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The auditory roles of the gas bladder and suprabranchial chamber in walking catfish (*Clarias batrachus*)

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Abstract

Background: The enhanced auditory abilities of certain fish are dependent on specialized hearing structures. Several gas-holding structures, including the suprabranchial chamber, otic gas bladder, and Weberian apparatus-linked gas bladder, have been demonstrated to improve the hearing ability of fish. The walking catfish (*Clarias batrachus*), a benthic species, is unique in that it has both a suprabranchial chamber (SC) and a Weberian apparatus-linked, encapsulated gas bladder (WGB). This study aimed to investigate the respective roles of these two structures in contributing to the overall hearing ability of walking catfish.

Results: The auditory evoked potentials method was used to measure hearing thresholds in intact fish and fish with a deflated WGB and/or SC. Gas removal from the WGB increased auditory thresholds at all frequencies tested (0.4 to 5.0 kHz), whereas injection of water into the SC shifted the threshold only at frequencies below 3.0 kHz. However, such a correlation was not observed for the SC at 4.0 and 5.0 kHz.

Conclusions: The findings indicated that both the encapsulated WGB and the SC had an accessory auditory role. However, WGB enhanced hearing abilities in the whole frequency range, whereas SC did not.

Keywords: Hearing ability; AEP; Catfish; Weberian apparatus; Suprabranchial chamber

Background

Physical restrictions prevent most fish from detecting all but low-frequency sounds (see review in Hawkins and Myrberg 1983). However, several non-related groups of teleost fishes have evolved mechanisms that enhance hearing ability; such mechanisms involve the transmission of the pressure component of passing sound waves to the inner ear via gas-holding structures (see reviews in Yan 2004; Ladich and Popper 2004). The Weberian apparatus, an ossicular chain that involve a set of minute bones originating from the first few vertebrae, which connect inner ear to the swim bladder, exists in all Otophysi, i.e., Characiformes, Cypriniformes, Gymnotiformes, and Siluriformes (Nelson 2006). The Weberian apparatus-linked gas bladder (WGB) is the best known of the

structures that aid hearing (see reviews in Fay and Popper 1975; Yan and Curtsinger 2000; Yan et al. 2000). Its auditory aid function has been proven in Brown bullhead (*Ameiurus nebulosus*), common roach (*Rutilus rutilus*) as well as gold fish (*Carassius auratus*) in early studies (Kleerekoper and Roggenkamp 1959; Laming and Morrow 1981; Fay and Popper 1974). After WGBwas mutilated or deflated, hearing thresholds of those species significantly increased compared to that of the intact fish. Furthermore, hearing abilities of Otophysi are highly dependent on the number of Weberian ossicles and the size of the gasbladder, what has been shown inseveral catfish species (Lechner and Ladich 2008).

Other gas-holding structures in the fish body, such as the suprabranchial chamber (SC) in Anabantoidei and the otic gas bladder in Mormyridae, connect directly to the inner ear and are thus also capable of enhancing hearing ability (Yan and Curtsinger 2000; Yan 2004). The SC is a respiratory organ found in gouramis (Anabantoidei) of the families Anabantidae, Helostomatidae, and Osphronemidae. Catfishes of the family Clariidae

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have a similar structure (SC) as well. The SC enhances the hearing ability in gouramis, what has been shown in several species such as paradise fish (*Macropodus opercularis*, Osphronemidae) (see reviews in Yan 1998), blue gouramis (*Trichogaster trichopterus*; Osphronemidae), dwarf gouramis (*Colisa lalia*; Osphronemidae), and kissing gouramis (*Helostoma temminckii*; Helostomatidae) (Yan 1998).

Certain benthic catfishes, such as Loricariidae and Callichthyidae, have small, paired, and encapsulated WGB (see reviews in Chardon et al. 2003). Lechner and Ladich (2008) suggested that although such bladders reduce buoyancy compared to the catfishes with single and large WGB, their auditory function ensured their retention through evolution. The auditory role of such bladders have been previously demonstrated in oto catfish (*Otocinclus affinis*; Loricariidae); hearing thresholds at 0.5 to 10.0 kHz are significantly increased by deflation of the *WGB* in this species (Botta 2009). Walking catfish (*Clarias batrachus*; Clariidae), on the other hand, possess both a SC and a single encapsulated WGB (Bridge and Haddon 1893; Alexander 1964; Chardon et al. 2003), and thus, auditory compensation in this species may differ to that in the oto catfish. The respective contributions of the WGB and SCto sound detection have not previously been compared in teleosts possessing both of these organs.

The main objectives of the present study were to (1) establish whether the encapsulated WGB and SC of walking catfish play a role in hearing enhancement, (2) determine whether the accessory auditory functions of those two structures are independent, and (3) investigate the frequency ranges in which they improve hearing ability. The auditory evoked potentials (AEP) method was used to determine the auditory function of the WGB and SC in walking catfish via shifts in hearing thresholds before and after gas removal.

Methods
Animal preparation and protocols
Walking catfish (*C. batrachus*) used in this study were obtained from a local aquarium shop. The standard lengths and weights of the catfish used were 50 to 65 mm and 11.0 to 16.5 g for experiment 1 and 60 to 110 mm and 14.0 to 25.5 g for experiment 2, respectively. Fish were housed in two 144 L aquaria with charcoal-filtered running water. The temperature was maintained at 25°C to 28°C, and fish were maintained under a natural photoperiod. Two sets of experiments were conducted. In experiment 1, the AEP method was used to measure the hearing thresholds of three experimental groups of catfish (SC deflated ($n = 5$), WGB deflated ($n = 5$), and both SC and WGB deflated ($n = 4$)) and a control group of intact catfish ($n = 5$). In experiment 2,

hearing thresholds were measured before and after gas removal from either the WGB ($n = 4$) or the SC ($n = 5$). After surgery, operated catfish were placed into an aquarium to enable their SC or WGB to be replenished with gas; after which, audiograms were subsequently measured again. The color patterns of each fish were recorded; hence, they could be identified easily by their unique patterns. The recovery period of the SC-deflated group was 3 days, which was followed by the previous report on gouramis SC-deflated experiment (Yan 1998). For the WGB-deflated group, digital X-rays (Intermedical Basic 4003) were used to inspect the WGBevery 3 days until it was completely replenished with gas; full recovery took 21 days (Figure 1). Furthermore, in experiment 2, initial and recovery fish in WGB-deflated group were shamoperated before AEP measurement.

