A NON-INVASIVE ELECTROPHYSIOLOGICAL STUDY ON THE ENHANCEMENT OF HEARING ABILITY IN FISHES

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1. ABSTRACT

Traditionally the study of fish hearing is achieved either by psychophysical method (e.g., behavioral training) or invasive electrophysiological recording (e.g., microphonics, single unit recording). A non-invasive, the auditory brainstem response (ABR) recording method is developed to study fish hearing. With the use of the ABR method and the removal of gas from various gas holding structures, it is proved that the mechanically coupled or directly linked gas holding devices are used by fish to enhanced hearing.

KEY WORDS: auditory brainstem response; neurophysiology; audiograms

2. INTRODUCTION

Scientific study on fish hearing dates back to the turn of the 20th century. Parker [1] was perhaps the first to conduct a well defined experiment investigating the hearing of cyprinid fish and the work was followed by Biglow [2]. Subsequently quantitative work on the range of frequencies over which fish can hear and on representatives of several different families was carried out by von Frisch and his associates [3-4]. Over the years several methods have been developed to investigate fish hearing. Behavioral methods usually involve training fish by using electric shock or food rewards to respond upon hearing a sound. In the classical conditioning method, fish respond with innate behavior such as stereotyped defense responses [5], cardiac suppression [6] or ventilatory suppression [7]. The drawbacks of the classical conditioning method include the stress caused by the electric shock, the response can be ambiguous (especially at threshold level) and is not applicable to species that can not be conditioned by the shock [8]. A second behavioral method is instrumental avoidance conditioning in which fish is trained and learn to cross a barrier in the tank upon hearing a sound to avoid electric shock [9]. The advantage of this method is that the response is unambiguous. However, due to free movement of the subject, the precise calibration of sound perceived by fish is difficult to obtain. In addition, the training may require excessive long time [10]. A third behavioral method is operant conditioning which involves positive reinforcement of training fish to peck paddles in response to sound [11-13]. The drawbacks of this paradigm are: 1) long duration of training for some species; 2) high degrees of variation in learning among individuals; 3) fish has to be large and responsive enough to perform the paddle pecking task. In addition, this method can only be applied to fish using striking mode for food gathering.

On the other hand, electrophysiological methods have less limitation associated with training subjects. Measurement of microphonics from auditory organs while presenting acoustic stimuli to the test subjects is widely used to measure auditory sensitivity of fishes [14]. In addition, single-unit recording is used to measure single nerve fiber discharge patterns [15]. Electrophysiological recordings allow faster data gathering than behavioral methods albeit with some constraints: 1) preparation is complex and invasive surgery is required; 2) the placement of electrodes is restricted to specific endoragns and thus responses recorded do not necessarily represent the whole auditory pathways. A third recording method is the auditory brainstem response (ABR)

which is a non-invasive far-field recording of synchronous neural activity in the eighth nerve and brainstem auditory nuclei elicited by acoustic stimuli [16].

The purpose of this paper is summarize the ABR recording technique developed in my laboratory and to share with colleagues the technical details of the protocol and the experience of using it over the past 5 years [17]. The results obtained from various experiments [18-21] on fish hearing carried out by this newly developed protocol are also summarized and to demonstrate that fish evolved to use various anatomical structures to enhance its hearing. New insights gained from the studies are shared to point to new directions of research on fish hearing.

