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DISSERTATION

Titel der Dissertation

Hearing in catfishes: Ontogenetic development, acoustic communication and albinism
(Hören bei Welsen: Ontogenetische Entwicklung, Lautkommunikation und Albinismus)

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Mag. Walter Lechner

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Doktor der Naturwissenschaften (Dr. rer.nat.)

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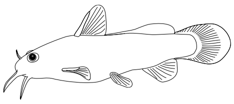

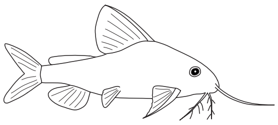

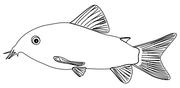

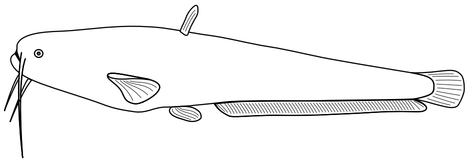

Summary

More than 30,000 species of fish inhabit our planet and many of them are able to produce sounds and communicate acoustically. Bony fishes show a high variety of sound producing mechanisms and also a great diversity in hearing abilities. While all fishes are able to perceive the particle motion component of sound, several groups have evolved adaptations to perceive sound pressure by coupling gas filled chambers to their inner ears. These fish groups, frequently termed "hearing specialists", are able to detect sounds of lower levels and higher frequencies in comparison to groups which lack hearing specializations. Otophysines are the largest group among "hearing specialists". They are characterized by the Weberian apparatus, a chain of ossicles ("Weberian ossicles" – tripus, intercalarium, scaphium and claustrum) which transmits oscillations of the bladder in a sound field to the inner ear. Otophysines comprise approximately 8,000 species and consist of four orders, Cypriniformes (carps, loaches and relatives), Characiformes (tetras), Gymnotiformes (South American knife fishes), and Siluriformes (catfishes). They inhabit freshwaters all over the world.

Catfishes which comprise more than 3,000 species are found in all continents but Antarctica and belong to the most successful groups of bony fishes. They reveal a high diversity in the anatomy of their Weberian apparatus and swimbladders and many of them are able to produce sounds utilizing their pectoral spines and/or their swimbladders.

While the anatomy and the homologies of the Weberian apparatus have been studied intensively since its discovery in the beginning of the 19th century, much less is known about the abilities to detect sounds and communicate acoustically. Only few papers deal with the ontogenetic development of hearing and sound communication in fishes. No work has been done so far to study the influence of genetic disorders such as albinism in fishes which potentially affects hearing in mammals.

The aim of this thesis is to investigate the ontogenetic development of hearing sensitivities, of the ability to detect communication sounds, of the Weberian apparatus, and of the influence of albinism on hearing in catfishes.

The development of acoustic communication was studied in the yellow marbled squeaker catfish *Synodontis schoutedeni* from 22 mm (young) to 126 mm standard length (adult). The noninvasive auditory evoked potential (AEP) recording technique was utilized to measure hearing abilities. The production of stridulatory sounds and the movement of pectoral fins were recorded using highly sensitive hydrophones and video technique. The sound pressure levels as well as various sound characteristics (temporal, spectral) were measured and fin movements analysed. The smallest juveniles showed the poorest hearing abilities of all size groups between 50 and 1,000 Hz and highest hearing sensitivity at 5 and 6 kHz. The duration of sounds emitted during abduction and adduction of pectoral spines, the pulse period within stridulation sounds and sound pressure level (in animals smaller than 58 mm) increased, while the dominant frequency of sounds decreased with size in animals larger than 37 mm. Comparisons between

audiograms and sound spectra revealed that the most sensitive frequencies correlate with the dominant frequencies of stridulation sounds in all *S. schoutedeni* size groups and that all specimens are able to detect sounds of all size groups.

The relationship between the ontogenetic development of the accessory hearing structures (Weberian ossicles) and development of hearing have been studied in the African bullhead catfish *Lophiobagrus cyclurus*. Specimens were studied from postlarval stages (11.3 mm standard length) to adults (85.5 mm). Hearing abilities were measured using the AEP recording technique. The morphology of the Weberian ossicles was assessed using dissections, histological sections, and X-ray computed tomography, along with 3D-reconstruction. In the smallest size group the tripus was not yet fully developed and the intercalarium and the interossicular ligaments were still missing. Smallest juveniles revealed lowest auditory sensitivity and were unable to detect frequencies higher than 2 or 3 kHz. In all larger stages investigated Weberian ossicles and interossicular ligaments were fully developed. Hearing sensitivity increased in larger specimens by up to 40 dB and frequencies were detected up to 6 kHz. In the size groups capable to perceive frequencies up to 6 kHz larger individuals revealed better hearing abilities at low frequencies (0.05 to 1 kHz), whereas an opposite trend was observed at the highest frequencies tested (4 to 6 kHz).

Albinism is often accompanied by hearing deficiencies in mammals. Our study is the first one looking at this phenomenon in non-mammalian vertebrates. We measured hearing sensitivities of normal coloured and albinotic specimens of the European wels *Silurus glanis* and the South American bronze catfish *Corydoras aeneus* utilizing the AEP recording technique. We observed that neither auditory sensitivity nor shape of AEP waveforms differed between normally pigmented and albinotic specimens at any frequency tested in two catfish species.

The data of the study in *Lophiobagrus cyclurus* indicate that the ability to detect sounds at low levels and high frequencies largely depends on the development of the Weberian apparatus in otophysine fish. A significant increase in auditory sensitivity was observed as soon as all Weberian ossicles and interossicular ligaments were present and the chain for transmitting sounds from the swimbladder to the inner ear was complete. The studies in *Lophiobagrus cyclurus* and *Synodontis schoutedeni* are the first to demonstrate that absolute hearing sensitivities change during ontogeny in otophysine fish. This is in contrast to the results of prior studies on two cypriniform fish species in which no such change could be observed. Furthermore, *Synodontis schoutedeni* can detect conspecific sounds at all developmental stages, again contrasting with all prior ontogenetic studies.

Finally, genetic disorders such as albinism probably do not affect hearing in fishes. This may be due to the lack of melanin in the inner ear of fishes.

This thesis contributes to our understanding of the development of hearing and acoustic communication in fishes, in particular in freshwater fishes. Nowadays, the growing pressure of humans on aquatic ecosystems and the influences of noise pollution on the acoustic scene of these habitats potentially affect acoustic orientation and communication of fishes. This may

have implications for distribution and population dynamics in particular in fish with a highly developed sense of hearing.

Zusammenfassung

Auf unserem Planeten leben heute mehr als 30000 Fischarten und viele von ihnen sind dazu imstande Laute zu produzieren und akustisch zu kommunizieren. Die Knochenfische zeigen eine große Diversität an Lautbildungs-Mechanismen und Hörfähigkeiten. Alle Fische sind in der Lage die Teilchenbewegungskomponente von Schall wahrzunehmen. Einige Gruppen haben zusätzlich Mechanismen entwickelt um die Schalldruckkomponente wahrnehmen zu können, indem sie gasgefüllte Räume mit dem Innenohr in Verbindung bringen. Diese Fischgruppen, häufig auch „Hörspezialisten" genannt, können Schall von geringeren Pegeln und auch höherer Frequenzen wahrnehmen, als jene Fische, welchen solche akzessorische Hörstrukturen fehlen. Die Otophysi sind die artenreichste Gruppe unter den "Hörspezialisten". Sie besitzen ein gemeinsames charakteristisches Merkmal, den Weberschen Apparat, eine Kette kleiner Knöchelchen („Webersche Knöchelchen" – Tripus, Intercalarium, Scaphium und Claustrum), welche Schwingungen der Schwimmblase zum Innenohr übertragen. Zur Überordung der Otophysi zählen etwa 8000 Arten in vier Ordnungen, den Cypriniformes (Karpfenartige), Characiformes (Salmlerartige), Gymnotiformes (Südamerikanische Messerfische) und den Siluriformes (Welse). Sie sind weltweit in Süßgewässern verbreitet.

Es gibt mehr als 3000 Arten von Welsen, und diese kommen auf allen Kontinenten mit Ausnahme der Antarktis vor. Welse zählen zu den erfolgreichsten Knochenfischgruppen. Sie zeigen eine große Diversität bezüglich der Ausbildung ihres Weberschen Apparates und ihrer Schwimmblase und viele Welsarten sind dazu imstande, mit ihren Brustflossenstacheln und/oder Schwimmblasen Laute zu erzeugen.

Die Anatomie und die Homologiebeziehungen des Weberschen Apparates waren seit seiner Entdeckung im frühen 19. Jahrhundert Gegenstand zahlreicher Forschungen; über die Fähigkeit Schall wahrzunehmen und akustisch zu kommunizieren ist hingegen bei Fischen weitaus weniger bekannt. Nur sehr wenige Arbeiten beschäftigen sich mit der Ontogenie des Hörens und der Lautkommunikation bei Fischen. Über den Einfluss von genetischen Anomalien wie Albinismus, der bei Säugetieren zu Hörbeeinträchtigungen führen kann, ist bisher bei Fischen gar keine Arbeit erschienen.

Das Ziel dieser Doktorarbeit ist es, die ontogenetische Entwicklung des Hörens und der Fähigkeit zur Lautkommunikation, des Weberschen Apparates, sowie den Einfluss von Albinismus auf das Hören bei Welsen zu untersuchen.

Am Marmorierten Fiederbartwels *Synodontis schoutedeni* wurde die Entwicklung der Lautkommunikation an Individuen von 22 mm (Jungtiere) bis zu 126 mm Standardlänge (voll ausgewachsen) untersucht. Das Hörvermögen wurde in dieser und den folgenden Untersuchungen mit Hilfe der nichtinvasiven Ableitung auditorisch evozierter Potentiale (AEP-Tech-

nik) gemessen. Die Laute der Welse wurde mit hochsensiblen Hydrophonen aufgezeichnet, ihr Schalldruckpegel ermittelt, die Bewegung der Brustflossen während der Produktion von Stridulationslauten mittels Videotechnik analysiert und verschiedene zeitliche und spektrale Schallcharakteristika ausgewertet. Die kleinsten Jungtiere zeigten das schlechteste Hörvermögen aller Größengruppen bei Frequenzen von 50 bis 1000 Hz, und das beste Hörvermögen bei 5 und 6 kHz. Die Dauer der während der Ab- und Adduktion der Brustflossenstachel erzeugten Laute, sowie deren Pulsperiode und der Schalldruckpegel (bei Tieren kleiner als 58 mm) nahmen mit der Größe zu, während die dominante Frequenz der Laute bei Tieren größer als 37 mm mit zunehmender Größe abnahm. Vergleiche zwischen Audiogrammen und Schallspektren ergaben, dass jene Frequenzen, bei denen die Welse am besten hörten, bei allen Größengruppen mit den dominanten Frequenzen der von ihnen produzierten Stridulationslaute korrelierten und dass alle Individuen die Laute aller anderen Größengruppen wahrnehmen konnten.

Der Einfluss der ontogenetischen Entwicklung der akzessorischen Hörstrukturen (Webersche Knöchelchen) auf das Hörvermögen wurde am Tanganjika-Stachelwels *Lophiobagrus cyclurus* untersucht. Diese Untersuchungen wurden an Tieren von gerade postlarvalem Stadium (11,3 mm Standardlänge) bis hin zu ausgewachsenen Individuen (85,5 mm) vorgenommen. Die morphologische Entwicklung der Weberschen Knöchelchen wurde mittels Sektionen, histologischer Schnittserien und Micro-Computertomgraphie untersucht und anschließend mittels 3D-Rekonstruktionen dargestellt. Der Tripus war bei der kleinsten Gruppe noch nicht vollständig entwickelt und die Interossicularligamente fehlten noch. Die kleinsten Individuen waren auch nicht dazu imstande Frequenzen über 2 - 3 kHz wahrzunehmen. Bei allen größeren Gruppen waren die Weberschen Knöchelchen und die Interossicularligamente vollständig entwickelt. Die Hörempfindlichkeit nahm bei größeren Individuen um bis zu 40 dB zu und es konnten Frequenzen bis 6 kHz wahrgenommen werden. Bei jenen Größengruppen, welche Frequenzen bis zu 6 kHz wahrnehmen konnten, zeigten größere Individuen ein besseres Hörvermögen bei tiefen Frequenzen (0,05 - 1 kHz), während das Gegenteil bei den höchsten Testfrequenzen (4 - 6 kHz) der Fall war; kleinere Individuen hören bei diesen Frequenzen besser.

Störungen der Melaninsynthese wie Albinismus gehen bei Säugetieren vielfach mit der Abnahme der Hörfähigkeiten einher. Unsere Untersuchungen sind die ersten, die dieses Phänomen bei einer anderen Wirbeltierklasse untersuchen. Wir bestimmten die Hörfähigkeiten von normal gefärbten und albinotischen Individuen des Europäischen Welses *Silurus glanis* und des südamerikanischen Metallpanzerwelses *Corydoras aeneus* mit der AEP Aufnahmetechnik und konnten bei keiner der getesteten Frequenzen, weder bezüglich Hörempfindlichkeit noch bezüglich der Form der AEP-Wellen, Unterschiede zwischen normal gefärbten und albinotischen Individuen feststellen.

Die Ergebnisse der Studie an *Lophiobagrus cyclurus* zeigen, dass bei Otophysen die Fähigkeit Schall geringen Pegels und hoher Frequenzen wahrzunehmen, stark von der Entwicklung des Weberschen Apparates abhängig ist. Sobald alle Weberschen Knöchelchen und Interossicularligamente entwickelt waren und die Knöchelchenkette zur Übertragung des

Schalls von der Schwimmblase zum Innenohr komplett war, konnte ein signifikanter Anstieg der Hörempfindlichkeit festgestellt werden. Die Untersuchungen bei *Lophiobagrus cyclurus* und *Synodontis schoutedeni* sind die ersten, die zeigen, dass sich das absolute Hörvermögen bei Otophysen während der ontogenetischen Entwicklung verändert. Dies steht im Gegensatz zu Ergebnissen früherer Studien an zwei Karpfenartigen, bei denen keine derartigen Veränderungen festgestellt werden konnten. Zusätzlich können *Synodontis schoutedeni* aller Entwicklungsstadien die Laute ihrer Artgenossen wahrnehmen, was wiederum im Gegensatz zu allen vorhergehenden ontogenetischen Studien steht. Schließlich scheinen Erbkrankheiten wie Albinismus das Hören bei Fischen nicht zu beeinflussen, was möglicherweise auf das Fehlen von Melanin im Fischohr zurückzuführen ist.

Diese Doktorarbeit ist ein wichtiger Beitrag zum Verständnis der ontogenetischen Entwicklung des Hörens und der Lautkommunikation bei Fischen, insbesondere bei Süßwasserfischen. Der zunehmende Einfluss des Menschen auf Gewässer auch durch akustische Umweltverschmutzung stellt eine potentielle Gefahrenquelle dar, die akustische Orientierung und die Lautkommunikation von Fischen behindern kann. Dies wiederum könnte Auswirkungen auf die Verbreitung und Populationsdynamik von Arten mit hoch entwickelten Hörfähigkeiten haben.

General introduction

Bony fishes are the most species-rich class of vertebrates. Approximately 30.000 species of Osteichthyes are recently known (Froese and Pauly, 2010), more than of all other vertebrates combined, and their diversity in matters of morphology and biology exceeds that of all other vertebrates by far. This diversity is also apparent in the different mechanisms for receiving and producing sounds.

Retzius (1881) showed already in the 19th century, that there is probably more anatomical variation in the ears across fish species than in all other groups of vertebrates. Three otolithic end organs in the inner ears of bony fishes are responsible for hearing. A calcareous otolith lies dense to the sensory epithelium of each end organ and the stimulation of the sensory hair cells in the epithelia results of a relative motion between epithelium and otolith. Because of their different densities epithelia and otoliths move at different amplitudes and phases (Hawkins, 1993; Popper and Schilt, 2008). In many fish species this is the only way for receiving sounds, what probably enables those fish to receive only the particle motion component, but not the pressure component of sounds and results in low hearing sensitivity and narrow range of detectable frequencies (Hawkins, 1993; Popper and Fay, 2010). But many groups have evolved different ways to couple gas-filled chambers to their inner ears, what enables them to detect sound pressure. These hearing specialisations increase both, the range of detectable frequencies and the hearing sensitivity (Hawkins, 1993; Ladich and Popper, 2004; Popper and Schilt, 2008). The Weberian apparatus of the otophysan fish (carps and relatives, tetras, South American knifefishes and catfishes), connecting the swimbladder to the inner ear with a chain of tiny ossicles, is probably the most elaborate accessory hearing structure. Several non-related taxa have evolved other hearing specializations, i.e. the labyrinth fishes (Anabantoidei) with their suprabranchial organ, an air-breathing chamber close to the inner ear, the elephantfishes (Mormyridae) with their otic bulla, an air bubble close to the inner ear, or squirrelfishes (Holocentridae), herrings (Clupeidae) and several other taxa, which evolved anterior extensions of the swimbladder to the ear (Braun and Grande, 2008; Ladich and Popper, 2004).

But fishes do not only show the highest variability among vertebrates concerning their hearing structures and hearing abilities, they also show the highest diversity in sound producing mechanisms of all vertebrates (Ladich and Fine, 2006). Beside accidentally generated sounds while swimming, feeding, or breathing a large number of fish species has evolved several different mechanisms to produce sounds for acoustic communication. The most common way of sound production in fish is oscillating the swimbladder in various ways, either directly by rapid contractions of intrinsic or extrinsic muscles or indirectly with several different bony skeletal elements moved by muscles (Ladich and Fine, 2006; Parmentier and Diogo, 2006). The second widely-used way for sound generation in fish is the production of stridulatory sounds. Stridulation sounds are produced by rubbing teeth, fin spines or other bony structures against each other (Fine and Ladich, 2003), i.e. rubbing the base of the pectoral fin spines within the pectoral girdle or pharyngeal teeth grating. In some common sound producing groups like gobies and loaches the sound producing mechanisms are still unidentified (Ladich and Fine, 2006) or have been identified lately as in the damselfish family (Parmentier et al., 2007).

Due to the large number of fish species and the huge variation in fish hearing organs, hearing capabilities of numerous taxa are still poorly or even not known. The exact influence of different structures involved in fish audition, such as otoliths, hair cells and many different kinds of hearing specialisations is still unanswered (Popper and Fay, 2010). Thus the field of acoustic communication in bony fishes is a field with numerous open questions. Correspondingly to this and as scientists still stick on fundamental questions of hearing and sound production in fish, studies on ontogenetic development of hearing and vocalisation are even scarcer. The ontogenetic development of acoustic communication has barely been studied in fish so far.

This doctoral thesis tries to answer some of those open questions, in particular the ontogenetic development of hearing, sound communication, and the morphological structures associated to hearing in fishes and the influence of pigmentation disorders on the development of hearing in Otophysi. Three main questions are tried to be answered in the studies included to this thesis:

1.: Are otophysan fish species which possess a highly developed hearing specialisation, namely the Weberian apparatus, able to communicate acoustically at earlier ontogenetic stages than non-otophysan species, such as the two species tested so far?

2.: Does the ontogenetic development of hearing show a consistent trans-species trend within an otophysan order and how is the ontogeny of hearing linked to the ontogeny of the accessory hearing structures?

3.: Are genetic disorders which result in pigmentation abnormalities and often hearing impairments in mammals also influencing hearing abilities in fish with highly developed auditory system?

In order to answer these questions catfish species were chosen for several reasons. Catfishes belong the otophysines, have well-defined accessory hearing structures and well-developed hearing abilities. Representatives of several families are well known for there sound producing mechanisms. Furthermore, many small catfish species can easily be kept in aquaria and some of them also bred in captivity. Additionally, it was possible to get normally pigmented and albinotic forms of two species belonging to quite different families of catfishes.

Catfishes belong to the superorder of otophysine fish, which represent with more than 8.000 extant species mainly in freshwaters the by far dominant fish group in freshwaters worldwide. Otophysi show an unique character in common, the so called Weberian apparatus, named in honour to a German anatomist, who first described it in the early 19th century (Weber, 1820). As mentioned above, a chain of ossicles, the Weberian ossicles, connects swimbladder and inner ear and transmits oscillations of the bladder in a sound field to the ear, what results in excellent hearing abilities of otophysine fishes.

Catfishes, with approximately 3.100 species in 36 families (Ferraris, 2007) mainly in freshwaters of all continents but Antarctica, belong to the most successful group of fishes. They show a huge variability concerning their ecology as well as their anatomy. Many catfish species belong to the best hearing fish we know, and many species are also able to produce sounds and communicate acoustically.

The great diversity in their biology is also obvious in their general anatomy and, especially interesting for this doctoral thesis, in their anatomy of structures related to hearing and sound production. Several authors studied the anatomy of the Weberian apparatus of different catfish species and have shown the different states of development in many families (e.g. Alexander, 1964; Alexander, 1965; Bridge and Haddon, 1889; Bridge and Haddon, 1892; Bridge and Haddon, 1893; Chardon, 1968; Chranilov, 1929; Sörensen, 1895). The chain of Weberian ossicles can consist of one to four ossicles in siluriforms, and the swimbladder can be free and well developed or highly reduced, paired and bony encapsulated. In a previous study we have shown, that large unpaired swimbladders and large numbers of Weberian ossicles significantly improve hearing in catfishes at higher frequencies (Lechner and Ladich, 2008). Representatives of many catfish families are known to be vocal, what even led to popular names like "talking catfishes" for doradids or "squeakers" for mochokids. Numerous catfish species are able to produce stridulation sounds by rubbing the spines of their pectoral fins within the shoulder girdle, and others are able to produce drumming sounds with their swimbladder, either directly with muscles or indirectly with the elastic spring apparatus, a bony structure attached to the bladder and moved by muscles (Fine and Ladich, 2003). Several groups are even able to produce sounds in both ways.