Removal of gas from the WGB and SC
Before undergoing surgery, catfish were anaesthetized with a 0.025% buffered MS-222 (ethyl 3-aminobenzoate methanesulfonic acid salt, Sigma, St. Louis, MO, USA) solution for 2 min.

Figure 1 Dorsal X-ray views of walking catfish after gas removal and recovery. (A) initial condition; **(B)** bladder deflated; **(C)** partially recovered (10 days after operation); **(D)** fully recovered (20 days after operation). The bladders are indicated by white arrows. The scale bar indicates 10 mm.

Unlike the paired, encapsulated bladders described in other benthic catfishes (Bridge and Haddon 1893; Alexander 1964; Lechner and Ladich 2008; Botta 2009), a single bladder with a shortened mid-ridge is present in walking catfish (Alexander 1964). In experiment 1, gas inside the WGB was drawn out slowly with an insulin syringe (Ultra-fine II U-100, Becton, Dickinson and Company, Franklin Lake, NJ, USA) from both sides of the lateral cutaneous area between the posterior of the operculum and the base of the pectoral fin, along the dorsal edge of the clavicle; this region was selected as it allowed the syringe to access the bladder while avoiding the thickened and calcified bony capsules (Alexander 1964). However, this operation was found to cause high mortality. In order to reduce stress and to improve survival, the gas removal protocol was modified in experiment 2 to use a 30-G insulin syringe needle (#305107, BD PrecisionGlide Needle, Becton, Dickinson and Company, Franklin Lake, NJ, USA) to pierce the bladder from one side of the lateral cutaneous area. The gas inside the WGB was then driven out by injecting Holmes and Stott freshwater teleost physiological saline (see review in Wolf 1963) from the other side of the bladder with a second U-100 insulin syringe, until gas bubbles stopped emerging from the first needle. The insulin syringe was initially loaded with 300 μl of saline (0.303 g; density = 1.010 g/ml), and the remaining saline was weighed after injection. The volume of saline injected into the WGB was calculated by subtracting the final weight from the initial weight (volume = weight/1.010), and this was considered to be equal to the volume of gas in the WGB. Digital X-rays were used to locate the bladder prior to surgery and to confirm gas removal afterward. The entire treatment was performed underwater to prevent air from re-entering the bladder via the incision. Fish were unable to maintain neutral buoyancy after the operation. X-ray examination confirmed that gas was not replenished during the experiment.

Gas was removed from the SC as described in the study of Yan (1998). Gas in the *SC* was driven out by water injected through soft polyethylene tubing (Clay Adams, inner diameter 1.19 mm) connected to a 20- to 200-μl pipette (Rainin, Mettler Toledo, Columbus, OH, USA). The fish were held upside down in water during the operation, and the tube was inserted deeply through both sides of the gill to reach the SC. Water was then injected slowly into the chamber until gas bubbles stopped emerging from the gill opening. The pipette was initially loaded with 200 μl of water (0.200 g; density = 1 g/ml), and the remaining water was weighed after injection. The volume of water injected into the SC was calculated by subtracting the final weight from the initial weight, and this was considered to be equal to the volume of gas in the SC. The fish were kept underwater during surgery and

AEP measurements to prevent the SC from refilling with gas.

Hearing threshold determination

The AEP recording protocol used in this study followed that of Kenyon et al. (1998). The fish were immobilized by intramuscular injection of gallamine triethiodide (Flaxedil; Sigma, St. Louis, MO, USA), a neuromuscular blocking agent, into the dorsal muscle (0.2 to 0.3 μg per gram of body weight). Treated fish were wrapped in wet Kimwipes (Kimberley-Clark Irving, TX, USA) to prevent scraping of the skin and were held in place with nylon mesh. The mesh was fixed to a stainless steel rod attached to a micromanipulator. Fish were suspended in a plastic tub (24 × 24 × 10 cm) filled with fresh water, with the top of the head kept at about 2 mm above the water surface. In order to keep the fish alive during the experiment, a small tube was used to irrigate the gills with oxygenated water during the recordings.

A recording electrode (a Teflon-coated silver wire, 0.25 mm in diameter with a 1-mm exposed tip) was placed on the midline of the skull between the eyes. A reference electrode of a similar specification was placed 0.5 cm ahead of the recording electrode. EEG paste (Ten20® conductive EEG paste, D.O. Weaver and Co., Aurora, CO, USA) was used to cover the area of skin attached to the electrodes, in order to enhance the conduction of evoked potentials from the brain to the electrodes. Eyeball rotation and barbel movement under tactile stimulation were used as indicators that the fish were still alive during the recordings.

The experimental setup was placed on a vibration-free air table (Vibraplane, Kinetic Systems, Boston, MA, USA) located inside a soundproof room (1.9 × 1.6 × 2.6 m) to minimize disturbances from noise and vibration. Signals recorded from the electrodes were amplified and converted to digital signals by a preamplifier (HS4, Tucker-Davis Technologies, Alachua, FL, USA) and transmitted from the soundproof room via an optical fiber. Sound stimuli at frequencies up to 2.0 kHz were produced using a 30-cm diameter woofer (4 to 8 Ω, Panasonic), while sounds at frequencies above 2.0 kHz were generated using a 12.5-cm diameter tweeter (7.25 Ω, Pyle). The speaker was suspended 1.25 m above the test subjects.

Sound stimuli and AEP waves were generated and recorded by a Tucker-Davis Technologies (TDT) (Gainesville, FL, USA) modular rack mount system, which was controlled by a TDT AP2 board with TDT BioSig software.

Sound stimuli at each frequency and pressure level consisted of 2,000 repeats of a 20-ms tone burst. Each stimulus was played twice for each test fish. In order to calibrate the sound pressure level of the stimulations in water, a hydrophone (Celesco LC-10) was placed near

the head of the fish. The signal from the hydrophone was amplified 100 times using a differential preamplifier (Grass P-15, bandpass, 10 to 10,000 Hz) and calibrated in absolute units (dB re 1 μPa).