3. MATERIALS AND METHODS

The block diagram in Figure 1 shows the overall layout of the ABR system. The system uses a Windows-based PC to control 7 electronic modules for signal conditioning which are manufactured by the Tucker Davis Technologies (Gainsville, Florida). Sound stimuli waveforms are generated with TDT "Sig-Gen" software and short (20 ms) pure tone bursts are presented with "Bio-Sig" software through DA1 (digital-analog converter) and a PA4 (programmable attenuator) to a power amplifier via speaker to broadcast stimuli to the subjects. A microphone is placed inside the soundproof chamber and in conjunction with MA1 (microphone amplifier) and MS1 (monitor-speaker) to monitor the presentation of acoustic signals inside the chamber.
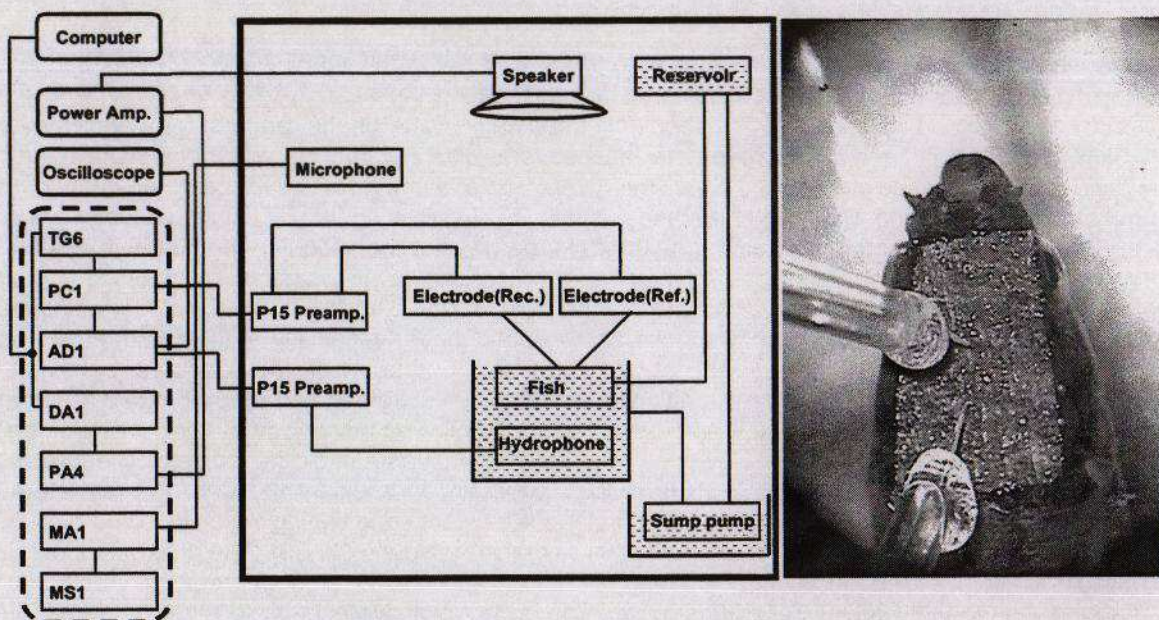

Figure 1. - Block diagram of the auditory brainstem response recording system. Components inside the soundproof chamber are enclosed by bold lines. Signal conditioning modules are enclosed by dashed lines. Image shows electrode positioning on fish.

Test subjects are sedated with the injection of gallamine triethioide (G-1137, Sigma Chemicals Co., St. Louis, Missouri, USA) and secured with the aid of a micromanipulator inside a rectangular 15-liter plastic tub filled with water. A reservoir placed inside the sound proof chamber provides gravity-feed highly oxygenated water to keep fish alive during the recording. The overflowed water is siphoned out to a tank that holds a sump pump to recycle water back to the reservoir. The height of the fish is adjusted so that the nape region is about 0.5-1.0 mm above the water. One Telfon-coated silver recording electrode is placed on the midline of the skull over the medulla

region while the reference electrode is placed about 5 mm anterior to the recording electrode. A hydrophone is placed inside the tub right next to the head region of the subject in order to calibrate the sound pressure level presumably perceived by the fish. The acoustically evoked potentials are recorded by electrodes and are amplified 100 x by a first stage Grass P-15 amplifier and are fed into a spike conditioner (PC1) and amplified 100 x again. The recorded brainwaves are routed into the computer and are averaged by "Bio-Sig" software. A TG6 (timing generator) is used to synchronize A/D and D/A conversion. The oscilloscope is used to monitor real time brainwaves and the output of the hydrophone.