This thesis comprises studies on the ontogenetic development of hearing, sound production and sound communication in the African mochokid squeaker catfish *Synodontis schoutedeni*, on the ontogenetic development of the Weberian ossicles combined with development of hearing abilities in the African claroteid catfish *Lophiobagrus cyclurus*, and on the influence of albinism on the development of hearing in the European silurid *Silurus glanis* and the South American callichthyid *Corydoras aeneus*. The ontogenetic development of the Weberian ossicles, hearing abilities and sound production have been examined in several studies so far. But parallel development of hearing and sound production has been studied only in two fish species, in the croaking gourami *Trichopsis vittata* (Wysocki and Ladich, 2001) and in the Lusitanian toadfish *Halobatrachus didactylus* (Vasconcelos and Ladich, 2008), but not in any otophysan fish species. Parallel investigations of the development of ossicles and hearing hav only been conducted in one species, the zebrafish *Danio rerio* (Higgs et al., 2003). No study has been conducted so far, examining the influence of pigmentation disorders on the development of hearing in any non-mammalian vertebrates. Pigmentation disorders such as albinism are known to have some potential negative effects on mammal hearing.

Studies on the ontogenetic development of hearing in non-otophysine fish, in particular in several perciform, clupeiform and batrachoidiform species, always found an increase in hearing acuity with growth (Higgs et al., 2004; Iwashita et al., 1999; Kenyon, 1996; Sisneros and

Bass, 2005; Vasconcelos and Ladich, 2008; Wysocki and Ladich, 2001), with the exeption of Egner and Mann (2005), who found a slight decrease in hearing sensitivity at low frequencies during ontogeny of the damselfish *Abudefduf saxatilis*, and Belanger et al. (2010), who found no differences in hearing abilities of different size stages in the round goby *Neogobius melanostomus*.

In contrast to the conclusions of all the studies mentioned above, I guessed to find a different change of hearing with size in catfishes, a decrease of hearing sensitivities with size in higher frequencies. This assumption is based on data of several further studies on hearing in fishes. In the loricariid catfish *Ancistrus ranunculus* I have found bigger specimens not to be able to respond to 5 kHz tone bursts at the maximum sound pressure level we could provide with our equipment, while only slightly smaller specimens definitely showed responses (Lechner and Ladich, 2008). Comparing hearing abilities of the pimelodid catfish *Pimelodus pictus* used in three studies (Amoser and Ladich, 2003; Ladich, 1999; Wysocki et al., 2009) indicates similar trends – the smallest specimens tested by Wysocki et al. (2009) showed lower hearing thresholds at the highest frequencies tested than the largest fish tested by Ladich (1999); and finally data of hearing in gouramis (Ladich and Yan, 1998; Wysocki and Ladich, 2001) also show a slight trend of better high frequency hearing in smaller specimens.

The study in the squeaker catfish *Synodontis schoutedeni* was the first conducted of the three studies contributing to this thesis. In the smallest size group tested I have used bigger specimens of *S. schoutedeni* than I have used in the second study of *L. cyclurus*. The reason for this and the reason for choosing those two species is based on the fact, that most catfish species are extremely difficult to breed in aquaria. Only several species of the catfish families Loricariidae and Callichthyidae, both showing highly reduced, paired and bony encapsulated swimbladders, are regularly bred in captivity. Reproducing and raising species with large single bladders, like representatives of the families Mochokidae (e.g. *Synodontis schoutedeni*) and Claroteidae (e.g. *Lophiobagrus cyclurus*) is still extremely rare and challenging. Fortunately, my friend Oliver Drescher could breed the mochokid *S. schoutedeni* and the bagrid *Lophiobagrus cyclurus* successfully in 2007 and 2008, respectively and I got the offspring for my studies. Because the young catfish are highly sensitive and I got only about 30 specimens of the first spawn (*S. schoutedeni*), which had survived the first weeks of life, I did not dare to do the tests immediately. I waited till the specimens were at least about 2 cm of standard length (SL) before testing them for hearing abilities and recording stridulation sounds. Loosing the baby catfishes would have been the early end of this study. But those tests with the young squeakers worked perfectly, and according to that I wanted to test even smaller catfish specimens. About one year later I again got about 30 specimens of catfish offspring, this time *L. cyclurus*, and I dared to do hearing measurements in specimens only shortly after larval stage, with about 12 mm SL, what again worked well.

In order to investigate if otophysines start to communicate acoustically at earlier stages than non-otophysines I investigated the development of sound production in parallel with the development of hearing abilities in the squeaker catfish *Synodontis schoutedeni*. Sound production is known from numerous fish species, and the ontogenetic development of sound production has been studied in several fish groups, as gurnards, mormyrids, croaking gouramis, damselfish and toadfish (Amorim and Hawkins, 2005; Crawford, 1997; Henglmüller and Ladich, 1999; Ladich et al., 1992; Myrberg et al., 1993; Parmentier et al., 2009; Vasconcelos and Ladich, 2008; Wysocki and Ladich, 2001). The changes in sound production seem to follow a consistent pattern, namely dominant frequencies of sounds decrease and in most species sound pressure levels, total duration of sounds and pulse periods within sounds increase with size. In the only two species in which the relationship of ontogenetic development of hearing and sound production has been tested so far, in the croaking gourami *Trichopsis vittata* (Wysocki and Ladich, 2001) and the Lusitanian toadfish *Halobatrachus didactylus* (Vasconcelos and Ladich, 2008), the smallest size groups tested could not detect conspecific sounds.

The study in the squeaker catfish *S. schoutedeni* was the first to investigate the development of sound communication in an otophysine fish species. Squeaker catfishes are able to produce stridulation sounds with their pectoral spines and drumming sounds with their elastic spring mechanism which vibrates the swimbladder (Fine and Ladich, 2003; Kaatz, 1999). The study presented was done investigating stridulation sounds, because all size stages of *Synodontis schoutedeni* are highly active in producing this type of sound (i.e. as fright reaction when handled by the experimenter). On the contrary, swimbladder sound production has rarely been observed in mochokids and is probably limited to intraspecific agonistic interactions between adults (Kaatz, 1999). I assumed the sound characteristics will follow the scheme shown in the studies mentioned above. But as *S. schoutedeni* is a species which hears very well (Lechner and Ladich, 2008) and which produces quite loud stridulation sounds, I hypothesize that early stages of *S. schoutedeni* are able to detect conspecific sounds due to the Weberian apparatus.

Studies on the ontogenetic development of the Weberian apparatus have been conducted in several otophysans, in cypriniform and characiform species (see Hoffmann and Britz, 2006 for an overview of the numerous studies), and in few catfish species of the families Ariidae, Bagridae, Callichthyidae, Clariidae, Ictaluridae and Siluridae (Bamford, 1948; Coburn and Grubach, 1998; Grande and Shardo, 2002; Ichiyanagi et al., 1997; Ichiyanagi et al., 1993; Radermaker et al., 1989). Analysing and comparing the data of those studies is rather difficult, because not all authors indicate if they used standard or total length for measurements and not all authors describe the ossicles at each developmental stage of the species. Furthermore, the comparison of different species with very different maximum sizes or age classes is probably problematic, too. Anyway, those studies indicate that the tripus is the first ossicle to differentiate, followed by intercalarium and scaphium, and that the claustrum is the last to form (Grande and Young, 2004).

But it remains unclear when the Weberian apparatus is completely developed (including the connecting interossicular ligaments) and when it starts to transmit sounds from the swimbladder to the ear. This is exactly the point I wanted to work on. My intent was not to give an exact description of all ontogenetic stages of the Weberian ossicles in a catfish species, but to do a comparative study on the ontogenetic development of the Weberian ossicles and hearing, using as many and as small size stages as possible and describing the Weberian ossicles as detailed as possible. I hypothesize that catfishes in which the Weberian ossicles and interossicular ligaments are not fully developed will have lower hearing sensitivities (absolute sensitivies and frequency range). Interrupting the ossicular chain by extirpation of the tripus in adult goldfish resulted in a hearing loss at all frequencies and in the inability to detect higher frequencies (Ladich and Wysocki 2003). Only one study in the zebrafish, conducted by Higgs et al. (2003), investigated both - ossicle and hearing development - so far. The authors give a rather rough description of the Weberian ossicles in the different size stages tested and in this and few further studies in otophysines (Higgs et al., 2001; Popper, 1971) the authors could not find any size related changes of hearing thresholds in the test specimens. Nevertheless Higgs et al. (2003) found a broadening of the range of detectable frequencies in the zebrafish, what could be an indication for a not fully developed Weberian apparatus in the smallest size stages tested.

The third study contributing to this doctoral thesis tries to answer the question if pigmentation disorders such as albinism influence the development of hearing in fishes. Albinism is a genetic disorder in the pigment system, where the synthesis of melanin is reduced or lacking. Several different types of total and partial albinisms and related pigmentation disorders have been described and there are indications, that albinism is linked to hearing impairments at least in mammals (Lezirovitz et al., 2006); already Charles Darwin mentioned "deaf blue eyed white cats" (Darwin, 1859). Various studies in men and further mammals lead to quite different and contradicting results. No consistent pattern can be concluded from those studies. Thus, there are multiple different kinds of albinism and many studies do not even name the kind of albinism of the study animals. This could be a reason for those contradictory findings. Undoubted, melanin plays an important role in the mammalian cochlea and therefore a lack of melanin could show severe affects on hearing (Bartels et al., 2001). A recent study of Dutton et al. (2009) in the zebrafish shows, that knocking out the Sox10 gene results in lack of body pigmentation and abnormalities in ear development. Sox10 gene mutations cause Waardenburg syndrome in humans, an inherited genetic disorder which may cause hearing loss and partial albinism. Comparing albinotic and normally pigmented specimens of the same fish species can easily answer this question, but this has never been done before. Only Popper (1970) compared hearing abilities of the colorless Mexican blind cave fish *Astyanax mexicanus* and it's eyed and pigmented congeners and could not find any differences. But those cave fish are no real albinos and show, besides lacking eyes and pigments, several further sensory adaptations to subterranean life. Bigelow's (1904) report of a deaf albino goldfish in the early 20th century was a further reason for me to conduct this study and answer this question. I hypothesize that pigmentation disorders will only slightly or not at all affect hearing in fishes due to the fact that hearing disorders seem to be linked to the cochlea in species investigated so far and fish lack a cochlea.

In order to test this assumption catfishes were chosen because of their very high hearing acuity, and because any effects of albinism probably could be observed easier in species having highly developed hearing abilities than in bad hearing ones. Even though many fish are offered as “albino” in ornamental fish trade, it was not that easy to get real albinos, lacking body and eye pigmentation. I have chosen to test catfish of both swimbladder types, the European Wels *Silurus glanis* with big, free swimbladder and four Weberian ossicles, and the South American bronze catfish *Corydoras aeneus* with highly reduced, paired and bony encapsulated bladders and only one single ossicle. A further interesting result of this study will be the hearing acuity of *S. glanis*. Even though the Weberian ossicles have been first described in this species in the early 19th century (Weber, 1820) and it is one of the most famous fish species worldwide, its hearing abilities have never been tested so far. Based on our previous study on swimbladders, ossicles and hearing (Lechner and Ladich, 2008), I expect *S. glanis* to have a good hearing acuity.

In all studies contributing to this thesis the auditory evoked potential (AEP) recording technique has been utilized to measure hearing abilities in the study specimens. This is a non-invasive electrophysiological technique, developed by Kenyon et al. (1998) and modified by Wysocki and Ladich (2005a; 2005b), where study specimens are not harmed. The AEP technique is perfectly qualified for my studies in ontogenetic development of hearing, where only a very limited and therefore very valuable number of study specimens was available and the same study specimens had to be tested several times. This would not have been possible with invasive methods, and not with behavioural methods for auditory measurements, either, where study specimens have to be trained for a long time period before testing. The anatomy of the Weberian ossicles of *Lophiobagrus cyclurus* has been investigated with several techniques, such as manual dissections, µCt-analyses, paraffin and semi-thin sectioning. Sound production of *Synodontis schoutedeni* was recorded underwater using hydrophones and video techniques. For the exact data of the procedures and techniques used see the material and methods sections of the three papers contributing to this doctoral thesis.

Summing up I tested three hypotheses to answer important questions on the development of hearing, sound production and acoustic communication in fishes.

I assume that all stages of *S. schoutedeni* tested, up from about 20 mm SL, are able to produce stridulation sounds and that the development of those sounds with growing up follows a consistent pattern already described in several studies. Because *S. schoutedeni* hears very well and produces loud sounds, it could be possible, that all size groups are able perceive the sounds of their congeners and communicate acoustically. This would be in contrary to the only two fish species tested on this so far. I checked the development of the Weberian ossicles in catfishes up from about 12 mm SL and assume a fully developed chain of ossicles to be necessary in the smallest catfishes tested for hearing abilities similar to their larger congeners. In catfishes with fully developed ossicles I slightly estimate better hearing acuities of smaller specimens in higher frequencies, based on the data of previous studies, and better hearing of bigger specimens in lower frequencies, as already shown in several further studies before. Concerning the study on hearing in albino fish I hypothesize that due to the lack of a cochlea in the fish's inner ear a lack of pigmentation might not result in hearing disorders despite the data in zebrafish. Anyway, both species tested, *S. glanis* and *C. aeneus*, should fit to our schema proposed in a further study – catfish with big, free bladders and several Weberian ossicles show a better high frequency hearing than species with reduced, paired and bony encapsulated bladders and a reduced number of ossicles.

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Ontogenetic development of auditory sensitivity and sound production in the squeaker catfish *Synodontis schoutedeni*

Walter Lechner*, Lidia Eva Wysocki, Friedrich Ladich

Abstract

Background: Surveys of ontogenetic development of hearing and sound production in fish are scarce, and the ontogenetic development of acoustic communication has been investigated in only two fish species so far. Studies on the labyrinth fish *Trichopsis vittata* and the toadfish *Halobatrachus didactylus* show that the ability to detect conspecific sounds develops during growth. In otophysine fish, which are characterized by Weberian ossicles and improved hearing sensitivities, the ontogenetic development of sound communication has never been investigated. We analysed the ontogeny of the auditory sensitivity and vocalizations in the mochokid catfish *Synodontis schoutedeni*. Mochokid catfishes of the genus *Synodontis* are commonly called squeakers because they produce broadband stridulation sounds during abduction and adduction of pectoral fin spines. Fish from six different size groups - from 22 mm standard length to 126 mm - were studied. Hearing thresholds were measured between 50 Hz and 6 kHz using the auditory evoked potentials recording technique; stridulation sounds were recorded and their sound pressure levels determined. Finally, absolute sound power spectra were compared to auditory sensitivity curves within each size group.

Results: The smallest juveniles showed the poorest hearing abilities of all size groups between 50 and 1,000 Hz and highest hearing sensitivity at 5 and 6 kHz. The duration of abduction and adduction sounds and the pulse period increased and sound pressure level (in animals smaller than 58 mm) increased, while the dominant frequency of sounds decreased with size in animals larger than 37 mm. Comparisons between audiograms and sound spectra revealed that the most sensitive frequencies correlate with the dominant frequencies of stridulation sounds in all *S. schoutedeni* size groups and that all specimens are able to detect sounds of all size groups.

Conclusions: This study on the squeaker catfish *S. schoutedeni* is the first to demonstrate that absolute hearing sensitivity changes during ontogeny in an otophysine fish. This contrasts with prior studies on two cypriniform fish species in which no such change could be observed. Furthermore, *S. schoutedeni* can detect conspecific sounds at all stages of development, again contrasting with prior findings in fishes.

Background

Fish possess a large diversity in hearing sensitivities. Several non-related groups of bony fish - often termed *hearing specialists* [1] - have evolved mechanisms which transmit vibrations of air-filled cavities to the inner ear. These mechanisms enable them to detect the pressure component of sound, enhance their absolute hearing sensitivity and broaden the range of detectable frequencies up to several kilohertz [2] versus several hundred Hertz in species without such specialization. Otophysi, a group of mainly freshwater fishes comprising the orders Siluriformes (catfishes), Cypriniformes (carps and loaches), Characiformes (tetras) and Gymnotiformes (South American knifefishes) are characterized by possessing such an accessory auditory structure, the Weberian apparatus. The Weberian apparatus consists of a chain of one to four ossicles that transmit oscillations of the swimbladder in the sound field directly to the inner ear. Catfishes, numbering more than 3,000 known species [3], show a high diversity in the structure of the Weberian apparatus and swimbladders. Their hearing ability

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* Correspondence: walter.lechner@univie.ac.at
University of Vienna, Department of Behavioural Biology, Althanstrasse 14, 1090 Vienna, Austria

depends on swimbladder size as well as on the number of Weberian ossicles [4].

Numerous studies have been conducted on the ontogenetic development of hearing and acoustic communication in mammals and birds [for example, [5-10]], but only a few have been carried out in fishes. Studies on the ontogeny of hearing in teleosts show varying results, ranging from no differences between two size groups of goldfish [11] and various size groups of the zebrafish *Danio rerio* [12], no change in absolute thresholds but expansion of the detectable frequency range in the zebrafish [13,14], up to an improvement of hearing abilities with size in the damselfish *Stegastes partitus* [15], the Red Sea bream *Pagrus major* [16], the labyrinth fish *T. vittata* [17] and the toadfishes *Porichthys notatus* [18] and *Halobatrachus didactylus* [19]. Egner and Mann [20] found a slight decrease in hearing sensitivity at lower frequencies during ontogeny of the damselfish *Abudefduf saxatilis*.

Ontogenetic development of sound production in fishes seems to follow a consistent pattern. Dominant frequencies of sounds decrease with size, for example in gurnards, mormyrids, croaking gouramis, damselfish and toadfish [17,19,21-26]. In most species tested in those studies, sound pressure levels, total duration of sounds as well as pulse periods within sounds increased.

The relationship between the development of hearing and sound production has been investigated in just two species so far. In both species - the anabantoid *Trichopsis vittata*, a hearing specialist, and the non-related batrachoidid *H. didactylus*, a hearing generalist - the smallest size groups were unable to detect conspecific sounds [17,19].

The present study is the first to investigate the ontogenetic development of hearing and sound production in an otophysine fish. The mochokid catfish *Synodontis schoutedeni* David 1936 emits stridulatory sounds in distress situations and during agonistic interactions by rubbing the spines of its pectoral fins in grooves of the shoulder girdle [27]. Therefore, mochokids are frequently called *squeakers*. *S. schoutedeni* shows a well-developed unpaired swimbladder, three Weberian ossicles and very good hearing sensitivities as compared to other species of catfish with a different swimbladder morphology [4]. We describe the developmental changes of temporal, spectral and intensity characteristics of stridulation sounds, analyse the development of auditory sensitivity with growth, and examine whether *S. schoutedeni* is able to communicate acoustically throughout its life history.

Results

Auditory sensitivity

Auditory evoked potentials were recorded in all test groups between 50 Hz and 6 kHz. Best hearing abilities were shown at frequencies between 0.3 and 1 kHz in all size groups, except the smallest group XXS. The largest group XL showed its lowest hearing threshold at 0.3 kHz (72.3 dB re 1 μPa), group XXS at 2 and 3 kHz (80.9 dB) (Figure 1, Table 1). Group XXS and XS showed poorest hearing abilities of all groups at 0.05 kHz. Group XXS showed lowest hearing abilities of all groups from 0.1 to 2 kHz, but showed best hearing abilities of all groups at the highest frequency tested (6 kHz). Group S had well-developed hearing abilities at low frequencies similar to larger size groups, but hearing sensitivity similar to that of group XXS and XS at the highest frequencies tested (5 and 6 kHz) (Figure 1, Table 1). Significant correlations between size and hearing thresholds existed at most frequencies. At lower frequencies (0.05 to 2 kHz), larger animals showed significantly better hearing, whereas at the highest frequencies tested (5 and 6 kHz) the opposite was the case: smaller animals had lower hearing thresholds. At 3 and 4 kHz no correlation was evident (Figure 2).

Sound production

Stridulatory sounds were emitted during abduction (forward movement) and adduction (backward movement) of pectoral spines in all groups tested as soon as specimens were handled (Figure 3). Swimbladder drumming sounds could not be detected or recorded. Sound pressure level (SPL) increased with size up to 58 mm standard length (SL) whereas no further increase was observed in larger-sized animals. SPLs of animals up to 58 mm SL were significantly lower than in larger specimens (Mann-Whitney-U test: U = 62.5, N = 40, $P < 0.01$). In larger fish, no further increase in SPL was evident (Figure 4A). Duration of adduction and abduction sound as well as pulse period (PP) increased with size (Figures 3 and 4B-D). The dominant frequency of sounds decreased significantly with size in fish larger than 37 mm SL, while no correlation was found in smaller ones (Figure 4E). However, the dominant frequency of fish up to 37 mm SL was significantly higher than in larger fish (Mann-Whitney-U test: U = 5, N = 40, $P < 0.01$). While bandwidth decreased with increasing size in fish up to 73 mm SL, no further decrease of bandwidth was observed in larger fish. The main energies of stridulation sounds produced by smaller specimens were more broad-banded than those of larger ones (Mann-Whitney-U test: U = 44, N = 40, $P < 0.01$; Figures 3 and 4F).

Comparison between audiograms and sound spectra

All size groups showed best hearing abilities in frequency ranges where main energies of stridulation sounds were concentrated, and all size groups were able to detect conspecific sounds (Figure 5). Thus, group XL detected sounds produced by group XXS and vice versa.

