratory, while those of adults were performed in semi-natural conditions in the intertidal area. For saccular potential recordings, both small and large juveniles and adults were shipped to the University of Washington (Seattle, WA, USA), where they were maintained in 40 litre and 80–250 litre tanks, respectively, under similar light and temperature conditions.

After auditory recordings fish were killed by immersion in a 0.025% ethyl p-aminobenzoate saltwater bath. For the adults, both sex and male types were confirmed (Modesto and Canário, 2003b).

Electrophysiological experiments followed National Institutes of Health guidelines for the care and use of animals and were approved by the University of Washington Institutional Animal Care and Use Committee.

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All behavioural experimental procedures comply with Portuguese animal welfare laws, guidelines and policies. All animals used in this study, including those shipped from Portugal to the University of Washington (USA), adapted rapidly to captivity and behaved normally in the stock tanks, which suggests that these animals were not exposed to overly stressful conditions.

Sound recordings

In order to investigate vocal differentiation among fry and juveniles, a group of each size class was placed in 50–60 litre recording tanks (fry: N=12–20, small juveniles: N=17, large juveniles: N=12 fish) provided with several shelters (halved ceramic flower pots), sand substrate, and maintained at 22±2°C. 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^{-1}) were placed in the middle of each recording tank at about 10–20 cm apart and ∼1–2 cm from the bottom. Hydrophones were high pass filtered (>10 Hz) for decoupling the DC component, and connected to an audiocapture device Edirol UA-25 (Roland, Osaka, Japan; 16 bit, 44.1 kHz acquisition rate per channel) and then to a laptop, to perform double-channel recordings down-sampled to 8 kHz by Adobe Audition 3.0 (Adobe Systems Inc., San José, CA, USA). Sound recordings were performed for 60 min. Fish behaviour was monitored throughout the recording session in order to verify the behavioural context of sound production. Food was provided at ∼30 min from the start of recording to stimulate social interactions and sound production. A total of 10 consecutive sessions (1−2 per day) were carried out for each group.

The vocal repertoire of toadfish adults was recorded under semi-natural conditions following the method described by Vasconcelos et al. (2010, 2012). Briefly, during the toadfish breeding season (May–June), males that spontaneously occupied six concrete shelters placed close to the lower limit of an intertidal area of the Tagus estuary were recorded with a similar audio chain as above: a High Tech 94 SSQ hydrophone placed next to each nest, connected to an audiocapture device (M-Audio Fast Track Ultra 8R, Cumberland, RI, USA) and then to a laptop controlled by Adobe Audition 3.0. Nest entrances were closed with a plastic net to prevent fish from escaping and to ensure male identity throughout the recordings. Plastic nets did not affect acoustic signals and allowed both the entrance of small prey and possible interactions with free-swimming conspecifics. We continuously recorded six type I adult males for 10 days and randomly selected a 1 h session from each day for sound analysis.

Sound recordings were analysed using Raven 1.2 for Windows (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY, USA). Toadfish vocalisations were identified based on previous works (Amorim et al., 2008; Vasconcelos et al., 2010). Sounds were classified into: single grunts, grunt trains, long grunt trains, double croaks and boatwhistles. In this study, croak-like sounds were classified as single grunts because in juveniles dominant frequencies are higher and single grunts are typically longer (Vasconcelos and Ladich, 2008). This makes it difficult to distinguish single grunts, commonly produced among juveniles, from croaks previously reported in adults (Amorim et al., 2008).

The calling rate for each sound type was determined per hour/session and then averaged per group for all size classes. In the recordings of fry and juveniles conducted in tanks, it was sometimes not clear which individual was vocalising. Therefore, calling rates were calculated based on the total number of sounds divided by the number of individuals present in the tank.

Although sound production was pooled from single groups for each size class, all animals used in this study were active and displayed social behaviour towards conspecifics, including the adults that attracted females to their nests. Therefore, we are confident that our sound recordings reflect size-specific socially relevant vocal activity. Also, data collected on sound production were not intended to thoroughly describe the association between specific vocalisations and the behavioural context, as this would have required a larger sample size. Our intent was instead to detect broad size-related differences in the vocal repertoire that gradually occur with fish development.