The recorded ABR traces of opposing polarities (one thousand sweeps each) are averaged together forming a 2000-sweep trace to eliminate any stimulus artifact. At each test frequency, this is done twice and is overlaid to examine if traces are repeatable. The lowest sound pressure level where a repeatable ABR can be obtained, as determined by overlaying replicate traces, is considered the threshold. In addition to the visual inspection method, a Spearman Rank Order correlation coefficient with r value less than 0.3 between two traces also indicates that two traces are not repeatable and is hence considered below the threshold.

Goldfish use Weberian ossicles to mechanically couple gasbladder to the inner ear. Gouramis holds air inside the suprabranchial chamber which is in close proximity to the inner ear. Mormyrid weakly electric fish has otic gasbladder tightly coupled to the saccule of inner ear. These tightly coupled gas-holding structures have been suggested to pickup pressure component of the passing sound waves and transmit it into the inner ear to enhance overall hearing. To test this hypothesis, goldfish (*Carassius auratus*), blue gourami (*Trichogaster trichopterus*), kissing gourami (*Helostoma temminckii*), dwarf gourami (*Colisa lalia*), and a mormyrid (*Brienomyrus brachyistius*), all have coupled gas-holding structures are used. In addition, the oyster toadfish (*Opsanus tau*) which does not any coupling between the gasbladder and the inner ear but with close proximity to each other is used as a control to demonstrate the effectiveness of mechanical coupling in hearing enhancement. Radiographs are taken for each species to help localizing the position of gas-holding structures. The baseline audiograms are taken with the ABR protocol. The gas inside the gas-holding structures is removed either by needle attached to syringe (for gasbladder) or flush out with water (for suprabranchial chamber). The audiograms are taken again after removal of gas.

4. RESULTS

Typical acoustically evoked brainwaves are illustrated in Figure 2. It consists of a series of pronounced peaks. When the stimuli is attenuated, the amplitudes of the evoked potentials are also reduced accordingly. The obvious sign of brainwave generated below threshold is when two traces can not replicate itself and in many places opposite polarities are observed (see Fig. 2, 60 dB traces).

The audiogram of goldfish clearly indicates that at least it can hear up to 4kHz and with best hearing frequency between 500 and 800 Hz (Figure 3; lower trace). Upon the removal of gas from the gasbladder, significant increase in thresholds is observed in all frequencies (Figure 3, upper trace). The removal of gas de-couples the
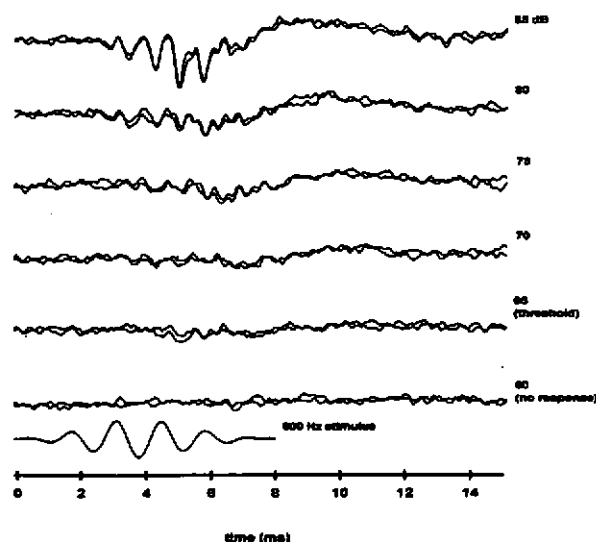

Figure 2 - Acoustically evoked brainwaves of goldfish in response to 600 Hz tone burst attenuated in 5-dB step. Averaged traces of two different runs for each level are overlaid. dB in relative scale.

mechanical link between the gasbladder and the inner ear. The removal of gas from suprabranchial chamber also caused significant elevation of thresholds in blue gourami, kissing gourami and dwarf gourami (Figure 4, 5, 6).
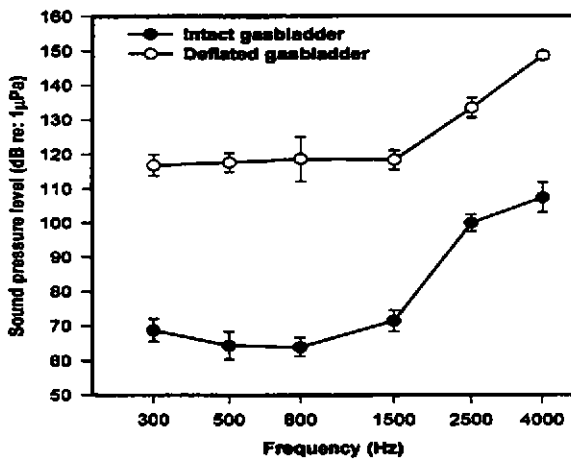