Each experiment started at the highest sound pressure level (SPL) to induce suprathreshold responses; the SPL was then reduced in 5 dB steps until traceable and repeatable waveforms were no longer detectable (Kenyon et al. 1998; Yan and Curtsinger 2000; Akamatsu et al. 2003). Since the main purpose of the present study was aimed at understanding the respective auditory roles of SC and WGB, therefore, frequencies lower than 0.4 kHz were not investigated in the present study because many studies have indicated that stimuli below 0.4 kHz could trigger evoked potentials of lateral line which could compound the intended studies of auditory functions (Harris and van Bergeijk 1962; Münz 1989; Kroese and Schellart 1992; Kenyon et al. 1998). In experiment 1, hearing threshold levels were determined at the following frequencies: 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 kHz. In experiment 2, tested frequencies have been simplified to reduce fish stresses and increase survival rate. They were determined at the following frequencies: 0.6, 1.0, 2.0, 3.0, and 5.0 kHz. SPLs were tested from the maximal output (about 150 to 142 dB for 0.4 to 1.0 kHz; 145 to 139 dB for 1.5 to 2.5 kHz; 135 to 131 dB for 3.0 to 5.0 kHz, in water) to the lowest threshold levels. At each frequency, the threshold shift (delta threshold) caused by the removal of gas from the WGB and/or SC was taken as their auditory aid ability.

The experiments were carried out following permission (RFiZOOYH2006120) issued from the Institutional Animal Care and Use Committee (IACUC) of Academia Sinica.

Statistical analysis

Comparisons between two independent samples were performed by analyzing the data with two-tailed Student's *t* test. Comparisons between multiple groups were performed using one-way ANOVA and Tukey's *post hoc* test ($p < 0.05$). Two-way ANOVA was applied to analyze the factors of stimuli frequencies and treatments. For experiment 2, hearing thresholds of each individual before and after gas removal were compared and threshold shifts were calculated. Relationships between gas volume in the gas bladder or suprabranchial chamber and hearing ability were determined by Pearson's correlation coefficients and linear regressions. All statistical tests were performed with SPSS v.14 (SPSS Inc., Chicago, IL, USA).

Results

Experiment 1

Figure 2 shows a series of AEP waveforms of walking catfish in response to 0.6 to 5.0 kHz tone bursts, at

sound pressure levels of 120 dB. A typical AEP waveform consisted of a series of peaks. In intact catfish (Figure 2, black lines), these peaks were readily apparent. However, the peaks in catfish with a deflated SC (Figure 2, red lines), WGB (Figure 2, blue lines), or both (Figure 2, green lines) were less prominent.

AEP thresholds for tone bursts between 0.4 and 5.0 kHz are shown for intact and SC- and/or WGB-deflated catfish in Figure 3. Two-way ANOVA showed that the threshold curves were highly frequency-dependent ($p < 0.01$), and treatments did have significant effect on the thresholds ($p < 0.05$). Furthermore, the clear interaction ($p < 0.05$) of those two factors indicated that the treatment effects on hearing thresholds were frequency-dependent as well.

The lowest hearing threshold of intact catfish was found to be 0.6 kHz, with 83.8 ± 2.2 dB (mean ± SE). For intact catfish, no significant differences ($p > 0.05$ for all comparisons) were detected at frequencies between 0.6 and 2.5 kHz; the mean threshold over these frequencies was 87.8 ± 3.1 dB. Thresholds increased gradually above 3.0 kHz (Figure 3).

At frequencies below 3.0 kHz, the hearing thresholds of s-deflated fish were significantly higher than those of the intact fish ($p < 0.05$ at 0.4 kHz and 3.0 kHz; $p < 0.001$ at 0.6 to 2.5 kHz), but such a difference was not observed at frequencies 4.0 and 5.0 kHz ($p > 0.05$) (Figure 3).

At frequencies between 0.4 and 1.5 kHz, the hearing thresholds of WGB-deflated fish were no different to those of the SC-deflated fish; however, above 2.0 kHz, the thresholds of WGB-deflated fish were higher than those of the SC-deflated fish ($p < 0.05$ at 2.0 and 2.5 kHz; $p < 0.005$ at other frequencies) (Figure 3).

The hearing thresholds of SC- and WGB-deflated fish were significantly higher than those of the intact catfish at all tested frequencies ($p < 0.05$ at 0.4 kHz; $p < 0.001$ at other frequencies) (Figure 3). Moreover, the thresholds of SC- and WGB-deflated fish were higher than those of the SC-deflated fish at frequencies above 0.6 kHz ($p < 0.05$ at 0.6 and 0.8 kHz; $p < 0.005$ at frequencies above 1.0 kHz) and higher than those of the WGB-deflated fish at frequencies below 2.5 kHz ($p < 0.05$ at all frequencies) (Figure 3).