Measuring peripheral auditory sensitivity

We used the evoked saccular potential recording method to measure auditory thresholds of populations of hair cells within the saccule based on previous studies (Sisneros, 2007; Vasconcelos et al., 2011). Briefly, fish were first anaesthetised in a 0.025% ethyl p-aminobenzoate saltwater bath and then immobilised by an intramuscular injection of pancuronium bromide: juveniles, ∼0.5 mg kg^{-1}; adults, 2–4 mg kg^{-1}. The saccule was then exposed by dorsal craniotomy and a barrier of denture cream was built up around the cranial cavity to allow the fish to be lowered below the water surface. A teleost ringer solution was used to prevent the cranial cavity from drying out and to dilute any bleeding.

Test subjects were placed in a round saltwater-filled tank (30 cm diameter, 24 cm high) and positioned 10 cm above an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA) that was embedded in gravel in the centre of the tank. During experiments, fresh seawater (at 21±1°C) was perfused through the fish's mouth and over the gills. The recording tank was placed on a vibration-isolated table housed inside an acoustic attenuation chamber (Industrial Acoustics, New York, NY, USA) with all the recording and stimulus generation equipment located outside the chamber.

Acoustic stimuli were generated via the reference output signal of a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA) that passed the stimulus signal through an audio amplifier to the underwater loud speaker. Prior to each experiment, we tested the speaker's frequency response by placing a mini-hydrophone (8103, Bruel and Kjaer; Naerum, Denmark) in the position later occupied by the fish's head, and then measured the peak-to-peak voltage on an oscilloscope. This peak-to-peak voltage was used in conjunction with an automated compensation script written for Matlab (MathWorks, Natick, MA, USA) to calibrate the speaker so that pressure level at all test frequencies was of equal amplitude within ±2 dB re. 1 µPa. This calibration procedure compensates for any resonant frequencies of the tank to ensure that all test frequencies used were of equal amplitude within 2 dB (i.e. the amplitude response of the speaker within the tank was flat across all test frequencies). Sound pressure measurements of the stimulus frequencies were measured using a spectrum analyser (Stanford Research Systems SR780). Auditory stimuli consisted of eight repetitions of single 500 ms tones from 75 Hz to 945 Hz (in 10–80 Hz increments) presented randomly at a rate of one every 1.5 s.

Saccular potentials were recorded with glass electrodes filled with 3 mol l^{-1} KCl (0.5–6 MΩ). Electrodes were visually guided and placed in the middle region of the saccular macula in either the left or right saccule. Analog saccular potentials were preamplified (5A microelectrode amplifier, Getting Instruments, San Diego, CA, USA), input into a lock-in amplifier (SR830, Stanford Research Systems) and then stored on a computer running a custom data acquisition Matlab script. The lock-in amplifier yields a DC voltage output that is proportional to the component of the signal whose frequency is locked to the reference frequency. The reference frequency was set to twice the stimulation frequency, the amplifier sensitivity set to 50 mV and the time constant to 100 ms. We used the 2nd harmonic of the stimulus (twice the stimulus frequency) as the reference frequency because the greatest evoked potential from the saccule in teleost fishes typically occurs at twice the stimulus frequency due to the nonlinear response and opposite orientation of hair cell populations within the saccule (Cohen and Winn, 1967). To estimate auditory thresholds, the saccular potentials were recorded in response to single tone stimuli that were reduced in 3 dB steps until the saccular response (mean voltage of eight evoked saccular potential measurements) was no longer above background noise (mean voltage measured without acoustic stimulation) ±2 s.d.

Background noise measurements were performed prior to the recording of each threshold-tuning curve. Noise measurements followed the same procedure of saccular potentials recordings except with the loud speaker turned off. The background noise levels were consistently measured from the recording electrode between 2–5 µV.

Although toadfishes possess no hearing specialisations and thus are primarily sensitive to particle motion (Fay and Edds-Walton, 1997), we report in this study auditory thresholds based on sound pressure levels (SPLs) for both technical reasons and comparison purposes with previous studies using batrachoidid fishes (e.g. Sisneros, 2007; Alderks and Sisneros, 2011; Vasconcelos et al., 2011). Our aim was to compare the saccular sensitivity between different size groups under identical experimental conditions. Therefore, the auditory thresholds presented should not be

considered as absolute values but instead as reliable data to perform quantifiable comparisons between the different size/age groups.