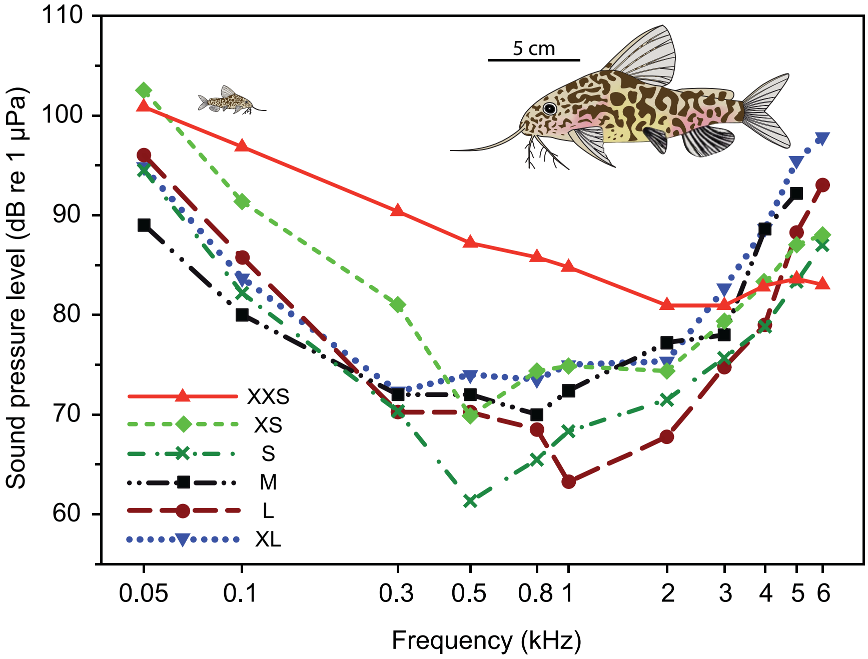

Figure 1 Auditory evoked potential audiograms of the size groups investigated. Mean hearing thresholds of the size groups XXS (N = 12), XS (N = 6), S (N = 6), M (N = 5), L (N = 4) and XL (N = 6) of *Synodontis schoutedeni* tested. Catfish pictures show representative specimens of group XXS (left) and XL (right) drawn in proportional scale for comparative purposes.

Discussion

Development of hearing

Changes in hearing abilities have been reported in several fish taxa [15,16,18,19]. The present study provides the first evidence that auditory thresholds change during ontogeny in an otophysine fish species (Figure 2), results that are in contrast with previous studies on two species of cypriniforms, namely the goldfish [11] and the zebrafish [14]. Several potential explanations can be forwarded for this discrepancy among otophysines. First, different species have been investigated, which even belong to different orders, namely Cypriniformes and Siluriformes. Furthermore, the authors of those studies did not calculate regressions of hearing abilities including all data from each individual study specimen, but instead compared the mean results of different size groups. Furthermore, the size differences of the goldfish used as well as the range of frequencies tested might have been too small: the specimens were 45 to 48 mm SL and 110 to 120 mm SL, and the five test frequencies ranged from 100 to 2,000 Hz. However, goldfish are able to detect sounds at least up to 4 kHz [28]. In contrast to the goldfish study, Higgs et al. measured early stages of zebrafish (10 mm) [14] and found an extension of the maximum frequency detectable from a 200 Hz upper limit in small specimens up to 4 kHz in large ones but no change in absolute thresholds. They argued that the development of the Weberian ossicles is responsible for this increase in the detectable frequency range. By contrast, Zeddies and Fay [12] found in their study on the development of startle response in zebrafish no change of stimulus thresholds and frequency bandwidth to which the zebrafish responded from five days post fertilization to adult. Similar to the observations of Zeddies and Fay [12] in zebrafish, we did not observe a change in the range of detectable frequencies. Based on Coburn and Grubach's [29] study on the ontogeny of the Weberian ossicles in several species of catfish we assume that all our tested animals possessed fully developed Weberian ossicles. Thus, the current study is the first to systematically demonstrate that auditory thresholds change with size in an otophysine fish (Figure 2), whereas the detectable frequency range does not change (Figures 1 and 2). Auditory sensitivity increased at lower frequencies up to 2 kHz and decreased at 5 and 6 kHz.

Some prior data on other catfish species are in agreement with these findings. In eight specimens of the loricariid catfish *Ancistrus ranunculus*, the four smallest specimens tested detected sound stimuli at 5 kHz, whereas only one of the larger ones responded to 5 kHz stimuli at the maximum SPL tested (129 dB re 1 µPa) [4]. This indicates that smaller individuals of this loricariid had higher auditory sensitivities at the highest

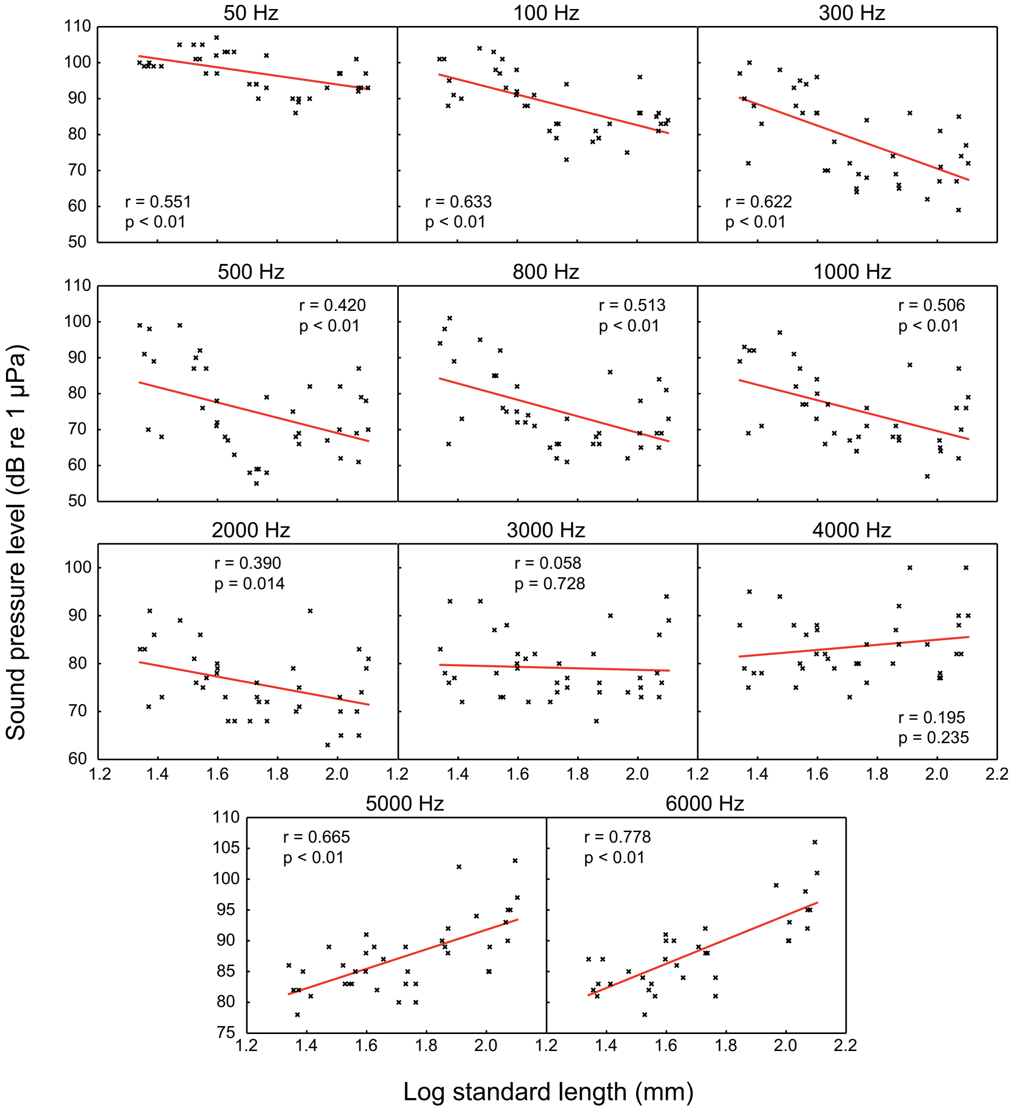

Figure 2 Correlations between auditory thresholds and fish size at frequencies tested. Semilog plots of hearing thresholds of each individual against log of standard-length at each frequency tested. N = 39 at each frequency except 6000 Hz (N = 34). Pearson's correlation coefficients and significances are given in graphs. Regression equations: x = log standard length, y = hearing threshold (dB re 1 µPa); 50 Hz: y = -1.92x + 117.79; 100 Hz: y = -21,33x + 125.27; 300 Hz: y = -29.91x + 130.33; 500 Hz: y = -21.32x + 111.67; 800 Hz: y = -22.84x + 114.81; 1000 Hz: y = -21.44x + 112.46; 2000 Hz: y = -11.57x + 95.77; 3000 Hz: y = -1.57x + 81.83; 4000 Hz: y = 5.34x + 74.30; 5000 Hz: y = 15.83x + 60.13; 6000 Hz: y = 19.68x + 54.78.

frequency tested. A similar observation can be made when comparing results of three studies on the pimelodid catfish *Pimelodus pictus* [30-32]. The smallest specimens tested by Wysocki et al. [32] had better hearing abilities at the highest frequencies tested (mean hearing threshold at 4 kHz: 75.3 dB re 1 µPa) than the largest fish tested by Ladich [30] (81.3 dB).

These data indicate that smaller individuals within a given species of catfish may hear better at higher frequencies. One possible reason could be that smaller specimens produce sounds of higher frequencies and are adapted to detect sounds of similar-sized conspecifics. Ladich and Yan [33] argued that the pygmy gourami, the smallest species investigated in their comparative study on labyrinth fishes (family Osphronemidae), heard better at 3 to 5 kHz than the larger two species. The authors argued that higher sensitivity at higher frequencies in the smallest species may reflect the higher

Table 1 Hearing threshold values.

f (kHz)	XXS	XS	S	M	L	XL
0.05	100.83 +/- 0.79	102.50 +/- 1.31	94.50 +/- 1.63	89.00 +/- 0.77	96.00 +/- 1.00	94.83 +/- 1.42
0.1	96.83 +/- 1.56	91.33 +/- 1.50	82.17 +/- 2.81	80.00 +/- 0.89	85.75 +/- 4.29	83.67 +/- 0.71
0.3	90.33 +/- 2.23	81.00 +/- 4.19	70.33 +/- 2.97	72.00 +/- 3.83	70.25 +/- 4.03	72.33 +/- 3.61
0.5	87.17 +/- 3.06	69.83 +/- 2.09	61.33 +/- 3.58	72.00 +/- 2.92	70.25 +/- 4.25	74.00 +/- 3.74
0.8	85.75 +/- 3.20	74.33 +/- 1.65	65.50 +/- 1.73	70.00 +/- 3.26	68.50 +/- 3.48	73.50 +/- 3.05
1	84.75 +/- 2.68	74.83 +/- 2.77	68.33 +/- 1.87	72.40 +/- 3.96	63.25 +/- 2.17	75.00 +/- 3.44
2	80.92 +/- 1.87	74.33 +/- 2.23	71.50 +/- 1.26	77.20 +/- 3.80	67.75 +/- 2.29	75.33 +/- 2.84
3	80.92 +/- 2.21	79.33 +/- 1.54	75.67 +/- 1.12	78.00 +/- 3.74	74.75 +/- 0.85	82.67 +/- 3.36
4	82.92 +/- 2.03	83.17 +/- 1.45	78.83 +/- 1.56	88.60 +/- 3.46	79.00 +/- 1.68	88.67 +/- 2.72
5	83.58 +/- 0.82	87.00 +/- 1.29	83.33 +/- 1.38	92.20 +/- 2.54	88.25 +/- 2.14	95.50 +/- 1.78
6	83.00 +/- 0.74	88.00 +/- 1.13	87.00 +/- 1.59	-	93.00 +/- 2.12	97.83 +/- 2.06

Mean hearing threshold values (+/- s.e.m., dB re 1 μPa) of the six size groups of *S. schoutedeni* at each frequency tested. f = frequency; for exact size ranges see material and methods; group M was not tested at 6 kHz.

resonance frequencies of their smaller-sized accessory hearing structures, the so-called suprabranchial chambers (paired air-filled chambers close to the inner ears).

Studies on the ontogenetic development of hearing in Perciformes (Pomacentridae - damselfishes, Osphronemidae - gouramis, Sparidae - sea breams) and Batrachoidiformes (Batrachoididae - toadfishes) [15-19] show a more consistent pattern. They reported mostly a moderate improvement of hearing with size, with one exception. Kenyon [15] observed a considerable 60 dB increase in hearing sensitivity in the bicolor damselfish *Stegastes* (syn. *Pomacentrus*) *partitus* at the most sensitive frequency (500 Hz) during ontogeny. Contrary to Kenyon's results, Egner and Mann [20] reported a decrease of hearing sensitivity in a representative of the same family. The sensitivity decreased in the sergeant major damselfish *Abudefduf saxatilis* with growth at 100 and 200 Hz. Iwashita et al. [16] observed an increase in hearing sensitivity in zero-, one- and two-year-old red sea bream *Pagrus major*, in particular between 100 and 300 Hz. In the croaking gourami *Trichopsis vittata*, different trends were found at different frequencies during development of hearing [17]. Hearing improved with size in the frequency range from 800 to 2,000 Hz, where the main energies of sounds were concentrated. An opposite trend was observed at 4 and 5 kHz. This decrease in sensitivity at highest frequencies in *T. vittata* is in agreement with the catfish data of the current study. In both toadfish species tested so far, the plainfin midshipman [18] and the Lusitanian toadfish [19], a small increase of hearing sensitivity with size was evident. In the Lusitanian toadfish, hearing improved with growth at three out of seven frequencies tested.

Development of sound production

Whereas different trends were found in the development of auditory sensitivities in different species, the development of sound characteristics shows more similar patterns among teleosts. These patterns agree with the current data in *Synodontis schoutedeni*.

The dominant frequency of sounds is negatively correlated with body size in representatives of all families investigated so far, for example, in pomacentrids - *Stegastes partitus*, *Dascyllus albisella*, *Amphiprion akallopisos*, *A. clarkii*, *A. frenatus*, *A. ocellaris*, [23,26,34,35], osphronemids - *Trichopsis vittata*, *T. pumila* and *T. schalleri* [17,21,22], sciaenids - *Cynoscion regalis* [36], triglids - *Eutrigla gurnardus* [25], and toadfishes - *Halobatrachus didactylus* [19]. Fine et al. [37] found a decrease of center frequency in the ictalurid catfish *Ictalurus punctatus*. In contrast, Ladich [38] reported such a correlation in only one out of six catfish species from three families. Only in the doradid *Platydoras armatulus* (formerly named *P. costatus*) did the dominant frequency decrease with size, while no such correlation was present in pimelodids and mochokids. The lack of a relationship might have been due to the difficulty of determining dominant frequencies in broad-band sounds and perhaps the small size differences of fish used in that study. In the present investigation, *S. schoutedeni* size differed more (1.02 g to 51.80 g vs. 1.7 to 5.6 g in [38]). A significant correlation between dominant frequency of sounds and size was found in animals larger than 37 mm SL. The decrease in dominant frequency may be an effect of longer pulse periods within sounds, and thus based on size-related changes in morphology [36]. However, these underlying structural changes seem not to apply to animals smaller than 37 mm in which dominant frequencies were significantly higher than expected by the linear regression. Smaller individuals of *S. schoutedeni* showed a more evenly distributed sound energy than large individuals. The frequency band 10 dB below the dominant frequency ranged from ca. 2,800 to 4,100 Hz in smallest fish down to ca. 370 to 1,500 Hz in largest ones (Figures 3 and

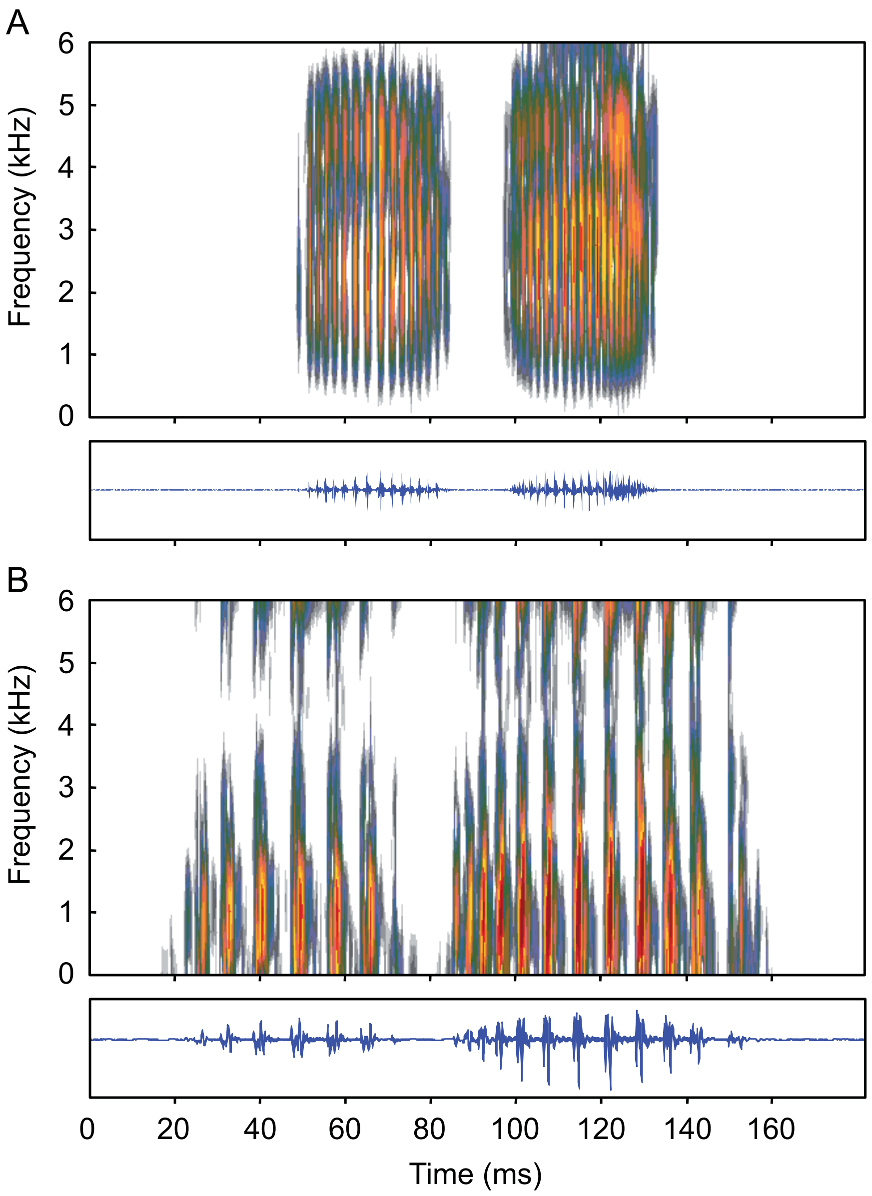

Figure 3 Sonagram and oscillogram of stridulation sounds. Sonagram (top) and oscillogram (below) of adduction sounds (left) and abduction sounds (right) of representatives of group XXS **(A)** and group XL **(B)**. Sampling frequency 44.1 kHz, filter bandwidth 650 Hz for XXS and 600 Hz for XL, 75% overlap, Hanning window.

4F). The decrease in bandwidth was significant from the smallest size group up to 73 mm SL; no further change was observed in larger animals. No such relationship has been described in other species so far, although a closer examination of sounds of other species might yield similar results. The power spectra of sounds of three species of clownfishes *Amphiprion* spp. [26] indicate that such a relationship may also exist in other fish species.