Figure 3. Audiograms of goldfish with intact gasbladder (filled circles) and deflated gasbladder (open circles). Error bars indicate standard error. N=6
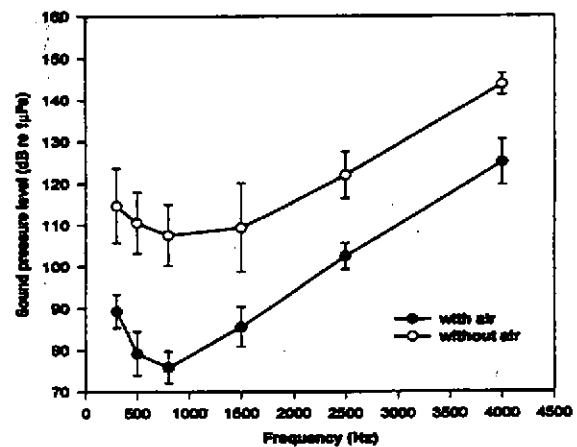

Figure 4. The audiograms of blue gourami before (solid circles) and after (open circles) removal of air bubbles. Error bars indicate standard error. N=5
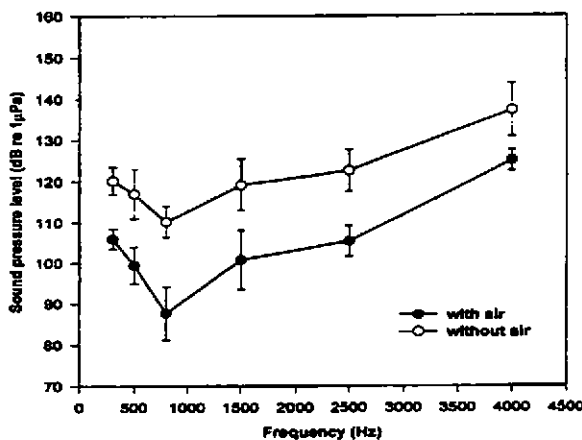

Figure 5. The audiograms of kissing gourami before (solid circles) and after

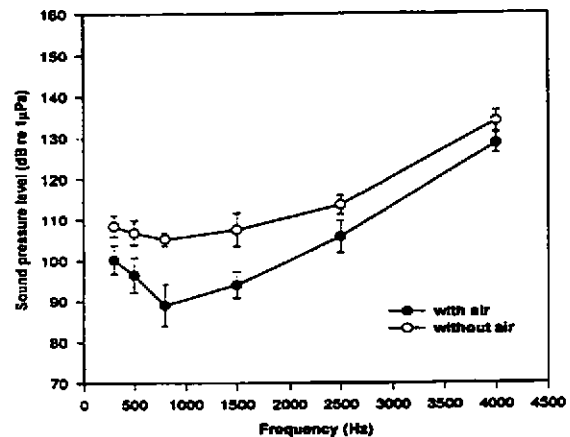(open circles) removal of air bubbles. Error bars indicate standard error. N=5

Figure 6. The audiograms of dwarf gourami before (solid circles) and after (open circles) removal of air bubbles. Error bars indicate standard error. N=5