Experiment 2

The baseline hearing thresholds observed in this experiment were not different to those recorded in intact fish in experiment 1. Catfish recovered from WGB deflation after 21 days and exhibited a significant increase in weight after recovery (initial, 19.1 ± 3.4 g; post-recovery, 27.2 ± 4.5 g; $p < 0.05$); on the other hand, no change in weight was observed after recovery from SC deflation. No significant differences in hearing thresholds were
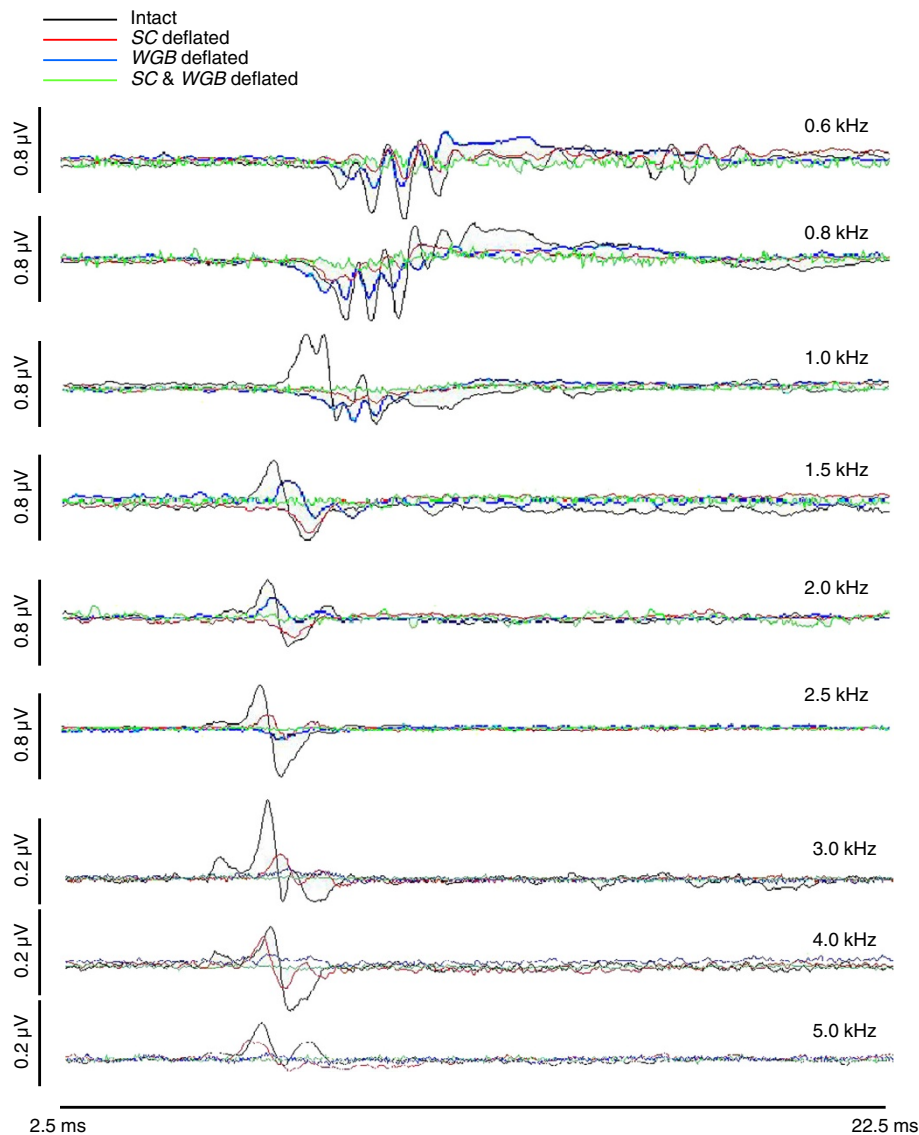
Figure 2 Examples of AEP traces from walking catfish stimulated with pulses of 120 dB of various frequencies of sounds (re 1 μPa).
Signals were captured 2.5 to 22.5 ms after sound stimulation. Scale bars on the left indicate signal amplitudes (0.8 μV at frequencies 2.5 kHz and higher; 0.2 μV at frequencies 3.0 kHz and lower). Black lines show the standard AEP waveforms of a normal walking catfish at different frequencies. Red and blue lines show the waveforms after gas removal from the suprabranchial chamber (SC) or bladder (WGB), respectively. Green lines show the waveforms of fish in which both structures were deflated.

observed between baseline and recovery for both the SC-deflated (Figure 4A; $p > 0.05$) and WGB-deflated groups (Figure 4B; $p > 0.05$), indicating that hearing ability was restored after recovery.

Flushing out all gas bubbles from the SC significantly increased thresholds at 0.6, 1.0, and 2.0 kHz ($p < 0.05$ at 0.6 and 2.0 kHz; $p < 0.005$ at 1.0 kHz) but not at higher frequencies ($p > 0.05$) (Figure 5A). At 0.6, 1.0, and 2.0 kHz, the mean threshold shift was 21.8 ± 3.4 dB, and no significant difference was observed among these frequencies (Figure 5A). On the other hand, an increase in auditory threshold was observed at all frequencies after gas removal from the WGB

($p < 0.005$) (Figure 5B). The maximum shift in the hearing threshold after WGB deflation (32.7 ± 1.9 dB) was observed at 2.0 kHz, and this was significantly higher than at other frequencies (19.1 to 25.6 dB) ($p < 0.005$ as compared to 1.0 kHz; $p < 0.05$ as compared to other frequencies) (Figure 5B).

The mean SC and WGB total volumes for walking catfish were 359 μl (ranging from 290 to 420 μl) and 243 μl (ranging from 150 to 300 μl), respectively. No significant correlation was observed between SC volume and its ability to enhance hearing ($p > 0.2$ at all frequencies) (Figure 6). However, significant positive correlations
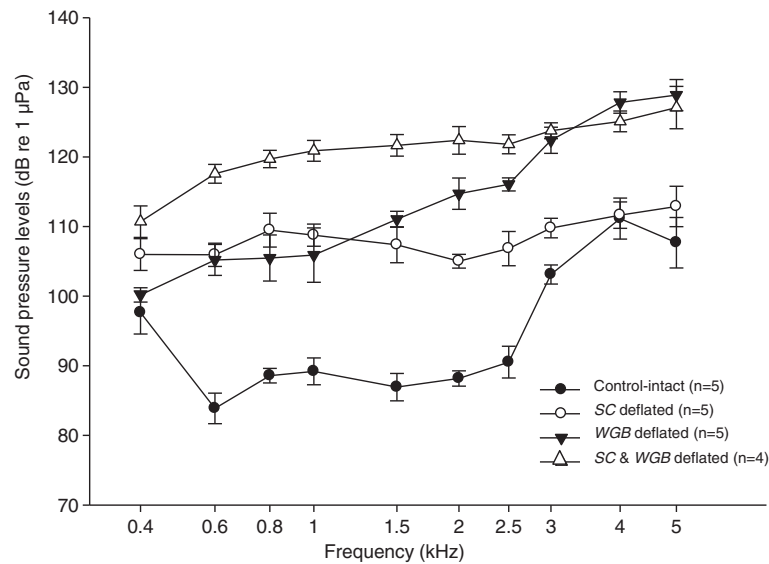
Figure 3 Audiograms of walking catfish at the indicated frequencies. Audiograms are shown for intact catfish (control) and fish with a deflated suprabranchial chamber (SC) and/or bladder (WGB). Data are shown as means ± SE (statistical analysis is described in the 'Results' section).

between WGB volume and threshold shifts after gas removal were observed at 3.0 and 5.0 kHz ($p < 0.05$) (Figure 7).