The sound pressure level (SPL) of vocalizations increases with size in species from different taxa such as in the tigerfish *Therapon jarbua* [39], the osphronemid *T. vittata* [17], the sciaenid *C. regalis* [36], the toadfish *H. didactylus* [19], and in the mochokid catfish *S. schoutedeni* (current study). Interestingly, while SPLs increased rapidly in animals below 58 mm SL, no further increase of SPLs occurred in the larger specimens (Figure 4A). The amplitude of sounds depends on

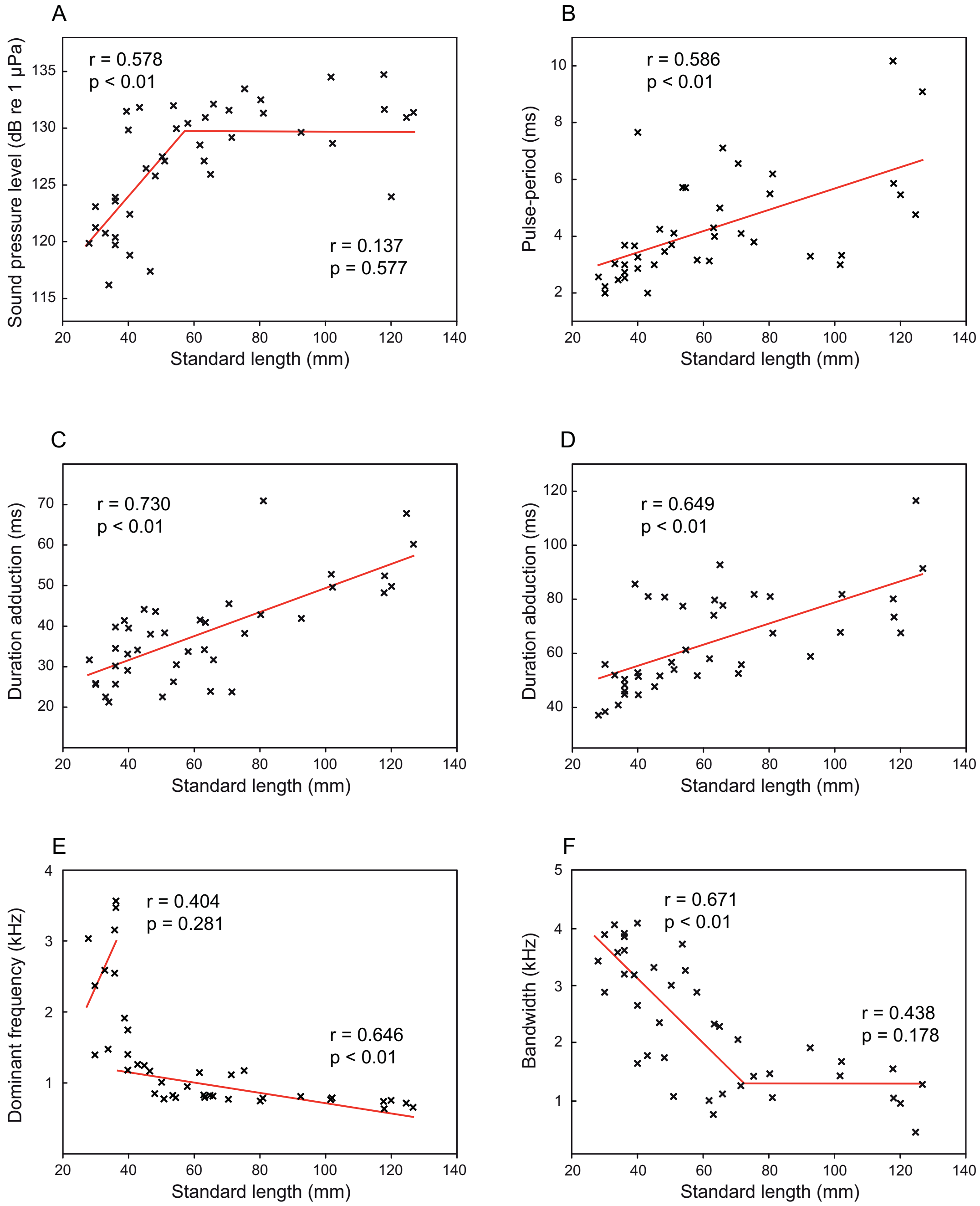

Figure 4 Correlations between sound characteristics and standard length. Plots of sound characteristics against standard length. Pearson's correlation coefficients and significances are given in graphs. **4A**: Segmented linear regression plot SPL against SL, breaking point 57.6 mm SL, regression equations (y = SPL): SL < 57.6 mm: N = 21, SPL = 0.36 SL + 109.76, SL > 57.6 mm: N = 19, SPL = 0.02 SL + 129.10; **4B**: Pulse-period (adduction) against SL: N = 40, pulse period = 0.04 SL + 1.93; **4C**: Duration of adduction sounds against SL: N = 40, duration = 0.30 SL + 19.59; **4D**: Duration of abduction sounds against SL: N = 40, duration = 0.39 SL + 39.69; **4E**: Segmented linear regression plot of dominant frequency against SL, breaking point 36.91 mm SL: SL < 36.91 mm: N = 9, dom. freq. = 101.35 SL - 741.89, SL > 36.91 mm: N = 31, dom. freq. = - 7.04 SL + 1469.41; **4F**: Segmented linear regression plot bandwidth of sounds 10 dB below SPL of peak frequency against SL, breaking point 73.43 mm SL: SL < 73.43 mm: N = 24, bandwidth = - 53.42 SL + 5174.77, SL > 73.43 mm: N = 11, bandwidth = - 9.36 SL + 2210.15. Regression lines in 4A, 4E and 4F were drawn according to the results of the segmented linear regression calculation. Note two p and r values (one for each regression) in figures 4A, 4E and 4F.

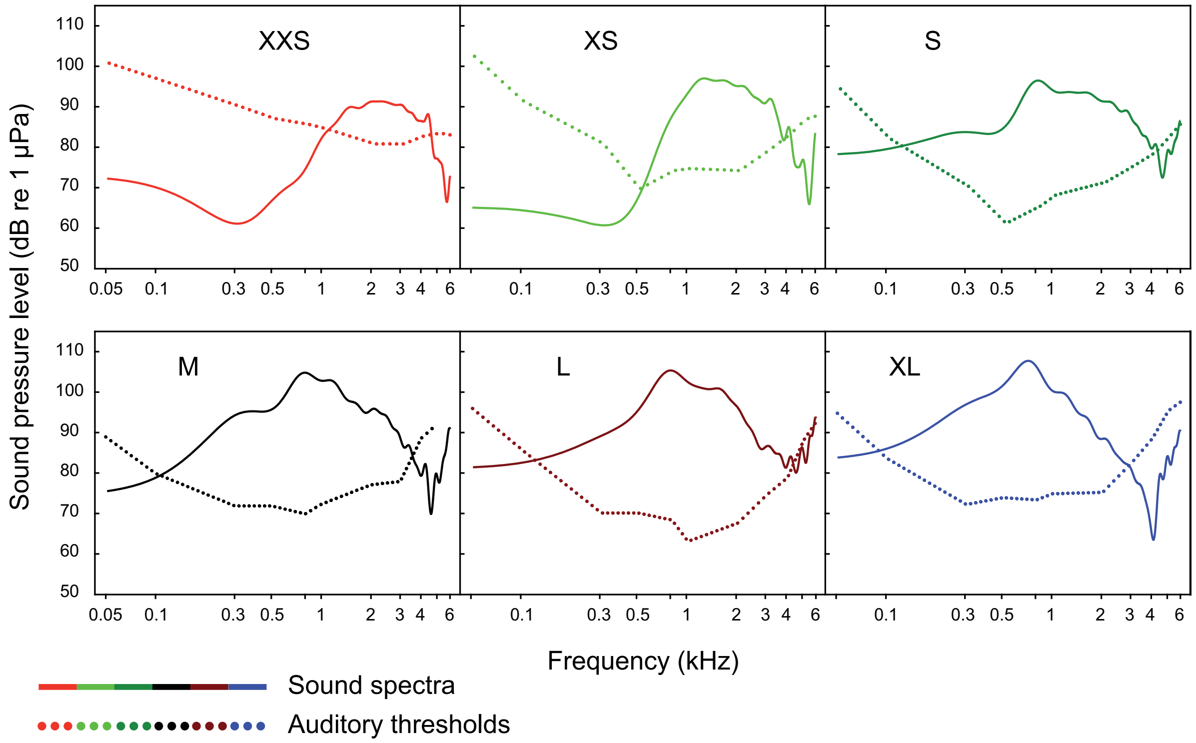

Figure 5 Relations between auditory thresholds and sound spectra. Cepstrum-smoothed sound power spectra of stridulatory sounds calculated for a distance of 5 cm from the hydrophone compared to auditory thresholds in the six size groups tested. For size-ranges see material and methods.

anatomical constraints and on how long and hard fish press the dorsal process of the pectoral spine against the groove of the shoulder girdle [37,40]. This could cause intraindividual variation of SPLs and a lack of correlation in larger animals.

Temporal characteristics of sounds such as duration of abduction and adduction sounds and pulse period of sounds increase with size in *S. schoutedeni*. This can be explained by the growth of the dorsal process of the pectoral spine and the fact that a full pectoral sweep takes longer in larger fish than in a smaller one [37,40]. Increases with size have also been observed for pulse period in the Lusitanian toadfish [19], pulse duration in weakfish [36], and grunt duration in the grey gurnard [25].

Comparison between hearing abilities and spectra of stridulation sounds

In all tested size groups of *S. schoutedeni*, the main energies of stridulation sounds correspond to the most sensitive frequency range of hearing (Figure 5). Despite some uncertainty of sound level measurements in aquaria as compared to free field conditions our data show that all groups are apparently able to detect sounds produced by specimens of the other groups, which is in contrast to prior findings in other teleost species. In croaking gouramis and Lusitanian toadfish, individuals of the smallest size groups were probably unable to detect sounds produced by similar-sized conspecifics [17,19] based on a comparison between absolute sound spectra and audiograms at a communication distance of 3 to 10 cm. The reason for this difference between *S. schoutedeni* and the latter two perciform species is two-fold: First, *S. schoutedeni* shows much higher hearing sensitivities than the other two species investigated, namely the croaking gourami and the Lusitanian toadfish [17,19]. Early stages of the mochokid catfish are more sensitive at the dominant frequencies of conspecific sounds than in the other two species. Secondly, the SPLs of juveniles' sounds are much higher than those of juveniles of the other two species. Thus, all squeaker catfish can detect sounds of conspecifics either uttered during agonistic intraspecific interactions or in a distress context [41].

Conclusions

The current study shows varied changes in auditory sensitivity during the ontogenetic development of *S. schoutedeni*. Hearing sensitivities increase with growth in the frequency range where main energies of sounds are concentrated, whereas the opposite is the case in the highest tested frequencies (5 and 6 kHz). The ontogenetic development of sounds follows fish-typical patterns, namely an increase in sound level and duration, and a decrease in dominant frequency of sounds. Contrary to previous studies, the present data show that the squeaker catfish can communicate with conspecifics of all size groups at short distances either during agonistic encounters or when being attacked by predators. These enhanced sound-detecting abilities of otophysines enable catfish to orient and communicate acoustically at much earlier stages of development.

Methods

Animals

For hearing measurements, fish were grouped into six size groups, XXS (SL = 21.9 to 36.5 mm, N = 12), XS (SL = 39.5 to 45.3 mm, N = 6), S (SL = 51.0 to 58.1 mm, N = 6), M (SL = 71.1 to 81.0 mm, N = 5), L (SL = 92.6 to 102.6 mm, N = 4) and XL (SL = 116.0 to 126.8 mm, N = 6). Hearing thresholds of group M were taken from Lechner and Ladich [4]. For sound recordings, corresponding groups were defined. Because not every specimen vocalized during the sound recording procedures, minimum and maximum size ranges differed slightly: XXS: SL = 28.0 to 36.0 mm, N = 9; XS: SL = 39.0 to 45.0 mm, N = 6; S: SL = 46.7 to 58.1 mm, N = 7; M: SL = 61.8 to 81.1 mm, N = 10; L: SL = 92.6 to 102.2 mm, N = 3; XL: SL = 117.8 to 126.8 mm, N = 5.

Fishes of groups M (hearing tests) to XL were wild caught from Malebo Pool (Congo River, Democratic Republic of the Congo) and obtained from a tropical fish supplier (Transfish, Munich, Germany). After a quarantine of three weeks, fish were acclimated to our aquaria for at least two months prior to the first experiments. Specimens of groups XXS, XS, S and the specimens of group M used for sound recordings were aquarium reared. For a detailed description of the breeding procedure (breeding occurred spontaneously and was not induced by hormone injections) of the tested specimens see Drescher [42]. Because breeding and rearing mochokid catfishes in aquaria is very difficult, we had only a small number of offspring and we were also interested in measuring fish of later stages, we did not test any specimens smaller than 22 mm SL.

Fish were kept in planted aquaria with a sand bottom equipped with roots and clay or bamboo tubes as shelters. Only external filters were used. In order to provide a quiet environment, we did not use any internal filters or air stones. Temperature was kept at 25 ± 1°C and a 12 h: 12 h L: D cycle was maintained. Fish were fed frozen chironomid larvae and artificial food (granulate, flakes and tablets); the small specimens of groups XXS, XS and S were also fed Cyclop-Eeze® (freeze-dried copepods, Argent Chemical Laboratories, Redmond, WA, USA). Since fry and juveniles grow very unequally despite identical conditions of husbandry [13,43], we classified the tested specimens as different size groups rather than age groups. Standard length (SL) was measured as *standard length 2* following Holcik et al. [44]. Using total length or body mass instead of SL for analyses did not change the results.

All experiments were performed with the permission of the Austrian Federal Ministry for Education, Science and Culture (GZ 66.006/2-BrGT/2006).

Auditory sensitivity measurements

Hearing thresholds were obtained using the AEP recording technique developed by Kenyon et al. [28] and modified by Wysocki and Ladich [45,46]. Only a brief description of the technique is given here. Test subjects were mildly immobilized with Flaxedil (gallamine triethiodide; Sigma-Aldrich, Vienna, Austria) diluted in a Ringer solution. The dosage applied (3.5 to 5.4 μg g^{-1}) allowed fish to still perform slight opercular movements but not to initiate significant myogenic noise that could interfere with the AEP recordings. All auditory measurements were carried out in a bowl-shaped plastic tub (diameter 33 cm, water depth 13 cm, 1 cm layer of gravel), which was lined inside with acoustically absorbent material (air-filled packing wrap) to decrease resonances and reflections [47]. The tub was positioned on an air table (TMC Micro-g 63-540, Technical Manufacturing Corporation, Peabody, MA, USA), which rested on a vibration-isolated plate of concrete. A sound-proof chamber, constructed as a Faraday cage (interior dimensions: 3.2 m × 3.2 m × 2.4 m), enclosed the whole setup. The subjects were placed at the water surface in the center of the tub. The contacting points of the electrodes were maximally 1 to 2 mm above the water surface. Tissue paper (Kimwipes®, Kimberly-Clark Corporation, Irving, TX, USA) was placed on the fish head to keep it moist and ensure proper contact of electrodes. Respiration was achieved through a temperature-controlled (25 ± 1°C), gravity-fed water circulation system using a pipette inserted into the subject's mouth. The AEPs were recorded by using silver wire electrodes (0.38 mm diameter) pressed firmly against the skin: the recording electrode was placed over the region of the medulla and the reference electrode cranially between the nares. Shielded electrode leads were attached to the differential input of an AC preamplifier (Grass P-55, Grass Technologies, West Warwick, RI, USA, gain 100 x, high-pass at 30 Hz, low-pass at 1 kHz), with a ground electrode placed in the water near the fish's body. A hydrophone (Brüel and Kjaer 8101, Naerum, Denmark; frequency range 1 Hz to 80 kHz ± 2 dB; voltage sensitivity -184 dB re 1 V μPa^{-1}) was placed close to the head on the right side of the animals (approximately 1 cm away) in order to determine absolute stimulus SPLs. A custom-built preamplifier was used to boost the hydrophone signal. Both presentation of sound stimuli and AEP waveform recording were achieved using a modular rack-mount system (Tucker-Davis Technologies (TDT) System 3, Gainesville, FL, USA) controlled by a PC containing a TDT digital signal processing board and running TDT BioSig RP software (Tucker-Davis Technologies, Gainesville, FL, USA).

Presentation of sound stimuli

Hearing thresholds were determined at 0.05, 0.1, 0.3, 0.5, 0.8, 1, 2, 3, 4, 5 and 6 kHz. The duration of sound stimuli increased from two cycles at 50 Hz and 100 Hz up to eight cycles at 4 kHz and above. Rise and fall times increased from one cycle at 50 to 300 Hz up to three cycles at frequencies from 2 to 6 kHz. All bursts were gated using a Blackman window. Data for group M were taken from Lechner and Ladich [4], and this group was not tested at 6 kHz. For each test condition, one thousand stimuli were presented at opposite polarities, that is, 90° and 270°, and were averaged together by the BioSig RP Software, yielding a 2000-stimulus trace to eliminate any stimulus artefact. The SPL was reduced in 4-dB steps. Close to hearing threshold, this procedure was performed twice and the AEP traces were overlaid to visually check if they were repeatable. The lowest SPL at which a repeatable AEP trace could be obtained, as determined by overlaying replicate traces, was defined as the threshold [see also [48]]. Sound stimuli waveforms were created using TDT SigGen RP software (Tucker-Davis Technologies, Gainesville, FL, USA). Tone-bursts were presented through two speakers (Fostex 256 PM-0.5 Sub and PM-0.5 MKII, Fostex Corporation, Tokyo, Japan). These were positioned 0.5 m above the water surface.

Sound recordings and sound pressure level measurements

The experiments were performed in a test tank (50 cm × 27 cm × 30 cm; length × width × height; water depth 25 cm) whose bottom was covered with sand. The aquarium walls, except for the front glass, were lined on the inside with air-filled packing wrap in order to reduce resonances and reflections. Prior empirical tests using white noise and pulsed sounds had shown that lining reduced artefacts such a high frequency resonances, which are a known phenomenon of small tank acoustics in this setup [17]. Akamatsu et al. [[49], see their Figure 7c] and our own recordings (Figure 5) revealed resonance frequencies at 4 kHz and higher frequencies. Video and audio signals were stored synchronously on the hard disc of a DVD hard disc recorder (Panasonic DMR-EX95V, Panasonic Corporation, Osaka, Japan). A hydrophone (Brüel & Kjaer 8106, sensitivity -174 dB re 1 V per l Pa) was positioned about 5 cm off the center of the aquarium and connected to a microphone power supply (Brüel & Kjaer 2804) whose output was sent to the hard disc recorder. Simultaneous video recordings of fin movements were carried out by a video camera (Sony CCD-VX1E, Sony Corporation, Shinagawa, Tokyo, Japan) connected to the DVD hard disc recorder. Sound pressure levels (SPLs) were measured in parallel with the sound recordings, with the hydrophone power supply additionally connected to a sound level meter (Brüel & Kjaer Mediator 2238). Fish were held about 5 cm away from the hydrophone in the middle of the tank, with one pectoral fin fixed either by the frame of a fish net or the fingers of the testing person to avoid overlap of sounds produced by the left and right pectoral fins. According to Akamatsu et al. [49] a short recording distance reduces the effects of tank acoustics on SPLs and signal distortions. Sounds produced by both fins were recorded in each fish. All experiments were carried out in a walk-in soundproof room, which was constructed as a Faraday cage. Test tanks were placed on a vibration-isolated floor.

Sound analysis

The VLC Media Player (VideoLAN, Club VIA, Châtenay Malabry, France, released under the GNU General Public License) was used to assign single sounds to right and left fins and to either abduction (of the body) or adduction (to the body) movement of the fins. All sounds were sampled at 44.1 kHz. Temporal characteristics of sounds were analysed using Raven Pro 1.3 for Windows (Bioacoustics Research Program, Cornell Laboratory of Ornithology, Ithaca, NY, USA) and spectral characteristics were analysed using STx 3.7 (Acoustics Research Institute, Austrian Academy of Sciences, Vienna, Austria).

Ten abduction sounds and 10 adduction sounds were analysed per individual with respect to total duration and pulse period (except for four specimens of group XS, four specimens of group S and one specimen of group

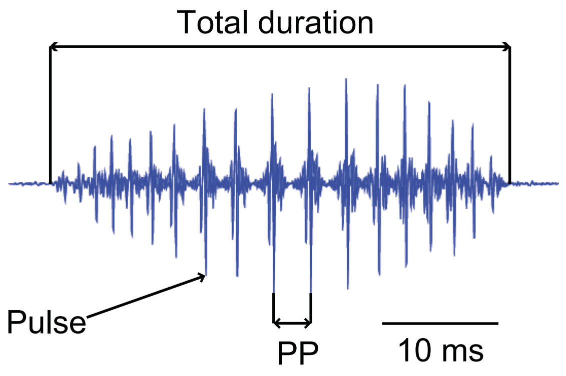

Figure 6 Temporal sound characteristics of single calls. Oscillogram of a single stridulation sound of *S. schoutedeni* showing temporal sound characteristics analysed (PP = pulse period).

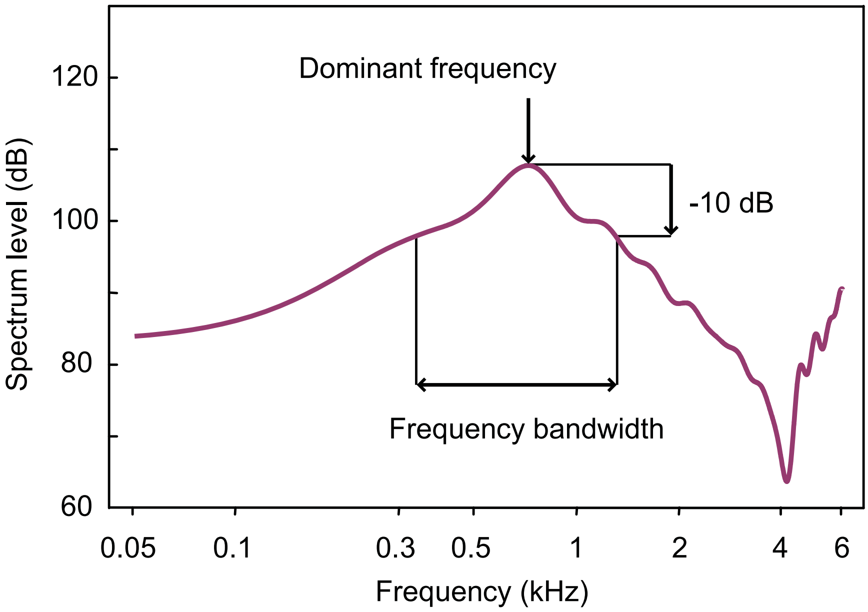

Figure 7 Cepstrum-smoothed power spectrum of stridulatory sounds of a size group indicating the calculation of the frequency bandwidth used for analysis. The minimum and maximum frequencies 10 dB below the dominant frequency were determined and the bandwidth calculated.

M, where only one to five abduction and adduction sounds could be analysed because of a lack of vocalising activity in these individuals) (Figure 6). The dominant frequency of sounds for each individual was determined from the sound power spectrum calculated from all stridulatory sounds emitted by one specimen. A sound file made up of vocalisations emitted by all specimens of a size group was created separately to calculate group-specific cepstrum-smoothed sound spectra [50]. Absolute sound spectra of the recordings were calculated as described previously [46,51]. A value *bandwidth -10 dB* was calculated for each size group, characterising the frequency range of sounds at a level 10 dB below the spectral sound level of the dominant frequency (Figure 7).

Statistical analyses

All data were tested for normal distribution utilising Shapiro-Wilk's test. When data were normally distributed, parametric statistical tests were applied. Mean hearing thresholds were determined for each size group and at each frequency, and audiograms were drawn using SigmaPlot 10.0 (Systat Software/Cranes Software Inc., Bangalore, India and San Jose, CA, USA). Means of sound characteristics were calculated for each fish and used for further analyses. Relationships between fish size (SL or logSL) and hearing thresholds or sound characteristics were determined by Pearson's correlation coefficients and linear regressions. The statistical tests were performed with the software SPSS 17.0 (SPSS Inc., Chicago, IL, USA). If it was evident that data points in a graph showed two different distribution patterns, segmented linear regressions and breakpoints were calculated with the software SegReg (R. J. Oosterbaan, Wageningen, The Netherlands).