Statistical analysis

Saccular sensitivity threshold curves and normalised iso-level curves from different size groups were compared by repeated-measures ANOVA, analysing auditory thresholds or iso-level responses to several frequencies (75–425 Hz) in each fish (within subject factor) of different size groups (between-subject factor). LSD *post hoc* tests were used to verify pairwise group-specific differences. The threshold values at the best frequency (BF, defined as the frequency that evoked the lowest saccular potential threshold) were compared across groups with one-way ANOVA.

To analyse the development of the acoustic repertoire, calling rates for each sound type from different size groups were compared with Kruskal–Wallis, followed by pairwise comparison *post hoc* tests to verify group specific differences. Parametric tests were used only when data were normally distributed and variances were homogeneous. The statistical analysis was performed using IBM SPSS v22 (IBM, Edicott, NY, USA) and Statistica 7.1 (StatSoft, Tulsa, OK, USA).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.O.V. was responsible for conception of the study and experimental design, performed most of data collection and analysis, and drafted the article. P.W.A. contributed to auditory data collection and scientific drawings. A.R. contributed to vocal data collection. P.J.F., M.C.P.A. and J.A.S. were involved in the conception of the study and supervision of the experimental work. All authors revised the manuscript prior to submission.

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References

Alderks, P. W. and Sisneros, J. A. (2011). Ontogeny of auditory saccular sensitivity in the plainfin midshipman fish, *Porichthys notatus*. *J. Comp. Physiol. A* **197**, 387-398.

Amorim, M. C. P. and Hawkins, A. D. (2005). Ontogeny of acoustic and feeding behaviour in the grey gurnard, *Eutrigla gurnardus*. *Ethology* **111**, 255-269.

Amorim, M. C. P., Simões, J. M. and Fonseca, P. J. (2008). Acoustic communication in the Lusitanian toadfish, *Halobatrachus didactylus*: evidence for an unusual large vocal repertoire. *J. Mar. Biol. Assoc. UK* **88**, 1069-1073.

Aronov, D., Andalman, A. S. and Fee, M. S. (2008). A specialized forebrain circuit for vocal babbling in the juvenile songbird. *Science* **320**, 630-634.

Barimo, J. F. and Fine, M. L. (1998). Relationship of swim-bladder shape to the directionality pattern of underwater sound in the oyster toadfish. *Can. J. Zool.* **76**, 134-143.

Bass, A. H. (2008). Steroid-dependent plasticity of vocal motor systems: novel insights from teleost fish. *Brain Res. Rev.* **57**, 299-308.

Bass, A. H., Gilland, E. H. and Baker, R. (2008). Evolutionary origins for social vocalization in a vertebrate hindbrain–spinal compartment. *Science* **321**, 417-421.

Boatright-Horowitz, S. S. and Simmons, A. M. (1995). Postmetamorphic changes in auditory sensitivity of the bullfrog midbrain. *J. Comp. Physiol. A* **177**, 577-590.

Brainard, M. S. and Doupe, A. J. (2002). What songbirds teach us about learning. *Nature* **417**, 351-358.

Caiger, P. E., Montgomery, J. C., Bruce, M., Lu, J. and Radford, C. A. (2013). A proposed mechanism for the observed ontogenetic improvement in the hearing ability of hapuka (*Polyprion oxygeneios*). *J. Comp. Physiol. A* **199**, 653-661.

Chagnaud, B. P. and Bass, A. H. (2014). Vocal behavior and vocal central pattern generator organization diverge among toadfishes. *Brain Behav. Evol.* **84**, 51-65.

Cohen, M. J. and Winn, H. E. (1967). Electrophysiological observations on hearing and sound production in the fish, *Porichthys notatus*. *J. Exp. Zool.* **165**, 355-369.

Corwin, J. T. (1983). Postembryonic growth of the macula neglecta auditory detector in the ray, *Raja clavata*: continual increases in hair cell number, neural convergence, and physiological sensitivity. *J. Comp. Neurol.* **217**, 345-356.

Costa, J. L. (2004). A biologia do xarroco, *Halobatrachus didactylus* (Bloch & Schneider, 1801), e o seu papel na estruturação e funcionamento das comunidades em que se insere; referência especial à população do estuário do Mira. Ph.D. thesis, Universidade de Lisboa, Lisbon, Portugal.

Derégnaucourt, S., Saar, S. and Gahr, M. (2009). Dynamics of crowing development in the domestic Japanese quail (*Coturnix coturnix japonica*). *Proc. R. Soc. B Biol. Sci.* **276**, 2153-2162.