Discussion

Hearing thresholds of walking catfish

The AEP waveforms shown in Figure 2 were clearly modulated by frequency; with the reduced amplitude and shorter latency time of AEP waveforms at higher frequencies, these are the two hallmarks of typical AEP waveforms (Kenyon et al. 1998). Moreover, we observed that the AEP thresholds increased with frequency (i.e., hearing abilities decreased), with a clear shift occurring at 3.0 kHz (Figure 3). The most sensitive frequency range of hearing in walking catfish was between 0.6 and 2.5 kHz, which is largely consistent with the sensitive frequency range observed in other catfishes (Figure 8) (data re-plotted from Tavolga 1982; Ladich 1999; Lechner and Ladich 2008; Botta 2009; Wysocki et al. 2009; Lechner et al. 2010; Lechner et al. 2011; Zebedin and Ladich 2013). However, certain differences were observed between 0.8 and 2.0 kHz stimulation. While the mean auditory thresholds in catfish with a free bladder were between 77 and 81 dB, those with an encapsulated bladder were between 90 and 102 dB (data from Tavolga 1982; Ladich 1999; Lechner and Ladich 2008; Botta 2009; Wysocki et al. 2009; Lechner et al. 2010; Lechner et al. 2011; Zebedin and Ladich 2013). Despite the encapsulation of gas bladders that may help keep surrounding tissue separate from the moving part of the bladder wall (Alexander 1966), the bony encapsulation results in a decrease in sensitivity probably because the walls of this air-filled chambers are

not free to vibrate (Lechner and Ladich 2008; Kratochvil and Ladich 2000). The present study revealed that the sensitivity of walking catfish was similar to the sensitivity of catfishes having tiny and small encapsulated bladders, which was far poorer than what is found in free bladder catfishes (Figure 8).

Effects of gas in the SC and WGB on hearing

The removal of gas from the WGB or SC significantly reduced the amplitudes of the AEP waveforms in walking catfish. Moreover, these procedures resulted in a threshold increase at certain frequencies. When the fish were allowed to recover so that their deflated WGB or SC refilled with gas through normal physiological mechanisms, their hearing sensitivity was restored. This gas removal and replenishment experiment corroborated the conclusions of earlier studies (see review in Yan 2004); namely, that the gas inside these two structures enhances the hearing abilities of various fish species.

In experiment 2, gas removal from the WGB increased the hearing threshold at all frequencies tested (0.6 to 5.0 kHz), with a mean increase of 22.8 ± 4.1 dB (mean ± SE). These findings are largely in agreement with those in WGB-deflated oto catfish (0.5 to 1.0 kHz; 20.3 ± 2.1 dB) (Botta 2009). While gas removal in oto catfish primarily increased the threshold between 0.8 and 1.5 kHz (22.6 ± 0.9 dB), the major threshold shift in walking catfish occurred at higher frequencies (2.0 to 2.5 kHz) and to a greater extent (32.8 ± 1.9 dB). In addition to catfishes, certain Cypriniformes also possess an encapsulated bladder. For example, the cobitid,
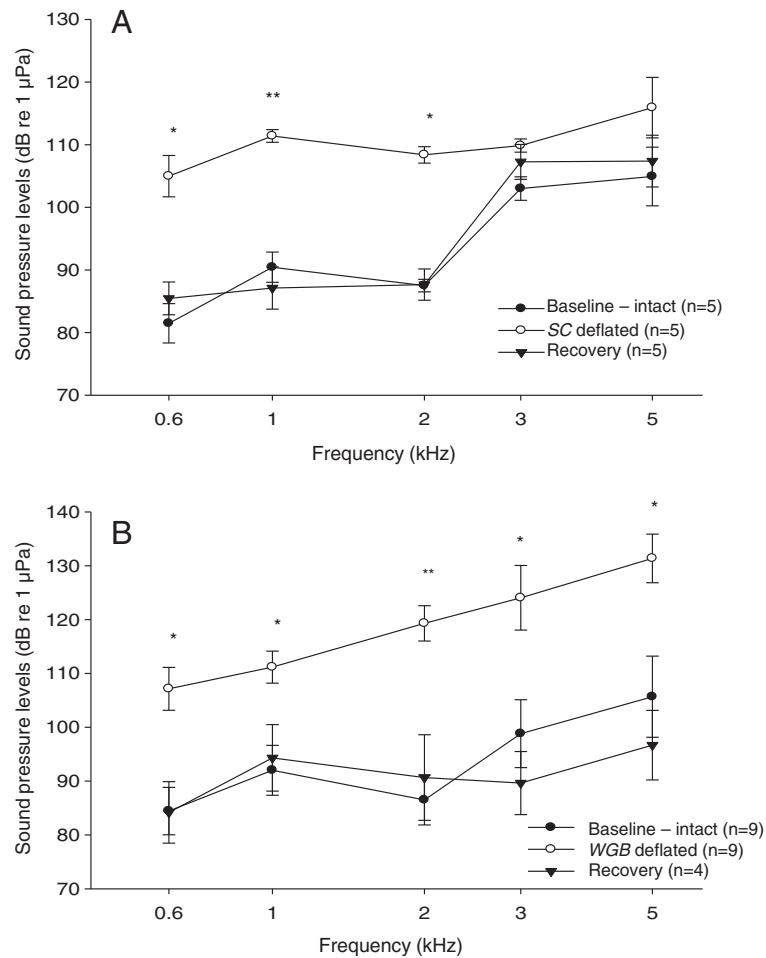
Figure 4 Audiograms of walking catfish at the indicated frequencies. The suprabranchial chamber (SC) **(A)** or bladder (WGB) was deflated **(B)** and then allowed to replenish with gas. Audiograms were determined before (baseline) and after (deflated) surgery and after recovery (recovery). Data are shown as means ± SE. Asterisk (*) indicates $p < 0.05$; double asterisks (**) indicate $p < 0.01$.

Yasuhikotakia modesta, has lateral trunk channels that stretch from its encapsulated bladder to the outer body wall and are filled with fat and lymph. When Kratochvil and Ladich (2000) manipulated these channels by filling with wettex tissue, it resulted to an increase in auditory thresholds indicating that their encapsulated bladder does have auditory function.