Abbreviations

AEP: auditory evoked potentials; dom. freq.: dominant frequency; DVD: digital versatile disc; L: D cycle: light: dark cycle; L: large; M: medium; PP: pulse period; S: small; SL: standard length; SPL: sound pressure level, XL: extra large; XS: extra small; XXS: extra extra small.

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Authors' contributions

FL and WL conceived the study. WL did measurements and analysis of auditory sensitivity. WL and LEW recorded the sounds, WL and FL analysed them. WL, LEW and FL did statistical analyses and wrote the paper. WL prepared the figures and graphs. All authors read and approved the final manuscript.

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Ontogenetic development of Weberian ossicles and hearing abilities in the African bullhead catfish

evaluation currently ongoing for publishing in PLoS ONE

Authors and affiliations

Walter Lechner*, University of Vienna, Department of Behavioural Biology, Austria
Egon Heiss, University of Vienna, Department of Theoretical Biology, Morphology Section, Austria
Thomas Schwaha, University of Vienna, Department of Theoretical Biology, Morphology Section, Austria
Martin Glösmann, University of Veterinary Medicine Vienna, Institute of Pathophysiology, Department of Biomedical Sciences, Austria
Friedrich Ladich, University of Vienna, Department of Behavioural Biology, Austria
* corresponding author

Abstract

Background

The Weberian apparatus of otophysine fishes facilitates sound transmission from the swimbladder to the inner ear to increase hearing sensitivity. It has been of great interest to biologists since the 19th century. No studies, however, are available on the development of the Weberian ossicles and its effect on the development of hearing in catfishes.

Methodology/principal findings

We investigated the development of the Weberian apparatus and auditory sensitivity in the claroteid catfish *Lophiobagrus cyclurus*. The studied specimens ranged from 11.3 mm to 85.5 mm in standard length. Morphology was assessed using sectioning, histology, and X-ray computed tomography, along with 3D-reconstruction. Hearing thresholds were measured utilizing the auditory evoked potentials recording technique. Weberian ossicles and interossicular ligaments were fully developed in all stages investigated except in the smallest size group. In the smallest catfish, the intercalarium and the interossicular ligaments were still missing and the tripus was not yet fully developed. Smallest juveniles revealed lowest auditory sensitivity and were unable to detect frequencies higher than 2 or 3 kHz; hearing sensitivity increased in larger specimens by up to 40 dB, and frequencies were detected up to 6 kHz. In the size groups capable of perceiving frequencies up to 6 kHz, larger individuals had better hearing abilities at low frequencies (0.05 - 1 kHz), whereas an opposite trend was observed at the highest frequencies (4 - 6 kHz).

Conclusions/significance

Our data indicate that the ability of otophysine fish to detect sounds at low levels and high frequencies largely depends on the development of the Weberian apparatus. A significant increase in auditory sensitivity was observed as soon as all Weberian ossicles and interossicular ligaments are present and the chain for transmitting sounds from the swimbladder to the inner ear is complete. This contrasts with findings in another otophysine, the zebrafish, where no changes in thresholds have been observed.

Introduction

Otophysine fish comprise four orders [Cypriniformes (carps and relatives), Characiformes (tetras), Gymnotiformes (South American knifefishes) and Siluriformes (catfishes)] containing approximately 8000 species (Nelson, 2006). This makes them the dominant freshwater fish-group worldwide. They possess a highly complex character, the Weberian apparatus, which was first described by Ernst Heinrich Weber in 1820 (Weber, 1820). It consists of the fused anteriormost vertebrae [“complex vertebra” (Wright, 1884)] and up to four Weberian ossicles which connect the swimbladder to the inner ear. The Weberian ossicles [tripus, intercalarium, scaphium and claustrum – see Dahdul et al. (2010) for a review of the different names used by several authors for the ossicles] transmit oscillations of the swimbladder in a sound field to the inner ear and enhance hearing sensitivity (Ladich and Popper, 2004; Stetter, 1929; von Frisch, 1923; von Frisch and Stetter, 1932). The Weberian apparatus shows a huge morphological variability, in particular within the order Siluriformes (catfishes) (for a review see Chardon et al., 2003). Catfishes inhabit all continents but Antarctica (Ferraris, 2007) and are (with the exception of cypriniforms) the most successful freshwater fish order (about 3100 species). Their hearing abilities depend on the size and number of Weberian ossicles as well as on swimbladder size (Lechner and Ladich, 2008).

The ontogenetic development of Weberian ossicles has been studied in several cypriniforms [(i. e. Coburn and Futey, 1996; Kulshrestha, 1977; Matveiev, 1929; Rosen and Greenwood, 1970; Watson, 1939), in particular in the zebrafish (Bird and Hernandez, 2009; Bird and Mabee, 2003; Grande and Young, 2004) - see Hoffmann and Britz (2006) for an overview of the numerous studies]. Further investigations have been conducted in several characiforms (Grande and Young, 2004; Rosen and Greenwood, 1970), and in a few catfish species [in the ariid *Galeichthys felis* (nowadays *Ariopsis felis*) (Bamford, 1948), the clariid *Clarias gariepinus* (Radermaker et al., 1989), the silurid *Silurus asotus* (Ichiyanagi et al., 1993), the bagrid *Pseudobagrus ichikawai* (nowadays *Coreobagrus ichikawai*) (Ichiyanagi et al., 1997), the callichthyid *Corydoras paleatus* and the ictalurids *Noturus exilis*, *N. miurus* and *Ictalurus punctatus* (Coburn and Grubach, 1998; Grande and Shardo, 2002)]. No studies, however, have been conducted on gymnotiforms. The bottom line of these studies is that the Weberian ossicles scaphium, intercalarium and tripus derive from the first, second and third vertebra, respectively, and that no clear information is available on the size or age at which the interossicular ligaments develop. The order of appearance of ossicles seems to follow a general pattern. According to Grande and Young (2004), the tripus is the first ossicle to differentiate, followed by the formation of the intercalarium and scaphium; the claustrum is the last to form. It remains unclear when the Weberian apparatus is completely developed (including interossicular ligaments) and starts to transmit sounds from the swimbladder to the ear.

Numerous morphological studies have been conducted on the ontogenetic development of the auditory periphery in fish, in particular on Weberian ossicles. In contrast, little information is available on the development of hearing in fishes, especially in otophysines. Studies in non-otophysine fishes regularly observed an increase in hearing sensitivity with size [Kenyon (1996) in the damselfish *Stegastes partitus*, Iwashita et al (1999) in the Red Sea bream *Pagrus major*, Wysocki and Ladich (2001) in the labyrinth fish *Trichopsis vittata*, Higgs et al. (2004) in the clupeid *Alosa sapidissima*, Sisneros and Bass (2005) in the plainfin midshipman *Porichthys notatus*, and Vasconcelos and Ladich (2008) in the Lusitanian toadfish *Halobatrachus didactylus*]. Only Egner and Mann (2005) found a slight decrease in hearing sensitivity at low frequencies during ontogeny of the damselfish *Abudefduf saxatilis*; Belanger et al. (2010) found no differences in hearing abilities of different size stages in the round goby *Neogobius melanostomus*.

Only few studies on the development of hearing have been conducted on otophysines. Popper (1971) compared hearing abilities of two size groups of goldfish (5 and 10 cm) and reported no differences in hearing acuity between those groups. In the cyprinid zebrafish *Danio rerio*, Higgs et al. (2001) (25 - 50 mm) and Zeddis and Fay (2005) (4 days post-fertilization to adult) used different techniques (AEP versus startle response) but found no differences in the stimulus levels and frequency bandwidth to which fish responded. In a subsequent study using 10 - 45 mm zebrafish, Higgs et al. (2003) again found no changes in the absolute hearing thresholds, but an expansion of maximum detectable frequency from 200 Hz to 4000 Hz. This increase of the range of detectable frequencies was attributed to the development of the Weberian ossicles. In a recent study on catfish, Lechner et al. (2010) found a significant frequency-dependent change in hearing thresholds with size in the African mochokid squeaker catfish *Synodontis schoutedeni*, but no change in the range of detectable frequencies.

The present study was designed to investigate the ontogenetic development of hearing abilities in parallel to the development of the auditory periphery (Weberian ossicles) in a representative of the order Siluriformes. We chose the African claroteid catfish *Lophiobagrus cyclurus* and started at earlier stages than in the study on *S. schoutedeni*.

Results

Development of Weberian ossicles

In the smallest size group XS, the chain of ossicles was not yet fully developed (Figure 1, Figure 3 A, B) and consisted of the claustrum, scaphium and tripus. The tripus on the right was bipartite, whereas the left one was a single ossicle and nearly completely attached to the swimbladder wall (Figure 1, Figure 3 A, B, Figure 4). The scaphium and claustrum were located lateral to the central nervous system (Figure 1). The intercalarium as well as the interossicular ligaments were missing (Figure 4) and thus no connection existed between the tripus and the scaphium at this stage (Figure 3 A, B, Figure 4). In contrast, specimens of size groups S to XL possessed a well-developed chain of Weberian ossicles consisting of the tripus, intercalarium, scaphium, claustrum and of interossicular ligaments (Figure 2, Figure 3 C, D, Figure 5). The intercalarium of *L. cyclurus* showed only a slight indication of a processus.

Development of hearing abilities

Group XS specimens had lower hearing abilities than those of the larger groups at each of the frequencies tested (Figure 6, Table 1), in particular at frequencies above 300 Hz. Only two out of six specimens responded to tone bursts at 3 kHz and none to tone bursts at 4, 5 and 6 kHz. Group XS showed its best hearing sensitivity at 300 Hz (106 dB re 1 µPa, Figure 6, Table 1). The lowest threshold from 50 Hz to 2 kHz of all size groups (81.6 dB re 1 µPa at 1 kHz) was found in group XL. At frequencies from 3 - 6 kHz, group S showed the highest sensitivity of all size groups (77.7 dB re 1 µPa at 4 kHz). The lowest hearing threshold of group M was 82.7 dB re 1 µPa and 4 kHz; the corresponding values of group L were 85 dB re 1 µPa at 3 kHz (Figure 6, Table 1). Significant correlations between size and hearing thresholds existed at most frequencies tested. At lower frequencies (50 Hz to 1 kHz), larger animals showed significantly better hearing abilities, whereas at the highest frequencies (4, 5 and 6 kHz) an opposite trend was found: smaller animals had lower hearing thresholds. At 2 and 3 kHz, no correlations were evident (Figure 7).

Discussion

Morphological development of Weberian ossicles

Several studies have addressed the ontogenetic development of siluriforms including the development and homology of the Weberian apparatus (for a review see Adriaens and Vandewalle, 2003). The present study for the first time discusses the development from a functional point of view.

The development of the Weberian apparatus in otophysan fish varies considerably. The first appearance and the sequence of appearance of the Weberian ossicles apparently varies between taxa.

In the zebrafish *Danio rerio* the first Weberian ossicle appears at approximately 5 mm length (SL) (Bird and Mabee, 2003; Grande and Young, 2004). Higgs et al. (2003) observed the chain of ossicles in 7-mm-long (TL) zebrafish and mentioned ‘gaps’ between ossicles. This indicates that a complete, uninterrupted connection between the swimbladder and the inner ear is missing in the zebrafish at this stage.

In the goldfish, the Weberian ossicles appear first at 10 mm (Watson, 1939). Interestingly, Rosen and Greenwood (1970) found no indications of a Weberian ossicle in the small characiform pencilfish *Poecilobrycon* (now *Nannostomus*) *harrisoni* at 7 mm SL.

In the zebrafish and the two characiform species *Brycon erythropterus* (nowadays *B. cephalus*) and the piranha *Serrasalmus* sp., the differentiation of ossicles progresses from posterior to anterior (Bird and Mabee, 2003; Grande and Young, 2004).

A number of investigations in catfishes show that the claustrum appears last [*Silurus asotus* – Ichiyangi et al. (1993); *Pseudobagrus ichikawai* – Ichiyanagi et al. (1997); *Ictalurus punctatus* – Grande and Shardo (2002)]. In *L. cyclurus* the intercalarium is the last of the four ossicles to appear. This is in contrast to the observations mentioned above and to the observations of authors studying Weberian ossicles in catfishes having an intercalarium (ictalurids, Coburn and Grubach, 1998; and silurids, Ichiyanagi et al., 1993).

Table 1. Hearing threshold values

Mean hearing threshold values (+/- s.e.m., dB re 1 µPa) of the five size groups of *L. cyclurus* at each frequency tested. f = frequency; for exact size ranges see material and methods.

f (kHz)	XS	S	M	L	XL
0.05	107.5 ± 0.89	105.1 ± 0.59	103.3 ± 0.78	105.4 ± 0.91	98.4 ± 0.61
0.07	108.3 ± 1.31	103.7 ± 1.90	104.0 ± 0.53	104.5 ± 0.73	98.4 ± 1.00
0.1	110.5 ± 1.82	103.4 ± 1.76	102.1 ± 0.40	103.5 ± 0.91	96.6 ± 1.19
0.3	106.0 ± 2.16	98.3 ± 1.57	97.9 ± 1.99	95.6 ± 1.79	93.4 ± 1.39
0.5	115.1 ± 0.96	96.4 ± 2.71	97.6 ± 1.43	89.6 ± 2.63	88.1 ± 1.52
0.8	122.3 ± 2.40	95.0 ± 1.91	94.0 ± 2.14	85.6 ± 2.62	84.7 ± 2.69
1	125.3 ± 1.28	92.9 ± 2.53	94.9 ± 1.53	86.1 ± 2.94	81.6 ± 2.06
2	130.1 ± 1.35	87.9 ± 2.57	86.7 ± 1.80	88.5 ± 2.35	84.6 ± 2.38
3	128 ± 6.00	83.3 ± 3.60	84.7 ± 3.41	85.0 ± 2.85	87.9 ± 1.83
4	-	77.7 ± 2.78	82.7 ± 1.89	89.6 ± 2.41	88.0 ± 3.01
5	-	87.7 ± 2.35	88.7 ± 1.97	92.5 ± 1.72	95.6 ± 2.40
6	-	85.4 ± 2.68	88.9 ± 2.15	94.1 ± 1.33	101.0 ± 1.51

Other studies fail to mention the sequence of ossicle appearance but do describe the appearance of all elements at a particular fish size. In the hardhead sea catfish *Galeichthys felis* the tripus, intercalarium, scaphium and the interossicular ligaments are developed at 14 mm total length (TL) (this species lacks a claustrum), but a "continuous stretch of tissue" between the bladder and inner ear is not yet developed at this stage (Bamford, 1948).

In *Clarias gariepinnus* the Weberian ossicles are fully developed at 15 mm TL (Radermaker et al., 1989). In *Corydoras paleatus*, which possesses only a single ossicle, this ossicle was found at 5 - 7.6 mm standard length (SL), but not fully ossified at this stage (Coburn and Grubach, 1998). In ictalurids, ossification was observed at stages larger than 11.1 mm (SL) in *Ictalurus punctatus* and larger than 8.3 mm in the genus *Noturus* (Coburn and Grubach, 1998). We cannot determine exactly at which size the chain of ossicles is fully developed in *Lophiobagrus cyclurus*. According to our morphological and physiological data a fully developed ossicular chain including interossicular ligaments is present in group S (min. 24.0 mm SL).

The right tripus of *Lophiobagrus cyclurus* is not a single ossicle (like the left tripus) at the earliest analysed stage (group XS) but is bipartite, a situation similar to that described in the goldfish by Watson (1939). This is because the tripus derives from different sources (parapophysis and pleural rib of the third vertebra, the former giving rise to the articulating process, the latter to the transformator process). These different origins were discussed in ictalurids by Coburn and Grubach (1998) and in the cyprinid *Scardinius erythrophthalmus* by Matveiev (1929) [see also Dahdul et al. (2010) for a review on the development of the Weberian ossicles].

Most catfish in which an intercalarium is present show reduced or missing processes of the intercalarium (Chardon et al., 2003). The intercalarium of adult *L. cyclurus* also shows only a slight indication of a processus. This was described by Bornbusch (1995) as "condition 0" in silurids: interossicular portion compressed, with a slender ascending process extending posterodorsally beyond the interossicular ligament.

The ossification of Weberian ossicles has been discussed in several papers: Watson (1939) and Kulshrestha (1977) mentioned that the intercalarium (manubrium) is an ossification of the interossicular ligaments in two cypriniform species. We suppose that the intercalarium has a similar origin in *L. cyclurus.* Grande and Shardo (2002) found a full ossification of all ossicles at a size of about 15 mm in *Ictalurus punctatus*. In our study species *L. cyclurus*, specimens of the smallest size group XS (size 11.3 - 15.3 mm SL) did not yet have fully ossified ossicles. Our µCt scans showed only the small ossified parts of the ossicles and no cartilaginous areas, in contrast to the 3D reconstructions of the sections which showed ossified and cartilaginous parts. In the bagrid catfish *Pseudobagrus* (*Coreobagrus*) *ichikaway* the scaphium was ossified at 14.8 mm SL and all ossicles of the Weberian system at 25 mm SL (Ichiyanagi et al., 1997). This coincides with our observations and findings in specimens of groups XS and S of *L. cyclurus*, which show ossified ossicles and well-developed hearing beginning with stage S. We suppose that *P. ichikaway* possesses well-developed hearing capabilities, similar to *L. cyclurus*, at this stage.

Development of auditory sensitivities

The present study reveals a relationship between hearing acuity and the development of the Weberian apparatus. Specimens of the smallest stage lacked a fully developed chain of Weberian ossicles and interossicular ligaments, suggesting that transmission of swimbladder oscillations to the inner ear is probably reduced or not yet possible. This may have affected absolute hearing thresholds and reduced the ability to detect higher frequencies (3 - 6 kHz). Surprisingly, the lack of a complete chain of Weberian ossicles had quite different effects in the zebrafish (Higgs et al., 2003). Those authors showed that the development of Weberian ossicles affected the bandwidth of detectable frequencies rather than absolute thresholds. The youngest zebrafish detected sounds up to 200 Hz (versus 2 kHz in our catfish), while older zebrafish detected frequencies up to 4 kHz. In contrast, we found that hearing sensitivity in *L. cyclurus* changed significantly at all frequencies. At this point we cannot explain the different results in zebrafish and *L. cyclurus*.

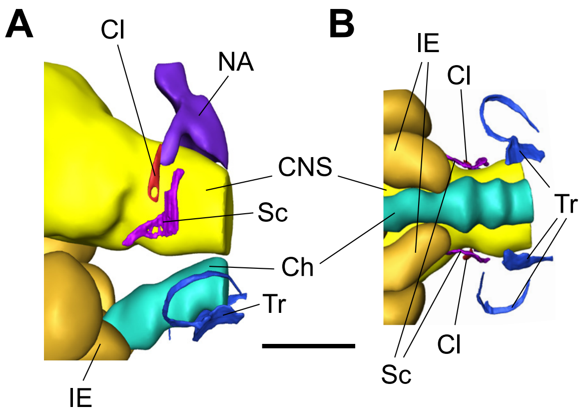

Figure 1. Weberian ossicles and surrounding tissue structures of a specimen of group XS

3D-reconstruction of the posterior skull region of a 11.3 mm SL specimen of *Lophiobagrus cyclurus* based on serial semithin sections. Weberian ossicles (tripus, scaphium, claustrum), inner ear, parts of CNS, chorda and vertebral column are shown. (A) lateral view, (B) ventral view. Ch – Chorda, Cl – Claustrum, CNS – Central nervous system, IE – Inner ear, NA – Neural arch, Sc – Scaphium, Tr – Tripus, scale bar = 300 µm: anterior is to the left, posterior to the right, (A): dorsal above, ventral below.

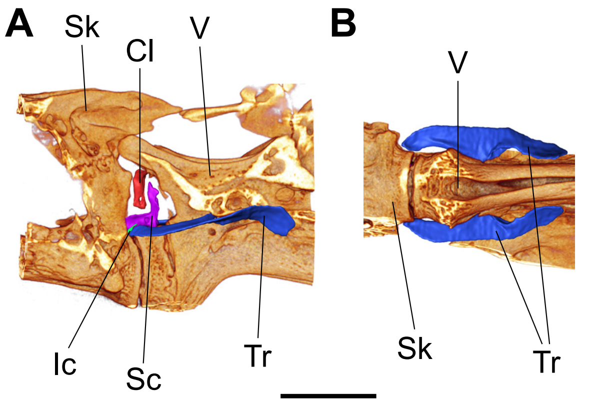

Figure 2. Weberian ossicles and surrounding structures of a specimen of group XL

3D-reconstruction of the cranial region of the vertebral column of an 85.5 mm SL specimen of *Lophiobagrus cyclurus* based on an image stack from a µCT scan. Weberian ossicles (tripus, intercalarium, scaphium and claustrum) and parts of the skull and vertebral column are shown. (A) lateral view, (B) ventral view. Light regions indicate areas where bony parts (shields formed by transverse processes of the vertebrae and processes of skull bones) are left away to provide a better view of the ossicles. Cl – Claustrum, Ic – Intercalarium, Sc – Scaphium, Sk – Skull, Tr – Tripus, V – Vertebral column; scale bar = 3 mm; anterior is to the left, posterior to the right, (A): dorsal above, ventral below.