Dmitrieva, L. P. and Gottlieb, G. (1994). Influence of auditory experience on the development of brain stem auditory-evoked potentials in mallard duck embryos and hatchlings. *Behav. Neurol. Biol.* **61**, 19-28.

Doupe, A. J. and Kuhl, P. K. (1999). Birdsong and human speech: common themes and mechanisms. *Annu. Rev. Neurosci.* **22**, 567-631.

Fay, R. R. and Edds-Walton, P. L. (1997). Diversity in frequency response properties of saccular afferents of the toadfish, *Opsanus tau*. *Hear. Res.* **113**, 235-246.

Fine, M. L. (1989). Embryonic, larval and adult development of the sonic neuromuscular system in the oyster toadfish. *Brain Behav. Evol.* **34**, 13-24.

Fine, M. L., Economos, D., Radtke, R. and McClung, J. R. (1984). Ontogeny and sexual dimorphism of the sonic motor nucleus in the oyster toadfish. *J. Comp. Neurol.* **225**, 105-110.

Gauldie, R. W. (1988). Function, form and time-keeping properties of fish otoliths. *Comp. Biochem. Physiol. A Physiol.* **91**, 395-402.

Henglmüller, S. M. and Ladich, F. (1999). Development of agonistic behaviour and vocalization in croaking gouramis. *J. Fish Biol.* **54**, 380-395.

Higgs, D. M., Souza, M. J., Wilkins, H. R., Presson, J. C. and Popper, A. N. (2002). Age- and size-related changes in the inner ear and hearing ability of the adult zebrafish (*Danio rerio*). *J. Assoc. Res. Otolaryngol.* **3**, 174-184.

Higgs, D. M., Rollo, A. K., Souza, M. J. and Popper, A. N. (2003). Development of form and function in peripheral auditory structures of the zebrafish (*Danio rerio*). *J. Acoust. Soc. Am.* **113**, 1145-1154.

Hollén, L. I. and Radford, A. N. (2009). The development of alarm call behaviour in mammals and birds. *Anim. Behav.* **78**, 791-800.

Jürgens, U. (2002). Neural pathways underlying vocal control. *Neurosci. Biobehav. Rev.* **26**, 235-258.

Kenyon, T. N. (1996). Ontogenetic changes in the auditory sensitivity of damselfishes (Pomacentridae). *J. Comp. Physiol. A* **179**, 553-561.

Kenyon, T. N., Ladich, F. and Yan, H. Y. (1998). A comparative study of hearing ability in fishes: the auditory brainstem response approach. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* **182**, 307-318.

Knapp, R., Marchaterre, M. A. and Bass, A. H. (1999). Early development of the motor and premotor circuitry of a sexually dimorphic vocal pathway in a teleost fish. *J. Neurobiol.* **38**, 475-490.

Lechner, W., Wysocki, L. E. and Ladich, F. (2010). Ontogenetic development of auditory sensitivity and sound production in the squeaker catfish *Synodontis schoutedeni*. *BMC Biol.* **8**, 10.

Lechner, W., Heiss, E., Schwaha, T., Glösmann, M. and Ladich, F. (2011). Ontogenetic development of Weberian ossicles and hearing abilities in the African bullhead catfish. *PLoS ONE* **6**, e18511.

Lombarte, A. and Popper, A. N. (1994). Quantitative analyses of postembryonic hair cell addition in the otolithic endorgans of the inner ear of the European hake, *Merluccius merluccius* (Gadiformes, Teleostei). *J. Comp. Neurol.* **345**, 419-428.

Lu, Z. and DeSmidt, A. A. (2013). Early development of hearing in zebrafish. *J. Ass. Res. Otolaryngol.* **14**, 509-521.

Miller-Sims, V. C. and Bottjer, S. W. (2012). Auditory experience refines cortico-basal ganglia inputs to motor cortex via re-mapping of single axons during vocal learning in zebra finches. *J. Neurophysiol.* **107**, 1142-1156.

Modesto, T. and Canário, A. V. M. (2003a). Hormonal control of swimbladder sonic muscle dimorphism in the Lusitanian toadfish *Halobatrachus didactylus*. *J. Exp. Biol.* **206**, 3467-3477.

Modesto, T. and Canário, A. V. M. (2003b). Morphometric changes and sex steroid levels during the annual reproductive cycle of the Lusitanian toadfish, *Halobatrachus didactylus*. *Gen. Comp. Endocrinol.* **131**, 220-231.