The present findings suggest that the accessory auditory function of the WGB is relative to its volume. At 3.0 and 5.0 kHz, a larger bladder may improve hearing ability more than a smaller one (Figure 7; 3.0 and 5.0 kHz). However, compared to other catfishes which have encapsulated, but smaller, bladder, i.e., *Ancistrus ranunculus*, *Dianema urostriatum*, *Hemiodontichthys acipenserinus* and *Hypoptopoma thoracatum* (Lechner and Ladich 2008), *Corydoras paleatus* (Ladich 1999) and *C. sodalis* (Lechner and Ladich 2008), *Otocinclus affinis* (Botta 2009), and *C. aeneus* (Lechner and Ladich 2011), the larger unpaired bladder in walking catfish did not

result to lower auditory thresholds at 3.0/5.0 kHz. In species with free bladder, larger specimens have better hearing ability both at low and high frequency. The hearing thresholds of the African bullhead catfish (*Lophiobagrus cyclurus*) are lower in larger individuals (>24.0 mm) than in smaller ones (<15.3 mm) with an immature ossicular chain. In the African bullhead catfish and squeaker catfish (*Synodontis schoutedeni*), all specimens with fully developed chain of Weberian ossicles show a size-dependent difference in hearing. Small fish with fully developed chain of ossicles hear better than large fish at higher frequencies and worse than large ones at lower frequencies (<1.0 kHz) (Lechner et al. 2010). The smallest ones, with not yet fully developed chain of ossicles, hear worse than all other groups at all frequencies tested (Lechner et al. 2011). Such relationships between size and hearing ability were not observed in adult goldfish (Popper 1971) or zebrafish (Higgs et al. 2003), both of which possess a well-developed Weberian
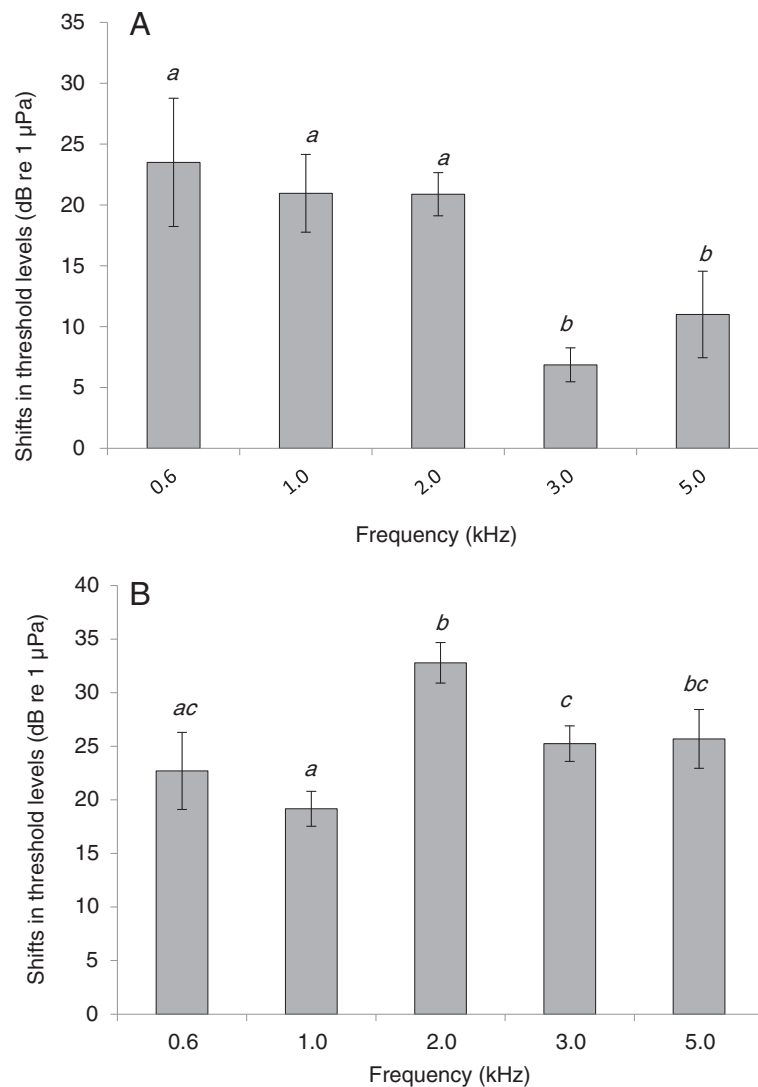
Figure 5 Auditory threshold shifts at the indicated frequencies in catfish after gas removal. (A) Deflation of the suprabranchial chamber.
(B) Deflation of the bladder. Data are shown as means ± SE. Bars with different letters indicate significant difference ($p < 0.05$).

chain. The differences between these species and walking catfish are probably not related to the development of the Weberian chain, as this structure should have been fully developed in the specimens used (Radermaker et al. 1989; see review in Coburn and Grubach 1998). As such, the underlying mechanism remains to be investigated.

The gas bubbles in the SC of gouramis enhance hearing between 0.3 and 4.0 kHz (Yan 1998). Our results showed that the gas in the SC of walking catfish only performed such hearing aid role at lower frequencies, i.e., <3.0 kHz. At frequencies between 0.6 and 2.0 kHz, gas removal from the SC in walking catfish resulted in a mean threshold increase of 21.8 ± 1.4 dB, a similar increase to that observed in SC-deflated kissing gouramis and dwarf gouramis at the same frequencies. However, threshold increases were not as great as those observed

in SC-deflated blue gouramis, which have a distinctly larger *SC*. The auditory capacity of the gourami SC is suggested to be relative to its gas volume, i.e., species with a larger SC exhibit enhanced hearing (Yan 1998). However, no significant correlation was found between SC volume and hearing function in walking catfish. It should be noted that the range of SC volumes (359 μl, range 290 to 420 μl) in the walking catfish used in experiment 2 was less than that previously reported in blue gouramis, dwarf gouramis, and kissing gouramis by Yan (1998) (204 μl, range 126 to 290 μl; 151 μl, range 68 to 292 μl; and 100 μl, range 72 to 184 μl, respectively).