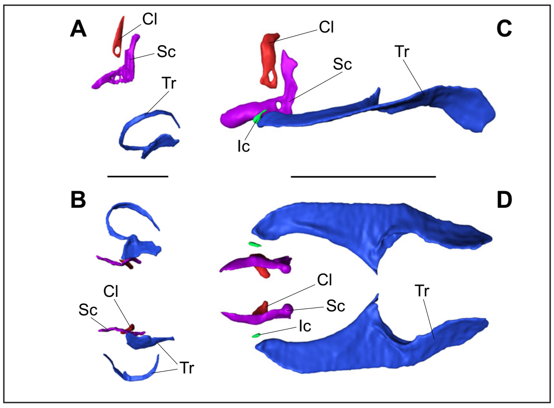

Figure 3. Comparison of Weberian ossicles
3D-reconstruction based on serial semithin section micrographs (A, B) and based on an image stack from a µCT scan (C, D) showing isolated Weberian ossicles. (A) shows a lateral and (B) a ventral view of a specimen of 11.3 mm SL (group XS) and (C) lateral and (D) ventral view of a 85.5 mm SL specimen (group XL). Cl – Claustrum, Ic – Intercalarium, Sc – Scaphium, Tr – Tripus; scale bars in A, B = 300 µm and in C, D = 3 mm; anterior is to the left, posterior to the right, (A), (C): dorsal above, ventral below.

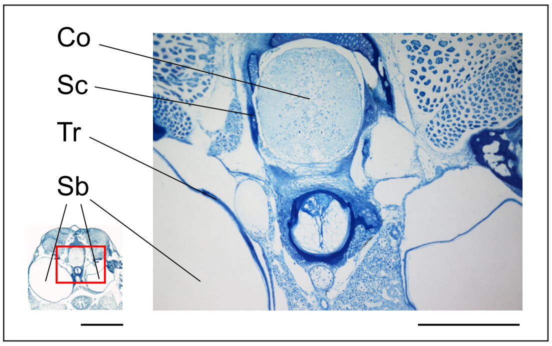

Figure 4. Semithin section in the region of the Weberian ossicles
Near-cross section of a specimen of group XS (SL = 11.3 mm) in the area of the anteriormost vertebrae and the Weberian ossicles. Ossified areas of scaphium and tripus are visible, but there is no indication of interossicular ligaments or intercalarium. Co – Spinal cord, Sb – Swimbladder, Sc – Scaphium, Tr – Tripus; scale bar = 300 µm (right) and 1 mm (left – for overview picture).

Our data support previous findings in the squeaker catfish *Synodontis schoutedeni* (Lechner et al., 2010). Within comparable size stages (> 21 mm to adult), smaller specimens of both catfish species show better high-frequency hearing (4 - 6 kHz in *L. cyclurus* and 5 - 6 kHz in *S. schoutedeni*), and larger specimens show better low-frequency hearing (50 Hz - 1 kHz).

Grande and Young (2004) stated that the Weberian ossicles in 6.6 - 14.5 mm zebrafish are "in position to receive sound vibrations". Higgs et al. (2003) found "large gaps between individual ossicles" up to 13 mm TL in zebrafish and an unbroken chain of ossicles at 19.5 mm TL. They argue that the change in hearing is driven by the development of auxiliary specializations. This agrees with our findings namely that the Weberian apparatus is not fully developed in the smallest stage and that the maximum detectable frequencies (2 - 3 kHz) are lower than in later stages. We cannot explain, however, why the smallest zebrafish did not respond to frequencies higher than 200 Hz. Higgs et al. (2003) discussed this issue and mentioned the possibility that the extension of the frequency range could be due to the addition of saccular and lagenar hair cells, and to a significant increase in the perimeter of both saccular regions. They also argued that it is unlikely that the changes in the maximum detectable frequency are due to selective addition of high-frequency hair cells in the sacculus.

In an earlier study on the goldfish, Popper (1971) detected no significant differences in hearing capabilities between fish of 5 and 10 cm SL. Popper's results can probably be explained by the fact that the major improvements in hearing take place in stages < 20 mm and that his specimens were larger. Nevertheless, that study shows that larger fish, which have larger inner ear sensory maculae and thus more hair cells, do not hear better.

Generally, changes in hearing abilities during ontogeny are known in several fish species (Egner and Mann, 2005; Iwashita et al., 1999; Kenyon, 1996; Sisneros and Bass, 2005; Vasconcelos and Ladich, 2008; Wysocki and Ladich, 2001). None of these studies, however, examined the changes in the auditory periphery or the inner ear, preventing conclusions on which factors are responsible for the changes in hearing. Only Higgs et al. (2004) discussed that structural changes in the utriculus might be correlated to the ability to detect ultrasound in the American shad *Alosa sapidissima*.

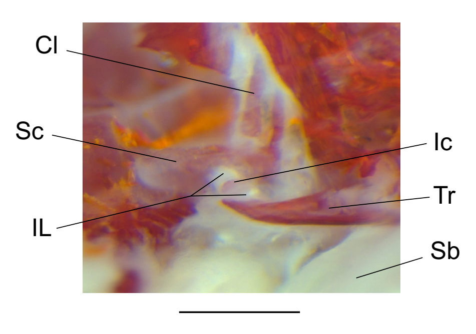

Figure 5. Chain of Weberian ossicles
Micrograph of an alizarin-stained specimen of 27.7 mm SL (group S) showing a complete chain of Weberian ossicles and interossicular ligaments. Surrounding bones and tissues have been removed. Cl – Claustrum, Ic – Intercalarium, IL – Interossicular ligaments, Sb – Swimbladder (part), Sc – Scaphium, Tr – Tripus (anterior part); scale bar = 500 µm; anterior is to the left, posterior to the right, dorsal above, ventral below.

The present study supports recent findings in the squeaker catfish *S. schoutedeni* (Lechner et al., 2010), where the first evidence of a change in auditory thresholds during ontogeny in an otophysine fish species has been reported. The results of hearing measurements at the low and mid-frequency range in *L. cyclurus* agree with most former findings in otophysine and non-otophysine fish, namely that sensitivity increases with size.-

The importance of the uninterrupted Weberian chain has been studied in adult otophysines by Poggendorf (1952) and Ladich and Wysocki (2003) by tripus extirpation in a catfish and a cyrinid, respectively. Poggendorf (1952) showed that bilateral removal of the tripus in the ictalurid *Ameiurus nebulosus* resulted in a frequency-independent decrease in hearing sensitivity. In contrast, Ladich and Wysocki (2003) showed that bilateral extirpation in the goldfish led to a hearing loss which increased with frequency and furthermore resulted in a narrower detectable frequency range. This frequency-dependency is more similar to our observations in *L. cyclurus* and supports our interpretation that hearing improvement depends on the presence of an uninterrupted Weberian chain of ossicles.

In summary, our study on the catfish *Lophiobagrus cyclurus* shows that the improved hearing abilities in this siluriform fish and probably in all otophysines depend on the development of the Weberian apparatus. We show that the freshly hatched catfish do not yet possess a fully developed Weberian apparatus, which probably reduces hearing acuity and the range of detectable frequencies. We furthermore illustrate that pronounced differences exist between the zebrafish and *L. cyclurus*.

Materials and methods

Animals

All *Lophiobagrus cyclurus* (Worthington and Ricardo, 1937) were aquarium bred and obtained from Oliver Drescher (Vienna, Austria). Fish were kept in planted aquaria with a sand bottom equipped with roots and clay or bamboo tubes as shelters. In order to provide a quiet environment, we used only external filters and no internal filters or air stones. Temperature was kept at 25 ± 1° C and a 12 h : 12 h L : D cycle was maintained. Fish were fed frozen chironomid larvae and artificial food (granulate, flakes and tablets); the small specimens of groups XS were also fed Cyclop-Eeze® (freeze-dried copepods, Argent Chemical Laboratories, Redmond, WA, USA). Since fry and juveniles grow very unequally despite identical conditions of husbandry (Fuiman et al., 1998; Higgs et al., 2001), we classified the tested specimens as different size groups rather than age groups, because size is more highly correlated with stage of osteological development than age (Bird and Mabee, 2003). Standard length (SL) was measured as "standard length 2" following Holcik et al. (1989). Using total length or body mass instead of SL for analyses did not change the results. For hearing measurements, fish were grouped into five size groups, XS (SL = 11.3 - 15.3 mm, 0.04 - 0.08 g, N = 8), S (SL = 24.0 - 34.7 mm, 0.33 – 0.69 g, N = 10), M (SL = 39.0 – 47.8 mm, 1.52 – 2.82 g, N = 7), L (SL = 54.5 – 66.3 mm, 4.60 – 7.85 g, N = 8) and XL (SL = 68.9 – 83.9 mm, 8.43 – 15.8 g, N = 7). Complete audiograms could not be obtained from each fish, in particular in the smallest size group. A minimum of six hearing thresholds was determined for each group and each frequency.

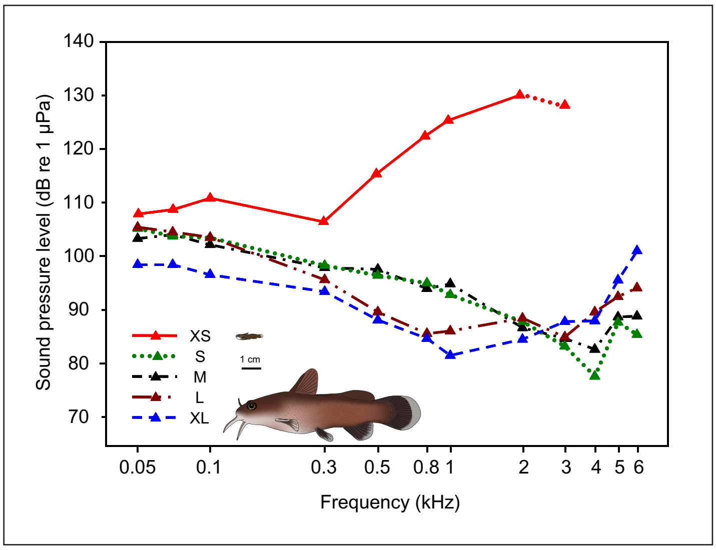

Figure 6. Auditory evoked potential audiograms of the five size groups

Mean hearing thresholds of representatives of size groups XS (N = 7), S (N = 7), M (N = 7), L (N = 8) and XL (N = 7) of *Lophiobagrus cyclurus*. Catfish pictures show representative specimens of group XS (upper) and XL (lower) drawn to scale for comparative purposes.

All hearing experiments were performed with the permission of the Austrian Federal Ministry of Science and Research (GZ 66.006/0023-II/10b/2008).

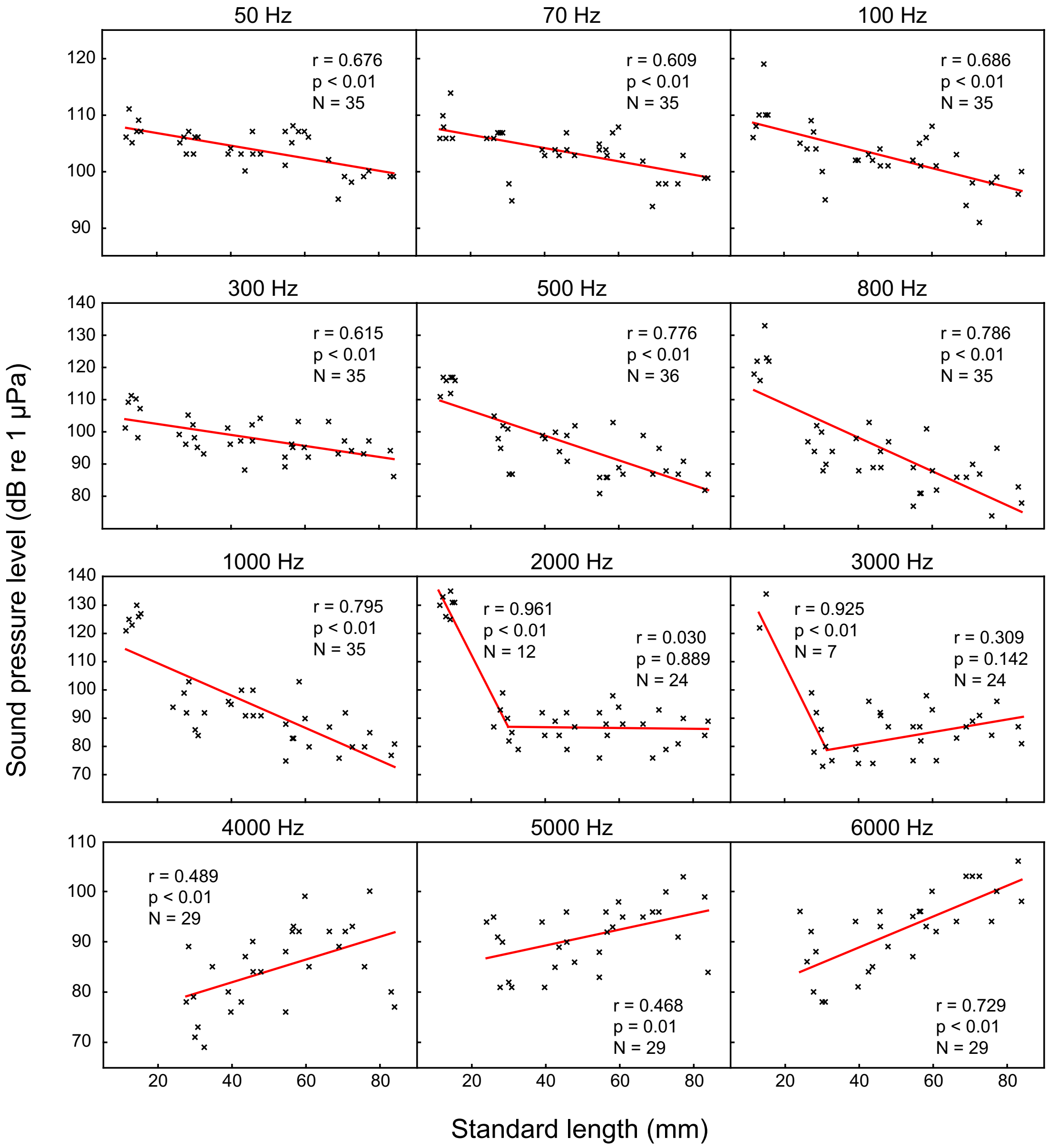

Figure 7. Correlations between auditory thresholds and fish size at each frequency tested

Plots of hearing thresholds of each individual against standard-length at each frequency tested. N-values, Pearson's correlation coefficients and significances are given in graphs. Regression equations: x = standard length, y = hearing threshold (dB re 1 µPa); 50 Hz: y = -0.11 x + 108.90; 70 Hz: y = - 0.12 x + 108.92; 100 Hz: y = - 0.17 x + 110.51; 300 Hz: y = - 0.17 x + 105.66; 500 Hz: y = - 0.38 x + 114.09; 800 Hz: y = - 0.52 x + 118.88; 1000 Hz: y = - 0.57 x + 120.92; 2000 Hz: breaking point (BP) = 30.18 mm, SL < BP: y = - 2.62 x + 164.96, SL > BP: y = 0.01 x + 85.64; 3000 Hz: BP = 30.77 mm, SL < BP: y = - 2.88 x + 167.74, SL > BP: y= 0.15 x + 76.68; 4000 Hz: y = 0.23 x + 72.87; 5000 Hz: y = 0.16 x + 83.01; 6000 Hz: y = 0.31 x + 76.61. Regression lines in 2000 Hz and 3000 Hz were drawn according to the results of the segmented linear regression calculation. Note two p and r values (one for each regression) in graphs of 2000 Hz and 3000 Hz.

Examination of Weberian ossicles

For morphological examinations, fish were sedated using an overdose of tricaine methanesulphonate (MS-222, Sandoz, Basel, Switzerland) and then immediately preserved in alcohol (70%), formalin (4%) or Bouin's solution (Romeis, 1989).

Dissections were carried out in alcohol-preserved specimens (of groups S, M, L and XL) with the aid of dissection microscopes (Wild M5, Wild Heerbrugg Ltd, Heerbrugg, Switzerland, and Nikon SMZ1500, Nikon Corporation, Tokyo, Japan). Bones and ossicles were stained in a solution of KOH (1%) and alicarin red. The photo of Weberian ossicles was taken using a Leica MZ16 F microscope (Leica Microsystems, Wetzlar, Germany) and a ProgRes C5 camera (Jenoptik, Jena, Germany); the photograph was edited with Adobe Photoshop CS5 (Adobe, San Jose, CA, USA).

Microcomputed tomography (µCT35, SCANCO Medical AG, Brüttisellen, Switzerland) was used to assess bone architecture using 3.5, 6, 10 and 15 µm isotropic voxel size (depending on the size of the scanned specimen). One specimen each of group XS, S, L and XL was scanned in 70% ethanol along the coronal axis. Images were acquired at 70 kV and 57 µA with a 0.18° rotation between frames. CT images were reconstructed in 2048 × 2048 pixel matrices using a standard convolution-backprojection procedure. The resulting greyscale image stack was cropped to the region of interest before importing it into 3D-reconstruction software Amira 4.1 (Mercury Computer Systems, Chelmsford, MA, USA). Bony structures were visualized as volume rendering using the Voltex tool and the VolrenGlow colormap of Amira. To accentuate the Weberian ossicles within the volume rendering, they were surface-reconstructed by labelling them with the magic wand tool of the Amira segmentation editor, with minor corrections conducted with the brush tool. A surface for each ossicle was created using Amira's SurfaceGen. Surfaces were optimised by iterated simplification and smoothing steps. Snapshots of the surface reconstruction were taken with Amira.

For paraffin-based histology, three animals of stage XS were fixed in Bouin's solution (Romeis, 1989) for 30 days, changing the solution twice a week. After complete fixation and decalcification, the samples were dehydrated in a graded ethanol-isopropanol series and embedded in paraffin. After polymerisation, 7-µm serial-sections were made on a Reichert-Jung 2030 rotary microtome (Reichert-Jung, Bensheim, Germany). The sections were mounted on glass slides and, after removing the paraffin, standard stained with Haematoxylin-Eosin (H-E) and Azan (after Kiernan, 2003; Romeis, 1989). Stained sections were analysed and documented by digital photography under a Nikon Eclipse E800 light microscope equipped with a Nikon DS-5MU1 digital camera (Nikon Corporation, Tokyo, Japan).

One specimen of group XS was decalcified for resin-embedding by storage in Bouin's solution for several weeks. After decalcification, the specimen was transferred to 70% alcohol, dehydrated in graded alcohol, and embedded in Agar low viscosity resin (Agar Scientific, Stansted, England) using acetone as intermediate. Ribbons of serial sections (2 µm section thickness) were obtained with a Histo Jumbo diamond knife on a Reichert Ultracut S microtome (Reichert-Jung, Bensheim, Germany) (Ruthensteiner, 2008). Sections were stained with Toluidine blue and micrographs were captured as described above for histological sections.

Images were reduced in size and converted to greyscales using Adobe Photoshop CS3 (Adobe, San Jose, CA, USA) before importing the image stack into Amira 4.1. The image stack was aligned with the AlignSlices tool of Amira. Structures, i.e. the Weberian ossicles, central nervous system, inner ear, chorda dorsalis and the first neural arch were labelled manually with a brush before creating a surface for each structure. Surface generation and optimization was conducted as mentioned for the reconstruction of ct-scanned specimens. and snapshots were taken with the Amira software.

Auditory sensitivity measurements

Hearing thresholds were obtained using the AEP recording technique developed by Kenyon et al. (Kenyon et al., 1998) and modified by Wysocki and Ladich (Wysocki and Ladich, 2005a; Wysocki and Ladich, 2005b). Only a brief description of the technique is given here. Test fish of groups S - XL were mildly immobilized with Flaxedil (gallamine triethiodide; Sigma-Aldrich, Vienna, Austria) diluted in a Ringer solution. The dosage applied (0.3 - 1.81 µg g-1) allowed fish to still perform slight opercular movements but not to initiate significant myogenic noise that could interfere with the AEP recordings. Specimens of group XS were not immobilized because of their small size. All auditory measurements were carried out in a bowl-shaped plastic tub (diameter 33 cm, water depth 13 cm, 1 cm layer of gravel), which was lined inside with acoustically absorbent material (air-filled packing wrap) to decrease resonances and reflections (Wysocki and Ladich, 2002). The tub was positioned on an air table (TMC Micro-g 63-540, Technical Manufacturing Corporation, Peabody, MA, USA), which rested on a vibration-isolated plate of concrete. A sound-proof chamber, constructed as a Faraday cage (interior dimensions: 3.2 m x 3.2 m x 2.4 m), enclosed the whole setup. The subjects were placed at the water surface in the center of the tub. The contacting points of the electrodes were maximally 1 - 2 mm above the water surface. Tissue paper (Kimwipes®) was placed on the fish head to keep it moist and ensure proper contact of electrodes. Respiration was achieved through a temperature-controlled (25 ± 1° C), gravity-fed water circulation system using a pipette inserted into the animal's mouth. The AEPs were recorded by using silver wire electrodes (0.38 mm diameter) pressed firmly against the skin: the recording electrode was placed over the region of the medulla and the reference electrode cranially between the nares. Shielded electrode leads were attached to the differential input of an AC preamplifier (Grass P-55, gain 100 x, high-pass at 30 Hz, low-pass at 1 kHz), with a ground electrode placed in the water near the fish's body. A hydrophone (Brüel and Kjaer 8101, Naerum, Denmark; frequency range 1 Hz to 80 kHz ± 2 dB; voltage sensitivity –184 dB re 1 V μPa^{-1}) was placed close to the head on the right side of the animals (~1 cm away) in order to determine absolute stimulus SPLs. A custom-built preamplifier was used to boost the hydrophone signal. Both presentation of sound stimuli and AEP waveform recording were achieved using a modular rack-mount system [Tucker-Davis Technologies (TDT) System 3, Gainesville, FL, USA] controlled by a PC containing a TDT digital signal processing board and running TDT BioSig RP software.