Moore, D. R. (2002). Auditory development and the role of experience. *Br. Med. Bull.* **63**, 171-181.

Moss, C. F., Redish, D., Gounden, C. and Kunz, T. H. (1997). Ontogeny of vocal signals in the little brown bat, *Myotis lucifugus*. *Anim. Behav.* **54**, 131-141.

Myrberg, A. A., Jr, Ha, S. J. and Shamblott, M. J. (1993). The sounds of bicolor damselfish (*Pomacentrus partitus*): predictors of body size and a spectral basis for individual recognition and assessment. *J. Acoust. Soc. Am.* **94**, 3067-3070.

Pereira, T. J., Silva, G., Costa, M. J. and Costa, J. L. (2011). Life strategies of *Halobatrachus didactylus* (Bloch and Schneider, 1801) in the Tagus estuary: comparison among different morphotypes. *Estuar. Coast Shelf Sci.* **93**, 328-335.

Popper, A. N. (1971). The effects of fish size on auditory capacities of the goldfish. *J. Aud. Res.* **11**, 239-247.

Popper, A. N. and Hoxter, B. (1984). Growth of a fish ear: 1. Quantitative analysis of hair cell and ganglion cell proliferation. *Hear. Res.* **15**, 133-142.

Reimer, K. (1995). Ontogeny of hearing in the marsupial, *Monodelphis domestica*, as revealed by brainstem auditory evoked potentials. *Hear. Res.* **92**, 143-150.
Rübsamen, R. (1992). Postnatal development of central auditory frequency maps. *J. Comp. Physiol. A* **170**, 129-143.
Sisneros, J. A. (2007). Saccular potentials of the vocal plainfin midshipman fish, *Porichthys notatus*. *J. Comp. Physiol. A* **193**, 413-424.
Sisneros, J. A. (2009). Adaptive hearing in the vocal plainfin midshipman fish: getting in tune for the breeding season and implications for acoustic communication. *Integr. Zool.* **4**, 33-42.
Sisneros, J. A. and Bass, A. H. (2005). Ontogenetic changes in the response properties of individual, primary auditory afferents in the vocal plainfin midshipman fish *Porichthys notatus* Girard. *J. Exp. Biol.* **208**, 3121-3131.
Tchernichovski, O., Mitra, P. P., Lints, T. and Nottebohm, F. (2001). Dynamics of the vocal imitation process: how a zebra finch learns its song. *Science* **291**, 2564-2569.
Vasconcelos, R. O. and Ladich, F. (2008). Development of vocalization, auditory sensitivity and acoustic communication in the Lusitanian toadfish *Halobatrachus didactylus*. *J. Exp. Biol.* **211**, 502-509.
Vasconcelos, R. O., Simões, J. M., Almada, V. C., Fonseca, P. J. and Amorim, M. C. P. (2010). Vocal behavior during territorial intrusions in the Lusitanian toadfish: boatwhistles also function as territorial 'keep-out' signals. *Ethology* **116**, 155-165.
Vasconcelos, R. O., Sisneros, J. A., Amorim, M. C. P. and Fonseca, P. J. (2011). Auditory saccular sensitivity of the vocal Lusitanian toadfish: low frequency tuning allows acoustic communication throughout the year. *J. Comp. Physiol. A* **197**, 903-913.
Vasconcelos, R. O., Carriço, R., Ramos, A., Modesto, T., Fonseca, P. J. and Amorim, M. C. P. (2012). Vocal behavior predicts reproductive success in a teleost fish. *Behav. Ecol.* **23**, 375-383.
Werner, Y. L., Montgomery, L. G., Safford, S. D., Igić, P. G. and Saunders, J. C. (1998). How body size affects middle-ear structure and function and auditory sensitivity in gekkonoid lizards. *J. Exp. Biol.* **201**, 487-502.
Wysocki, L. E. and Ladich, F. (2001). The ontogenetic development of auditory sensitivity, vocalization and acoustic communication in the labyrinth fish *Trichopsis vittata*. *J. Comp. Physiol. A Sens Neural Behav. Physiol.* **187**, 177-187.
Zeddies, D. G. and Fay, R. R. (2005). Development of the acoustically evoked behavioral response in zebrafish to pure tones. *J. Exp. Biol.* **208**, 1363-1372.