The respective auditory roles of the SC and WGB
Both the encapsulated WGB and SC improve the hearing ability of walking catfish. However, they appear to function
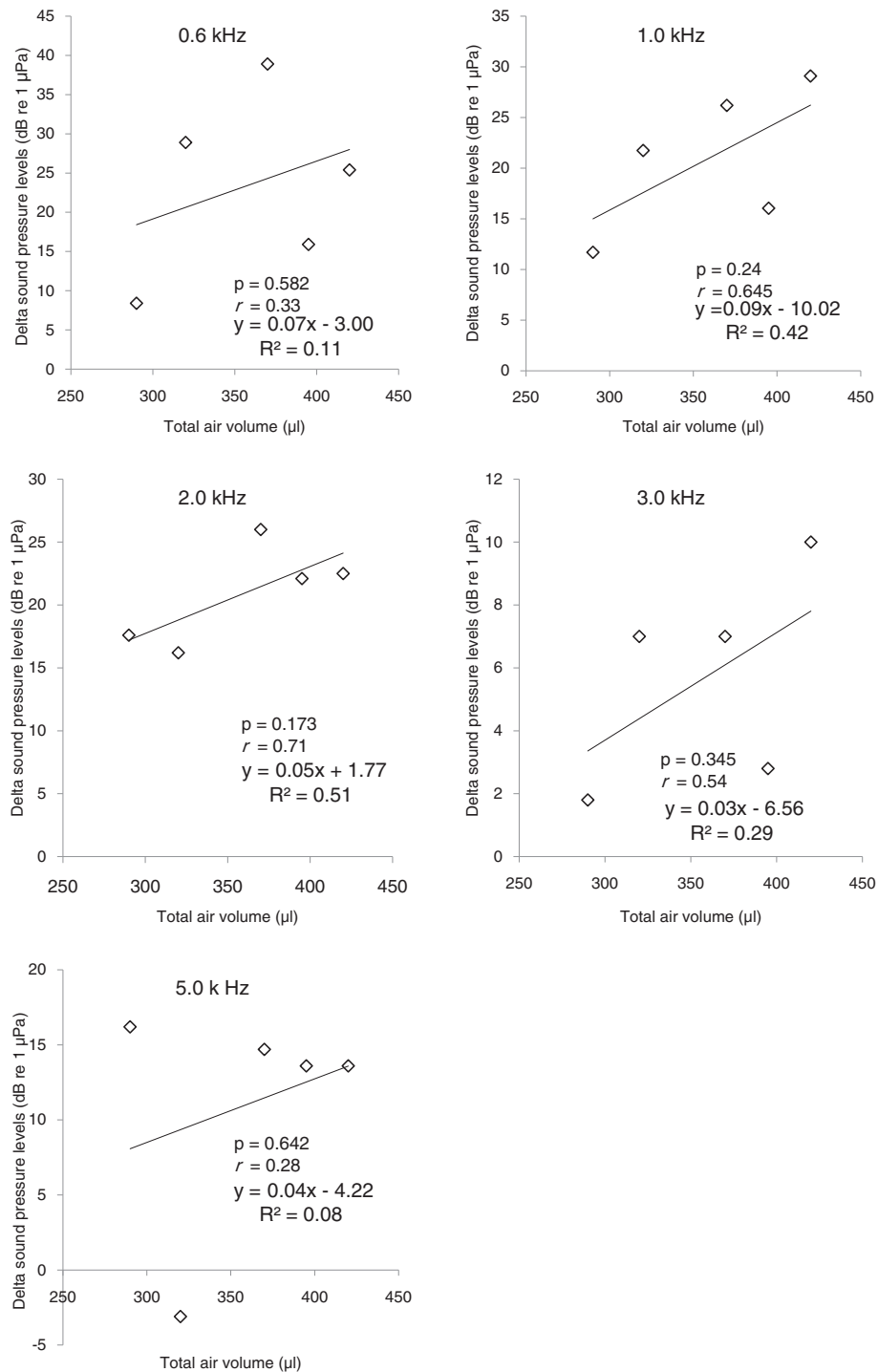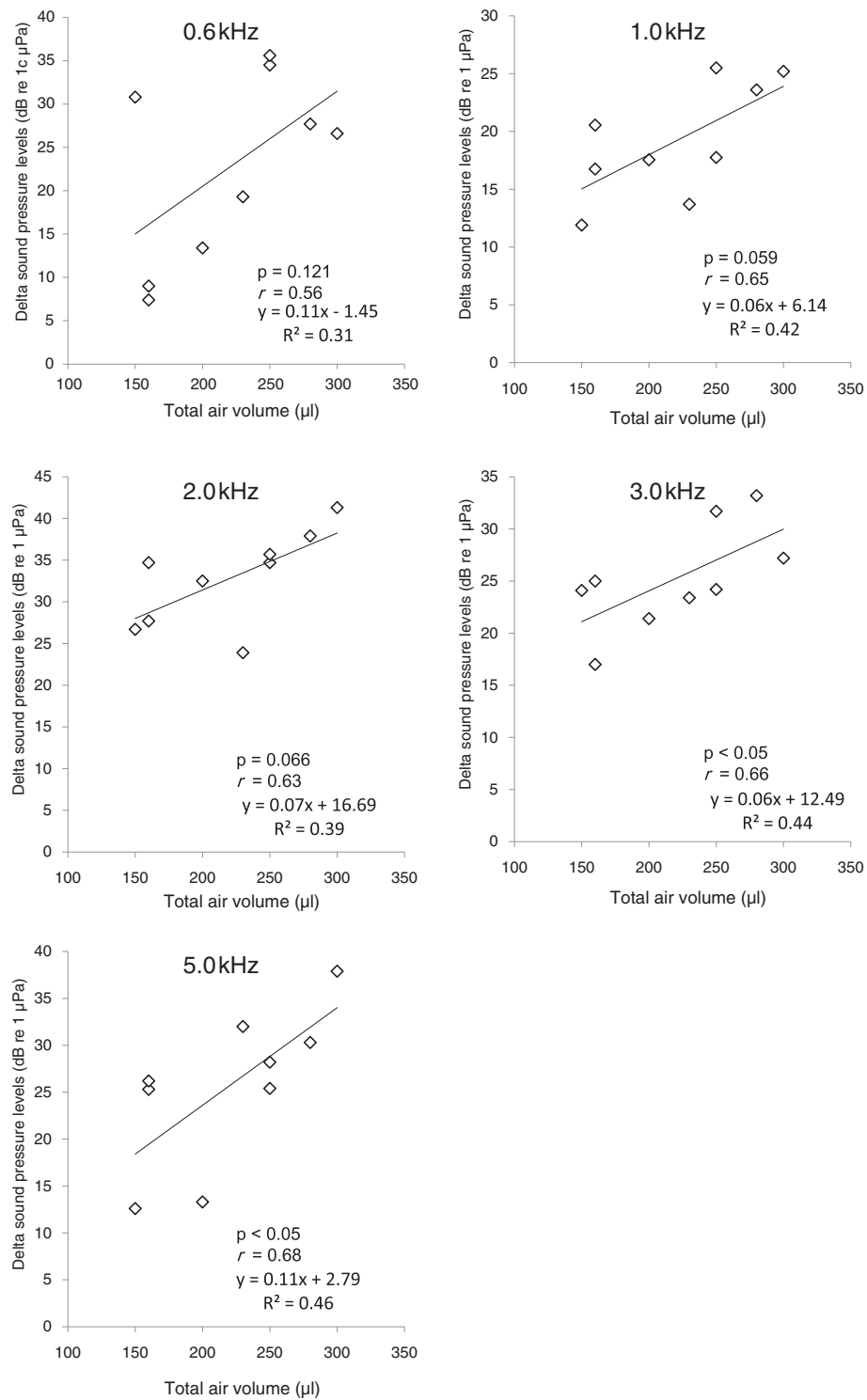
Figure 6 Correlations between auditory threshold shifts and suprabranchial chamber gas volume at the indicated frequencies.
Threshold shifts in each individual after gas removal were plotted against suprabranchial chamber volume. $N = 5$ for all frequencies. Regression equation with R squared (R^2) and Pearson's correlation coefficients (r) with p values are shown.