Presentation of sound stimuli

Hearing thresholds were determined at 0.05, 0.07, 0.1, 0.3, 0.5, 0.8, 1, 2, 3, 4, 5 and 6 kHz. The duration of sound stimuli increased from two cycles at 50 Hz, 70 Hz and 100 Hz up to eight cycles at 4 kHz and above. Rise and fall times increased from one cycle at 50 to 300 Hz, up to three cycles at frequencies from 2 to 6 kHz. All bursts were gated using a Blackman window. For each test condition, one thousand stimuli were presented at opposite polarities, i.e. 90° and 270°, and were averaged together by the BioSig RP Software, yielding a 2000-stimulus trace to eliminate any stimulus artifact. The SPL was reduced in 4-dB steps. Close to hearing threshold, this procedure was performed twice and the AEP traces were overlaid to visually check if they were repeatable. The lowest SPL at which a repeatable AEP trace could be obtained, as determined by overlaying replicate traces, was defined as the threshold (see also Ladich and Wysocki, 2009). Sound stimuli waveforms were created using TDT SigGen RP software. Tone-bursts were presented through two speakers (Fostex 256 PM-0.5 Sub and PM-0.5 MKII, Fostex Corporation, Tokyo, Japan). These were positioned 0.5 m above the water surface.

Statistical analyses

All data were tested for normal distribution using Shapiro-Wilk's test. When data were normally distributed, parametric statistical tests were applied. Mean hearing thresholds were determined for each size group and at each frequency, and audiograms were drawn using SigmaPlot 10.0 (Systat Software/Cranes Software Inc., Bangalore, India and San Jose, USA). Means of sound characteristics were calculated for each fish and used for further analyses. Relationships between fish size (SL) and hearing thresholds were determined by Pearson's correlation coefficients and linear regressions. The statistical tests were performed with the software SPSS 17.0 (SPSS Inc., Chicago, Illinois). When datapoints showed two different distribution patterns, segmented linear regressions and breakpoints were calculated using the software SegReg (R. J. Oosterbaan, Wageningen, The Netherlands).

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How do albino fish hear?

W. Lechner & F. Ladich

Department of Behavioural Biology, University of Vienna, Vienna, Austria

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Correspondence
Walter Lechner, Department of Behavioural Biology, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria. Tel: +43 1 4277 54430; Fax: +43 1 4277 9544
E-mail: walter.lechner@univie.ac.at

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Abstract

Pigmentation disorders such as albinism are occasionally associated with hearing impairments in mammals. Therefore, we wanted to investigate whether such a phenomenon also exists in non-mammalian vertebrates. We measured the hearing abilities of normally pigmented and albinotic specimens of two catfish species, the European wels *Silurus glanis* (Siluridae) and the South American bronze catfish *Corydoras aeneus* (Callichthyidae). The non-invasive auditory evoked potential (AEP) recording technique was utilized to determine hearing thresholds at 10 frequencies from 0.05 to 5 kHz. Neither auditory sensitivity nor shape of AEP waveforms differed between normally pigmented and albinotic specimens at any frequency tested in both species. *Silurus glanis* and *C. aeneus* showed the best hearing between 0.3 and 1 kHz; the lowest thresholds were 78.4 dB at 0.5 kHz in *S. glanis* (pigmented), 75 dB at 1 kHz in *S. glanis* (albinotic), 77.6 dB at 0.5 kHz in *C. aeneus* (pigmented) and 76.9 dB at 1 kHz in *C. aeneus* (albinotic). This study indicates no association between albinism and hearing ability. Perhaps because of the lack of melanin in the fish inner ear, hearing in fishes is less likely to be affected by albinism than in mammals.

Introduction

Albinism, a genetic abnormality of the melanin system in which the synthesis of this pigment is reduced or lacking, occurs in all classes of vertebrates and in invertebrates. A large diversity of pigmentation disorders is known. Albinism can generally be subclassified as oculocutaneous albinism (four subtypes OCA 1–4 are described) or ocular albinism (OA) depending on loss of melanin in the skin (hair) and eyes versus just in the eyes. Partial albinism describes diseases such as the Waardenburg syndrome, piebaldism and the Tietz–Smith syndrome, in which only part of the skin (hair) lacks pigmentation (Lezirovitz *et al.*, 2006). Inherited pigmentary abnormalities occasionally co-occur with hearing impairments in mammals, but no co-occurrence has been reported from other vertebrates. Darwin (1859) has already stated that 'cats which are entirely white and have blue eyes are generally deaf'. Their colour abnormality and deafness are caused by the dominant white gene *W* (Bergsma & Brown, 1971). Similarly, approximately one-fourth of Dalmatian dogs are at least unilaterally deaf due to genetic disorders (Shelton *et al.*, 1993), and spotting mutations in mammals affecting the coat are often associated with hearing impairments (Steel & Bock, 1983).

Several studies have been conducted in rodents such as gerbils, mice, rats and guinea pigs and in cats, leading to quite different results concerning the influence of albinism on hearing. Albino gerbils show significantly lower compound action potentials but equal auditory brain response (ABR) thresholds compared with normally coloured specimens (Szymanski, Henry & Buchting, 1994). In mice, Bartels *et al.* (2001) found no differences in hearing thresholds of normal and albinotic specimens, whereas Cable, Barkway & Steel (1992) stated that albinos with strial melanocytes show evoked potentials of lower amplitude. Significantly lower hearing thresholds have been reported for albinotic guinea pigs by Conlee *et al.* (1986, 1988), whereas Nuttall (1974) found no differences in cochlear microphonic potentials. Albino cats (true *cc* albinos and not dominant white *w*) occasionally show hearing anomalies (Creel, Conlee & Parks, 1983; Conlee *et al.*, 1984).

There are some indications for a relationship, but no general linkage, between OCA and hearing impairments in humans (e.g. Shiloh *et al.*, 1990; Amiel *et al.*, 1998; Lezirovitz *et al.* 2006).

Albinism has been reported in numerous fish species, including several catfishes, such as hagfish and lampreys (e.g. Braem & King, 1971), sharks and rays (e.g. Sandoval-Castillo, Mariano-Melendez & Villavicencio-Garayzar, 2006) and numerous bony fishes [i.e. in grunts (Abitia, Aguilar & Aguilar, 1995), or cyprinids (e.g. Ueda, Ishinabe & Jeon, 2007); for a review on albino catfish, see Dingerkus, Seret & Guilbert (1991)]. However, we are not aware of any study investigating the association between albinism and hearing sensitivities in non-mammalian vertebrates including fishes, except for an anecdotal report of a deaf albino goldfish in the early 20th century (Bigelow, 1904).

Popper (1970) showed that Mexican blind cave fish *Astyanax mexicanus* do not differ in hearing sensitivity from the normally pigmented and eyed surface-dwelling populations. Blind cave tetras are OCA albinos according to Protas *et al.* (2006). However, cave-dwelling (hypogean) forms of *A. mexicanus* are not just albinotic forms but differ in many traits from surface-dwelling sighted populations, in particular in their sensory system. They show eye regression, much more developed (lateral line) neuromasts and taste buds.

Dutton *et al.* (2009) mentioned that the inner ears develop abnormally in colourless *Sox10* zebrafish mutants. Mutations of this gene cause Waardenburg syndrome in humans.

In order to determine whether an association between pigmentation disorders (albinism) and auditory sensitivity in fishes exists, we compared the auditory evoked potentials (AEP) and hearing abilities in albinotic and pigmented individuals of two species of fishes possessing hearing specializations. We chose species having excellent hearing sensitivities (often termed hearing specialists) because we hypothesize that slight changes in hearing caused by albinism may affect hearing specialists to a higher degree than species having low hearing sensitivities. Two catfish species, namely the European wels *Silurus glanis* (Siluridae) and the South American bronze catfish *Corydoras aeneus* (Callichthyidae), were studied. These two species represent two different groups of catfishes. *Silurus glanis* has a large free swimbladder and four Weberian ossicles (Weber 1820; Bridge & Haddon, 1893), whereas *C. aeneus* has paired, tiny, bony, encapsulated swimbladders and a single ossicle (Huysentruyt & Adriaens, 2005; Lechner & Ladich, 2008). Based on previous studies (Lechner & Ladich, 2008), we expect that the anatomical differences between the two species will be reflected in different auditory sensitivities (at least in normally pigmented specimens) and may be potentially differently affected by albinism. Moreover, the current study is the first to test hearing in the catfish family Siluridae.

Materials and methods

Animals

Catfishes were obtained from ornamental fish suppliers (*C. aeneus* from Ruinemans Aquarium, Montfoort, the Netherlands, and *S. glanis* albinos from Hornbach, Brunn am Gebirge, Austria) and a fish hatchery (*S. glanis* normal from Fischzucht Pottenbrunn, Pottenbrunn, Austria) (Figs 1 and 2).

Fish were kept in planted aquaria with a sand bottom equipped with roots and clay or plastic tubes as shelters. Only external filters were used in order to provide a quiet environment. The temperature was maintained at 20 ± 1 °C for *S. glanis* and 25 ± 1 °C for *C. aeneus*. A 12 h:12 h L:D cycle was maintained. *Silurus glanis* were fed frozen food (chironomid larvae, smelt *Osmerus* sp., *Gammarus* sp. and turkey heart) and live earthworms, *C. aeneus* were fed frozen chironomid larvae and artificial food (granulate, flakes and tablets). Standard length (SL) was measured as 'standard length 2' following Holcik, Banarescu & Evans (1989): *Silurus* specimens ranged from 152 mm SL and 35 g body weight to 215 mm SL and 85 g, *Corydoras* specimens ranged from 32.7 mm SL and 1.53 g to 45.7 mm SL and 3.34 g.

Figure 1 Normally pigmented and albinotic specimens of *Silurus glanis*.

Figure 2 Normally pigmented and albinotic specimens of *Corydoras aeneus*.

Auditory sensitivity measurements

Hearing thresholds were obtained using the AEP recording technique developed by Kenyon, Ladich & Yan (1998), with slight modifications. The exact procedure, including the presentation of sound stimuli and statistical analysis is described by Lechner, Wysocki & Ladich (2010).

Hearing thresholds were determined at 0.05, 0.07, 0.1, 0.3, 0.5, 0.8, 1, 2, 3, 4 and 5 kHz. Test subjects were mildly immobilized with Flaxedil (gallamine triethiodide; Sigma-Aldrich, Vienna, Austria) diluted in a Ringer solution. The dosage applied was 2.12–3.39 $\mu g\,g^{-1}$ for *S. glanis* normal, 3.57–5.78 $\mu g\,g^{-1}$ for *S. glanis* albino, 0.41–0.62 $\mu g\,g^{-1}$ for *C. aeneus* normal and 0.55–0.85 $\mu g\,g^{-1}$ for *C. aeneus* albino. Water temperature during recordings was adjusted to 20 ± 1 °C for *S. glanis* and 25 ± 1 °C for *C. aeneus*.

Results

Pigmentation

Albino specimens of both species showed uniform body coloration from whitish pink in the ventral to yellowish orange in the dorsal region and the fins, combined with red eyes (Figs 1 and 2). Pigmented *S. glanis* had black eyes and a high variability in body pigmentation. Some individuals were dark brown with numerous black and only few silvery whitish blotches and dots, whereas in other specimens, the number of whitish blotches was much higher, covering most of the brown body (Fig. 1). Normal *C. aeneus* were uniformly coloured (black eyes and a yellowish-brown to delicate reddish-brown body colour with strong metallic glint on the sides and darker regions in the middle of the flanks) (Fig. 2). All catfish were acclimated to our aquaria for at least 3 weeks before testing.

AEP waveforms

The AEP of pigmented and albinotic groups of each species did not differ in the overall shape of waveforms, latency, amplitudes or in the number of positive or negative peaks (Figs 3 and 4).

Auditory thresholds

Hearing thresholds of *S. glanis* were the lowest between 300 and 1000 Hz; the lowest threshold was 78.4 dB at 500 Hz in pigmented *S. glanis* and 75 dB at 1000 Hz in albinos (Fig. 5, Table 1). Comparison of the audiograms of the two groups by a two-way ANOVA revealed no significant overall differences ($F_{1,132} = 0.854$, $P = 0.357$). Comparison of hearing thresholds at each frequency with an unpaired *t*-test showed no significant differences between normally coloured and albino fish ($P > 0.05$).

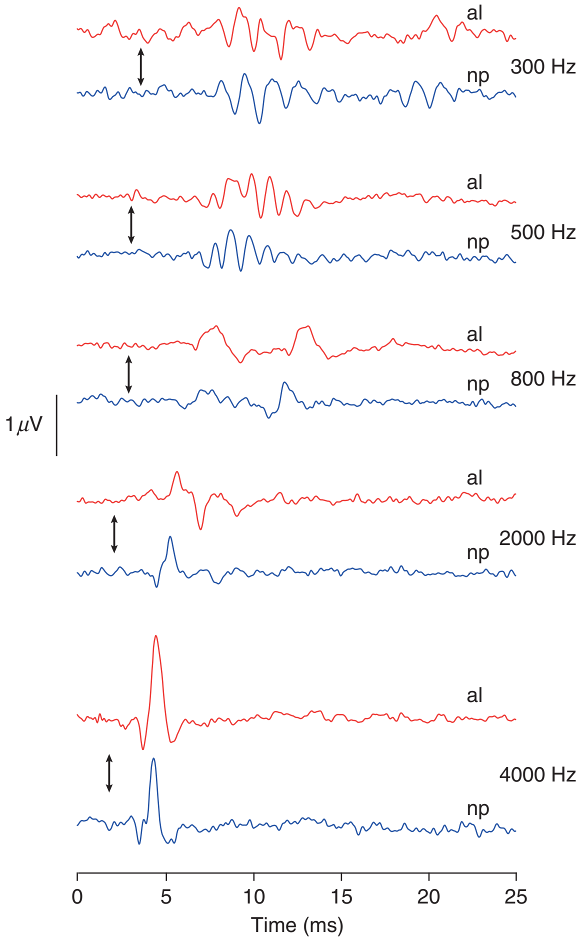

Figure 3 Waveforms of auditory evoked potentials at representative frequencies in normally pigmented (np) and albinotic (al) *Silurus glanis*. Arrows indicate stimulus onset.

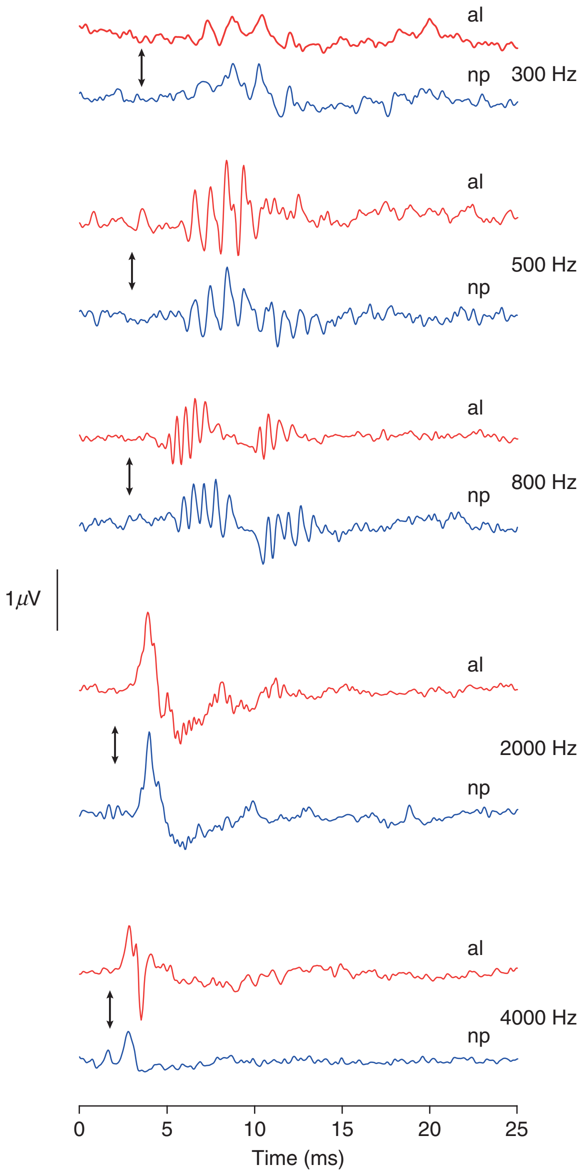

Figure 4 Waveforms of auditory evoked potentials at representative frequencies in normally pigmented (np) and albinotic (al) *Corydoras aeneus*. Arrows indicate stimulus onset.

In *C. aeneus*, auditory sensitivity was the highest between 300 and 1000 Hz. The maximum sensitivity was 77.6 dB at 500 Hz in pigmented and 76.9 dB at 1000 Hz in albinotic *C. aeneus* (Fig. 6, Table 1). Comparing the audiograms of the two groups by a two-way ANOVA revealed no significant overall differences ($F_{1,132} = 0.590$, $P = 0.444$). Comparing hearing thresholds at each frequency with an unpaired t-test showed no significant differences between the normally pigmented and the albino group ($P > 0.05$).

Discussion

AEP waveforms

AEP waveforms of the studied catfish species did not differ between normal and albinotic specimens of each species (Figs 3 and 4). This is in agreement with Dum, Schmidt & von Wedel (1980), who found no difference in the latency and amplitude of the individual peaks in the brain stem response of normal and albinotic guinea pigs. In contrast, Cable *et al.* (1992) reported that W^v/W^v mouse mutants showed lower or no endocochlear potentials. Significant differences have been observed in the ABRs of normal and albinotic humans (Creel *et al.*, 1980) and between normal and albinotic (real *cc* and not *W*, which is associated with deafness) cats (Creel *et al.*, 1983). This is most likely due to differences in auditory pathways and projections between nuclei in albino and pigmented animals (Moore & Kowalchuk, 1988). In general, the AEP waveforms in our catfish were in accordance with those in previous studies in fishes (e.g. Kenyon *et al.*, 1998; Ladich, 1999; Wysocki & Ladich, 2001). Latency and duration of responses decreased with increasing frequencies.

Hearing sensitivity

Hearing impairment and pigmentation disorders frequently occur together in many syndromes in mammals (Lezirovitz *et al.*, 2006). The exact mechanism has been discussed for many years (Garber *et al.*, 1982; Barrenas & Axelsson, 1992). The area primarily involved in many of the pigmentation disorders is the stria vascularis of the mammalian cochlea. Melanocytes within the stria vascularis generate the endocochlear potential (Cable *et al.*, 1992; Bartels *et al.*, 2001), and the absence or degeneration of melanocytes is assumed to be responsible for hearing abnormalities in albino mammals.

As noted above, comparative studies on hearing abilities in normally pigmented and albinotic specimens revealed

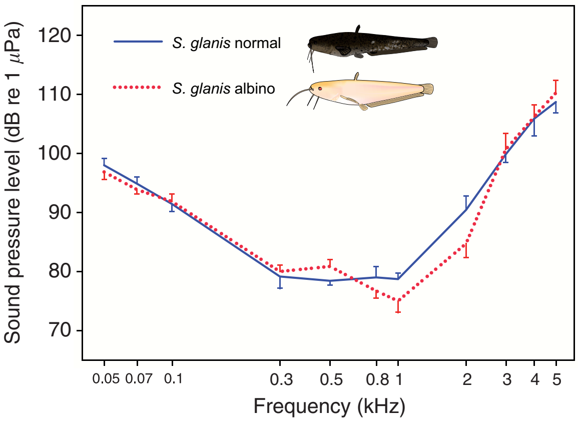

Figure 5 Mean (±SEM) hearing thresholds of normal and albino *Silurus glanis*.

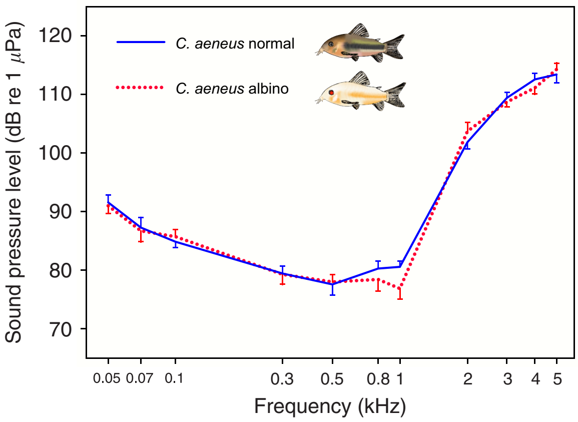

Figure 6 Mean (±SEM) hearing thresholds of normal and albino *Corydoras aeneus*.

Table 1 Mean hearing thresholds (±SEM) of the normal and the albinotic specimens in the two species studied

	Hearing thresholds (dB re 1 μPa)			
Frequency (kHz)	*Silurus glanis*	*Silurus glanis* albino	*Corydoras aeneus*	*Corydoras aeneus* albino
0.05	98.00 ± 1.11	96.86 ± 1.22	91.57 ± 1.29	91.00 ± 1.35
0.07	94.86 ± 1.16	93.86 ± 0.74	87.29 ± 1.76	86.71 ± 1.86
0.1	91.43 ± 1.29	91.86 ± 1.24	84.86 ± 0.99	85.71 ± 1.21
0.3	79.14 ± 1.97	80.00 ± 1.05	79.43 ± 1.27	79.29 ± 1.69
0.5	78.43 ± 0.75	80.86 ± 1.14	77.57 ± 1.85	78.00 ± 1.22
0.8	79.00 ± 1.86	76.71 ± 1.23	80.29 ± 1.23	78.43 ± 2.00
1	78.71 ± 1.02	75.00 ± 1.91	80.57 ± 1.00	76.86 ± 1.81
2	90.43 ± 2.39	84.71 ± 2.37	101.86 ± 1.14	103.71 ± 1.55
3	99.86 ± 1.39	100.71 ± 2.64	109.43 ± 0.97	108.71 ± 0.87
4	105.86 ± 2.87	106.14 ± 2.05	112.57 ± 1.04	111.14 ± 1.08
5	108.71 ± 1.81	110.29 ± 2.08	113.43 ± 1.41	114.29 ± 1.06

widely differing results in AEPs and hearing thresholds. A main reason for these inconsistencies may be the term 'albinism', which is much too sketchy to describe the heterogeneous reasons for defects in pigment production in vertebrates.

Besides the general differentiation between OA and OCA (Orlow, 1997), there are several subclassifications of these two types of albinism, and further pathological syndromes expressed as hypopigmentation (among other effects) are known.

OCA is a group of four autosomal recessive disorders (OCA1–OCA4) caused by either a complete lack or a reduction of melanin biosynthesis in the melanocytes, resulting in hypopigmentation of the hair, skin and eyes (Gronskov, Ek & Brondum-Nielsen, 2007). Studies on hearing in albino vertebrates normally use specimens with some type of OCA, but most studies do not name the type of albinism of the experimental animals. Our albino catfish show OCA2, as did the Mexican cave tetras (Protas *et al*., 2006; Jeffery, 2009).

In fish that lack a cochlea and a stria vascularis, melanocytes – in particular their absence in the inner ear – may play a less crucial role than in the mammalian ear. This may explain why our study animals did not exhibit pigmentation-related differences in hearing abilities. Nonetheless, hypopigmentation could potentially co-occur with hearing disorders in zebrafish mutants. Dutton *et al*. (2009) showed that knocking out the *Sox10* gene results in abnormalities in ear development and lack of body pigmentation, whereas the eyes remain normally pigmented.

Fish lacking pigmentation have been tested for hearing by Popper (1970) only. As in our study, hearing abilities of two closely related species, the Mexican blind cave fish *Astyanax jordani* and its eyed ancestor *A. mexicanus*, did not differ. Today, *A. jordani* is not regarded as a valid species, but as a blind, depigmented, cave-dwelling form of *A. mexicanus* (Jeffery, 2009). In fact, several cave populations evolved convergently from surface-dwelling populations of *A. mexicanus* (Jeffery, 2006) in different habitats. Contrary to the albino catfish of the current study in which lack of pigmentation is based on a genetic disorder, blind cave fish are adapted to cave dwelling and differ in several traits other than pigmentation from the surface-dwelling, pigmented populations. They exhibit major changes in their sensory system, including eye regression, larger lateral line neuromasts and modified taste buds (Bleckmann, 1993; Boudriot & Reutter, 2001; Montgomery, Coombs & Baker, 2001). According to Teyke (1990), the cupulae of neuromasts are about twice as long as in sighted populations, which (most likely) results in higher sensitivity of neuromasts and thus better orientation in caves. Boudriot & Reutter (2001) mentioned that taste buds in cave-dwelling fish contain significantly more axons than in sighted river fish, which indicates a compensatory improvement of chemoreception for prey detection.

In summary, based on our current results, we assume that albinism is not associated with hearing disorders in fishes.

Hearing in catfishes

The order of catfishes comprises more than 3000 species (Ferraris, 2007). Catfishes belong to otophysines and possess Weberian ossicles, which connect the swimbladder to the inner ear. Hearing abilities of many siluriform species are among the best in fishes, with regard to both the detectable frequency range and the absolute sensitivity. The two species chosen represent two different groups of catfishes. *Siluris glanis* has one large free swimbladder and four Weberian ossicles, whereas *C. aeneus* has paired, tiny, bony, encapsulated swimbladders and a single ossicle, which should result in better high-frequency hearing in *S. glanis* (Lechner & Ladich, 2008). While this is the case, the hearing acuity of *S. glanis* is quite low at high frequencies compared with other species with a well-developed swimbladder and ossicles. At 3 kHz and at higher frequencies, *S. glanis* exhibited higher hearing thresholds than all but one catfish species with free bladder tested so far, which belonged to the families Ariidae, Auchenipteridae, Heptapteridae, Malapteruridae, Mochokidae and Pseudopimelodidae (Lechner & Ladich, 2008; Lechner *et al*., 2010), Doradidae (Ladich, 1999), Ictaluridae (Poggendorf, 1952; Wysocki, Montey & Popper, 2009) and Pimelodidae (Ladich, 1999; Wysocki *et al*., 2009).

As expected, the hearing abilities of the callichthyid *C. aeneus* are similar to its congenerics *Corydoras paleatus* and *Corydoras sodalis* (Ladich, 1999; Lechner & Ladich, 2008), with *C. paleatus* having a lower high-frequency hearing sensitivity than the two other species.

In summary, the hearing abilities of the silurid *S. glanis* and the callichthyid *C. aeneus* fit the concept that hearing abilities in catfishes depend on the swimbladder size and the number of Weberian ossicles, as stated by Lechner & Ladich (2008). Albinism does not affect hearing in either of these two species.

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Concluding discussion

The three studies contributing to this doctoral thesis tried to answer several questions on the development of hearing in fishes in general and in catfishes in particular.

Since the methods used in these studies were well established, performing the studies was no problem in this matter. The AEP recording technique for measuring hearing abilities turned out to be even suitably for very small fish specimens. The specimens of group XS of *Lophiobagrus cyclurus* tested were probably the smallest fish ever tested for this (0.04 g and 11.3 mm SL). Sound recordings and analyses were no problem, anyway, because of the good instrumentation in our bioacoustics lab; and µCt-analyses in the bigger catfish specimens (with fully ossified skeletal parts) as well as sectioning techniques with the smallest specimens (with still partial cartilaginous skeleton) turned out to be well adapted techniques to answer my questions.

In the squeaker catfish *Synodontis schoutedeni* six size groups (XXS – XL) have been studied with respect to their ontogenetic development of hearing and sound communication. At lower frequencies an increase of hearing sensitivity with size could be found, what matches findings on most ontogenetic studies done on hearing in fish so far. On the contrary, at higher frequencies a decrease of sensitivity with size was observed. This is a very new finding and has never been observed in any fish species before. The ontogenetic development of sounds produced by *Synodontis schoutedeni* matches previous findings in further studies in terms of sound characteristics. The dominant frequency of sounds in specimens larger than 37 mm is negatively correlated with body size, which has been found in several studies before (i.e. Amorim and Hawkins, 1994; Colleye et al., 2009; Connaughton and Taylor, 1996; Henglmüller and Ladich, 1999; Ladich et al., 1992; Lobel and Mann, 1995; Myrberg et al., 1993; Parmentier et al., 2009; Vasconcelos and Ladich, 2008; Wysocki and Ladich, 2001). Sound pressure level increased with size in specimens up to 58 mm, such an increase of sound pressure level has also been found in tigerfish, croaking gouramis, sciaenids and toadfish (Connaughton and Taylor, 1996; Schneider, 1961; Vasconcelos and Ladich, 2008; Wysocki and Ladich, 2001). The duration of pulse periods of single ad- and abduction sounds also increased with size, what matches findings in toadfish, weakfish and grunts (Amorim and Hawkins, 2005; Connaughton and Taylor, 1996; Vasconcelos and Ladich, 2008). Those results in sound characteristics have been expected, but in *Synodontis schoutedeni* the main energies of stridulation sounds correspond to the most sensitive frequency range of hearing in all size groups tested and the sounds of all groups are of a high sound pressure level. Thus, all size groups are able to detect communication sounds of conspecifics of all sizes and communicate acoustically. These are very new findings and in contrast to prior observations in the croaking gourami (Wysocki and Ladich, 2001) and the Lusitanian toadfish (Vasconcelos and Ladich, 2008). In these two species earliest stages are not able to detect communication sounds of similar sized juveniles even at very close distance. This ability to communicate acoustically already at very small size stages is probably based on the extremely good hearing abilities of *S. schoutedeni* and the high sound pressure levels of the stridulation sounds produced already by the smallest size groups. The present study in *S. schoutedeni* is the first to show that individuals of all size stages are able to communicate acoustically in a fish species. Thus the hypothesis that fishes possessing Weberian ossicles (otophysines) communicate acoustically at earlier stages than groups lacking highly developed accessory hearing structures is supported by this study.

The study on the ontogenetic development of the chain of Weberian ossicles in *L. cyclurus* yielded a fully developed chain of Weberian ossicles in all size stages investigated with exception of the smallest catfish specimens studied. In the fish of groups S – XL (24 mm SL and larger) the chain consisted of four Weberian ossicles (tripus, intercalarium, scaphium and claustrum) and two interossicular ligaments, which connected tripus, intercalarium and scaphium. This is in agreement with all studies investigating the Weberian apparatus in basal catfish families (for a review see Chardon et al., 2003). In the specimens of the smallest size group investigated (XS, SL 11.3 – 15.3 mm) the chain of ossicles was not yet fully developed. An intercalarium was not yet present, the tripus was not yet fully developed and interossicular ligaments were still missing. On the other hand, the scaphium and claustrum were already fully grown in group XS and of same shape as in larger conspecifics. This is partly in contrast to previous studies. In three otophysine species of the orders Cypriniformes and Characiformes the differentiation of the ossicles has been observed to progress from posterior to anterior (Bird and Mabee, 2003; Grande and Young, 2004), and in studies on catfishes the claustrum has been observed to appear as the last ossicle (Ichiyanagi et al., 1997; Ichiyanagi et al., 1993). I could never detect a claustrum in the smallest sized *L. cyclurus*, neither in paraffin nor in semi-thin sections and 3-d reconstructions. This also contradicts observations of authors studying Weberian ossicles in catfishes having an intercalarium and thus four Weberian ossicles (Coburn and Grubach, 1998; Ichiyanagi et al., 1993). The results of the present study in *L. cyclurus* clearly show scaphium and claustrum as first ossicles to be fully developed, the tripus being not yet fully formed in the smallest size stages and the interossicular ligaments still missing. Even though this study does not provide a full ontogenetic series of the development of the ossicular chain in *L. cyclurus*, the results support my hypothesis. There is either a high variation in ossicular development in otophysine fish or the observations of previous authors, who mostly also gave only more or less rough insights in some developmental stages of the study animals, have to be verified.

Nevertheless, the results of hearing measurements in the different size groups of *L. cyclurus* fit perfectly to the observations made in the development of the Weberian ossicles. The smallest size group tested, which did not yet have a fully developed and connected ossicular chain, showed dramatically decreased hearing sensitivities compared to groups S – XL with fully developed chains. Additionally, specimens of group XS were not able to detect frequencies higher than 2 or 3 kHz. This supports to some small extent the observations of Higgs et al. (2003) in the zebrafish. They observed no changes in hearing sensitivities in different size stages, but a broadening of the range of detectable frequencies from 200 Hz in the youngest zebrafish tested up to 4 kHz in the oldest ones. All *L. cyclurus* tested were able to detect at least frequencies up to 2 kHz, and in contrast to Higgs et al. (2003) hearing sensitivity changed significantly at most frequencies during ontogeny of *L. cyclurus*. The results in the zebrafish study and in the present study in *L. cyclurus* indicate that a fully developed chain of Weberian ossicles is necessary for fully developed hearing capacities, but I cannot explain why the results found in the zebrafish and in the catfish studies differ in terms of absolute thresholds. I found a change in hearing sensitivity in all size stages of *L. cyclurus* investigated. In the groups with fully developed ossicular chain (S – XL) hearing sensitivities increase at lower frequencies, but decrease at the highest frequencies tested.

This matches the observation made in the first study contributing to this doctoral thesis in *Synodontis schoutedeni*. The smallest specimens tested in *S. schoutedeni* (group XXS) were about 22 mm SL, what can be compared to size group XS in the study in *L. cyclurus*. Therefore, I assume that the smallest squeaker catfishes tested possessed already a fully developed chain of ossicles. In both studies specimens of the largest size groups were full-grown (some of them the parents of the smallest catfish of the studies). The results of hearing development in *S. schoutedeni* and in the size groups S – XL of *L. cyclurus* are even. Smaller specimens show decreased hearing acuities in lower frequencies, but increased hearing in the highest frequencies tested. This is the first time such changes in hearing sensitivities during ontogeny of a fish species have been observed. Previous studies in non otophysine fish mostly have shown an increase of hearing sensitivities with size (Higgs et al., 2004; Iwashita et al., 1999; Kenyon, 1996; Sisneros and Bass, 2005; Vasconcelos and Ladich, 2008; Wysocki and Ladich, 2001), with the exception of the studies in *Abudefduf saxatilis* (Egner and Mann, 2005), where a slight decrease in hearing sensitivity at low frequencies has been observed, and in *Neogobius melanostomus* (Belanger et al., 2010), where no differences in hearing abilities of different size stages have been found. But the species investigated in those studies did not show any hearing specialisations and thus had only reduced high frequency hearing, with the exception of the croaking gouramis *Trichopsis vittata* studied by Wysocki and Ladich (2001). The results in the development of hearing in *L. cyclurus* (groups S – XL) and *S. schoutedeni* at low frequencies match the results of most of those studies, where an increase of sensitivity with size has been observed. The hearing abilities of the different size groups of *T. vittata* indicate a slight trend of better high frequency hearing in smaller specimens. The authors of this study did not calculate correlations between size and hearing abilities of each specimen, but only compared the abilities of the six size groups tested by a two-way analysis of variance (ANOVA). I recalculated their data which have been collected at the Bioacoustics Lab at the University of Vienna, to find out if hearing sensitivities were correlated to the size of gouramis. These calculations yield a significant increased hearing acuity of the smallest specimens in the highest frequencies tested, what matches the findings in the studies in *S. schoutedeni* and *L. cyclurus*. These results are additionally supported by the observations made in the different sized *Ancistrus ranunculus* and *Pimelodus pictus* (Amoser and Ladich, 2003; Ladich, 1999; Lechner and Ladich, 2008; Wysocki et al., 2009), as already mentioned in the introduction of this thesis. At this stage I cannot give any explanations for this better hearing of smaller specimens in high frequencies. Possible reasons could probably be found in the different development of hair-cells in the sensory epithelia of the inner ears, in different damping coefficients to high and low frequency sound waves of swimbladder walls or suprabranchial organ coating of different diameters, or of body tissue of varying thickness as occurring in different sized specimens. Ladich and Yan (1998) supposed in their study on gouramis, that the better high frequency hearing in smaller species (sic!) could be based on a higher resonance frequency of the air-filled suprabranchial organ in smaller-sized fish. Maybe better high frequency hearing of smaller catfish specimens is also linked to differences in resonance frequencies of swimbladders of different sizes.

Anyway, at this point I can provide possible explanations for my results and the results of most studies conducted on ontogenetic development of hearing so far, but I have no explanation for the results observed in the only otophysine species tested on this. In the zebrafish *Danio rerio* no changes in hearing sensitivities have been observed (Higgs et al., 2003; Higgs et al., 2001), what contradicts my findings.

The effect of albinism and related pigmentation disorders on development of hearing in vertebrates has not been fully explained yet. The results of studies in different mammalian species including men are somewhat converse, probably because study species with several different kinds of disorders have been examined. The third paper of this doctoral thesis studied a potential effect of albinism on hearing in fishes for the first time. The albino specimens of *Silurus glanis* and *Corydoras aeneus* used for this study showed OCA2 (oculocutaneous albinism of subtype 2), similar to Mexican blind cave fish (Jeffery, 2009; Protas et al., 2006). No hearing differences between normally colored and albino specimens could be determined in both species tested, neither in hearing sensitivity nor in the waveform of their auditory evoked potentials. These results accord to the results of Popper (1970), who had compared hearing abilities of blind, colorless cave tetras and pigmented, eyed surface populations of the same species. Nevertheless, cave tetras show major adaptations of their sensory system to cave life and therefore cannot be termed as albinotic forms of the surface populations. Dutton et al. (2009) showed, that genetically based pigmentation disorders can have a negative influence on the development of the ear in zebrafish. Such a dysplastic ear certainly would have effects on hearing abilities of the fishes. Therefore OCA2 albino fish probably do not have the same Sox10 mutation as the zebrafishes of this study. The results of the third study on the effects of albinism on hearing support my hypothesis that negative effects are rather unlikely due to the quite different anatomy of the inner ear of fishes as compared to mammals. However, it can be assumed that such effects may be found in other non-mammalian vertebrates. Hearing abilities of the two catfish species tested fit to the scheme proposed by Lechner and Ladich (2008). *Corydoras aeneus* with its reduced, paired and bony encapsulated swimbladders and single ossicles has reduced high frequency hearing compared to catfishes with free, single bladders and three or four ossicles. *Silurus glanis*, the first species of the catfish family Siluridae tested for hearing abilities, shows slightly advanced hearing acuities.

The present thesis is composed of three studies which try to answer several questions concerning hearing and sound communication in fishes. Each of the studies provides new findings to the field of fish bioacoustics. The ontogenetic development of the chain of Weberian ossicles seems to follow several different sequences in otophysine fish. In L*ophiobagrus cyclurus* the intercalarium and the interossicular ligaments evolve last, while scaphium and claustrum are the first to be fully developed. This is in contrast to previous observations. This study has also shown that a fully developed chain of Weberian ossicles is necessary for full hearing abilities in catfishes. In specimens with fully developed ossicular chain, the studies in *L. cyclurus* and in *Synodontis schoutedeni* show for the very first time a developmental change in hearing sensitivities during ontogeny in otophysine fish species. In lower frequencies an increase of sensitivity with size could be observed, what accords to the findings in several non otophysine fish species. But in the catfish used in those studies a decrease of hearing sensitivity with size in higher frequencies has been observed. Such a change has never been found in any fish before. Furthermore, *Synodontis schoutedeni* is the first fish species, in which an ability to communicate acoustically over all developmental stages has been shown. The ontogenetic development of the sounds of *S. schoutedeni* follows a pattern observed in several further sound producing fish before, but their high hearing acuity combined with the high sound pressure level of their stridulation sounds is probably the basis for these very new findings. Finally, the last paper of this thesis exhibits, that pigmentation disorders probably do not effect the development of hearing abilities in fishes, which again has never been shown before in a non-mammalian vertebrate.

All in all the findings of the studies presented in this doctoral thesis support the hypotheses postulated in the beginning and provide several valuable new data and answers to open questions to the wide field of fish bioacoustics.

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(of general introduction and concluding discussion. For the references of the three studies contributing to this doctoral thesis see the reference list of each paper.)

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Authors contributions

The following authors contributed to the three studies of this doctoral thesis:

- Walter Lechner (WL)$^{1)}$
- Friedrich Ladich (FL)$^{1)}$
- Lidia Eva Wysocki (LEW)$^{1)}$
- Egon Heiss (EH)$^{2)}$
- Thomas Schwaha (TS)$^{2)}$
- Martin Glösmann (MG)$^{3)}$

1) University of Vienna, Department of Behavioural Biology
2) University of Vienna, Department of Theoretical Biology, Morphology Section
3) University of Veterinary Medicine Vienna, Department of Biomedical Sciences

Lechner, W., Wysocki, L. E. and Ladich, F. (2010). Ontogenetic development of auditory sensitivity and sound production in the squeaker catfish *Synodontis schoutedeni*. *BMC Biology* 8, 10.
FL and WL conceived the study. WL did measurements and analysis of auditory sensitivity. WL and LEW recorded the sounds, WL and FL analysed them. WL, LEW and FL did statistical analyses and wrote the paper. WL prepared the figures and graphs. All authors read and approved the final manuscript.

Lechner, W., Heiss, E., Schwaha, T., Glösmann, M. and Ladich, F. (2010). Ontogenetic development of Weberian ossicles and hearing abilities in the African bullhead catfish. Submitted to *PLoS ONE*.
WL designed the study. WL did measurements and analysis of auditory sensitivity. WL did dissections. MG did MicroCt-scans. EH did paraffin based histology. TS did semithin sectioning. TS made the 3D reconstructions of MicroCt-scans and semithin sections. WL prepared the figures and graphs. WL, TS and FL analyzed the 3D reconstructions. WL and FL did statistical analyses. WL and FL wrote the paper. All authors read and approved the final manuscript.

Lechner, W. and Ladich, F. (2010). How do albino fish hear? *J. Zool.*, doi:10.1111/j.1469-7998.2010.00762.x.
FL and WL conceived the study. WL did measurements and analysis of auditory sensitivity. WL prepared the figures and graphs. WL and FL did statistical analyses and wrote the paper.

Curriculum vitae

Personal information:

- Name: Walter Lechner
- Nationality: Austrian
- Date and place of birth: January 8th, 1969, Steyr, Upper Austria
- Actual address: Stumpergasse 48/23, 1060 Vienna, Austria

Education:

- 1975 – 1979: Elementary school, VS Steyr-Gleink
- 1979 – 1987: Grammar school, BRG Steyr
- June 15th, 1987: General qualification for university entrance (Matura), BRG Steyr
- 1988 – 1994 and 2004 – 2010: University of Vienna
- November 27th, 2007: Mag. rer. nat. (MSc) at the University of Vienna; diploma thesis: „Size matters – the effect of the swim bladder on hearing abilities in catfishes“

Military service:

- 1987/88: LWSR 22, Karlkaserne, Vienna

Employment:

- 1990 – 1998: Wesser GmbH, Stuttgart, Germany
- 1998 – 2008: Kober GmbH, Aalen, Germany
- 2008 – 2009: AC Nielsen, Vienna
- Since January 2010: Research assistant (as doctoral student and subsequent post doctoral researcher) at the University of Vienna, project “Ecoacoustical constraints on the evolution of hearing and sound communication in catfishes”, funded by the Austrian Science Fund (FWF)

Teaching experience:

- Winter semester 2008/09, 2009/10, 2010/11: „Bioakustisches Projektpraktikum“, University of Vienna
- Summer semester 2009, 2010: „Übungen zur Physiologie der Tiere 2 - Sinnes-, Nerven- und Muskelphysiologie“, University of Vienna

Scientific publications and talks:

Papers in scientific journals

Lechner, W. and Ladich, F. (2008). Size matters: diversity in swimbladders and Weberian ossicles affects hearing in catfishes. *J. Exp. Biol.* 211, 1681-1689.

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.

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Talks

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