at a different range of frequencies. In walking catfish, the WGBaids hearing at a broad range of frequencies, while the auditory role of the *SC* is restricted to lower frequencies. A clear synergistic effect of these two structures on

hearing was observed between 0.6 and 2.5 kHz, but this was not the case at higher frequencies (>3.0 kHz).

In the present study, the average volume of gas in the WGB was found to be slightly, but not significantly,

Figure 7 Correlations between auditory thresholds shifts and bladder gas volume at the indicated frequencies. Threshold shifts in each individual after gas removal were plotted against bladder volume. $N = 9$ for all frequencies. Regression equation with R squared (R^2) and Pearson's correlation coefficients (r) with p values are shown. Significant correlations ($p < 0.05$) were observed at 3.0 and 5.0 kHz.
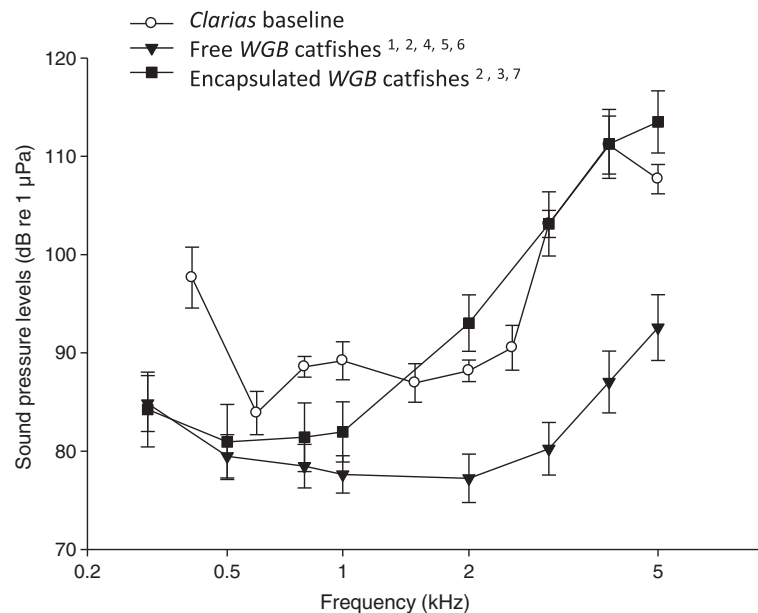
Figure 8 Audiograms of intact walking catfish (*Clarias batrachus*) (open circles), catfish with encapsulated bladders, and catfish with free bladders. The data for the latter two groups were re-plotted by the means thresholds of each investigated species published previously. Eight species of catfish with encapsulated bladders in solid squares: superscripted two, *Corydoras paleatus* from the study of Ladich 1999; superscripted three, *Ancistrus ranunculus, Corydoras sodalis, Dianema urostriatum, Hemiodontichthys acipenserinus, Hypoptopoma thoracatum* from the study of Lechner and Ladich 2008; superscripted four, *Otocinclus affinis* from the study of Botta 2009; superscripted eight, *Corydoras aeneus* from the study of Lechner and Ladich 2011. Sixteen species with free bladders in reversed triangles: superscripted one, *Arius felis* from the study of Tavolga 1982; superscripted two, *Platydoras armatulus* from the study of Ladich 1999; superscripted three, *Ariopsis seemanni, Batrochoglanis raninus, Malapterurus beninensis, Synodontis schoutedeni, Trachelyopterichthys taeniatus* from the study of Lechner and Ladich 2008; superscripted five, *Ictalurus punctatus* from the study of Wyoscki et al. 2009; superscripted six, *Synodontis schoutedeni* from the study of Lechner et al. 2010; superscripted seven, *Lophiobagrus cyclurus* from the study of Lechner et al. 2011; superscripted eight, *Silurus glanis* from the study of Lechner and Ladich 2011; superscripted nine, *Acanthodoras spinosissimus, Agamyxis pectinifrons, Amblydoras affinis, Hemidoras morrisi, Megalodoras uranoscopus, Oxydoras niger* from the study of Zebedin and Ladich 2013. Data at the frequencies below 300 kHz are not shown.

smaller than that in the SC. Unlike the SC, the bladder in walking catfish does not connect directly to the inner ear but is linked by a Weberian chain. The importance of the Weberian chain linkage in otophysines has been demonstrated in goldfish, in which extirpation of the tripus results in a frequency-dependent decrease in hearing sensitivity (from 7 dB at 100 Hz to 33 dB at 2 kHz) and a loss in the ability to detect 4 kHz tone bursts (Ladich and Wysocki 2003). Our findings suggest that the ossicular linkage between the bladder and the inner ear may be beneficial to high frequency sound transmission, and thus, the SC functions more effectively at higher frequencies.

Conclusions

To summarize, the SC and encapsulated WGB have synergistic roles in the enhancement of hearing in walking catfish. Although the SC functions effectively at lower frequencies, it is unable to replace the role of the WGB at higher frequencies. Therefore, although the remaining bladders that create buoyancy may be disadvantageous for a benthic species, their role in hearing enhancement may have ensured their retention during evolution.

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