The presence of otic gasbladder on each side of ear provides an unique chance to examine how individual otic gasbladder plays a role in modulating the overall hearing ability of mormyrid weakly electric fish (*Brienomyrus brachyistius*).
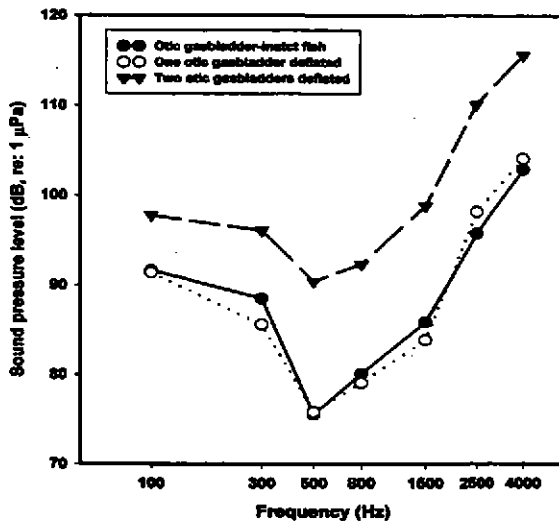
Figure 7. The audiograms of mormyrid weakly electric fish (*Brienomyrus brachyistius*) obtained from three experimental conditions. Solid circles: otic gasbladder intact fish (N=4). Open circles: one side of otic gasbladder deflated (N=4). Solid triangles: two sides of otic gasbladders deflated (N=4).
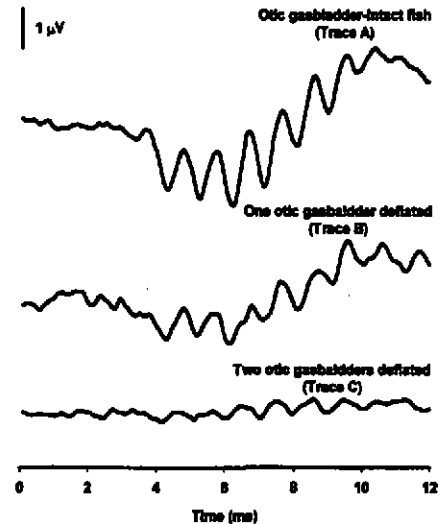

Figure 8. The acoustically evoked brainwaves of *Brienomyrus brachyistius* in response to 500-HZ, 125-dB tone burst. Trace A: otic gasbladder intact fish; Trace B: one side of otic gasbladder deflated; Trace C: two sides of otic gasbladder deflated.

The results in Figure 7 indicate that significant increase of hearing thresholds are observed after both sides of otic gasbladder are deflated. However, removal of gas from just one side of otic gasbladder does not result in any significant change of threshold when compares to the baseline data. The acoustically evoked brainwaves in Figure 8 also illustrate the reduction of potentials after both otic gasbladders are deflated (Trace C). Only moderate reduction of potentials is seen in fish with one side of otic gasbladder deflated.
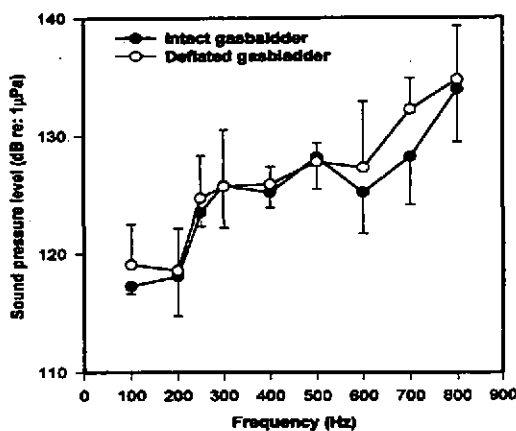

Figure 9. The audiograms of oyster toadfish before (solid circles) and after (open circles) removal of air bubbles. Error bars indicate standard error. N=5

The oyster toadfish is the only species examined that does not have any mechanical coupling between gas holding device (e.g., gasbladder) and the inner ear. In terms of relative distance between the gas holding device and the inner ear, the toadfish has the closest distance between two structures among 6 species examined. Nevertheless, the removal of gas from gasbladder of toadfish does not result in any change of hearing threshold. Based on the results obtained from 6 species examined, it is concluded that mechanical coupling between gas-holding device and inner does play a major role in enhancing overall hearing ability of certain groups of fish.

5. DISCUSSION

The conventional psychophysical mrthods (e.g., heart beat rate conditioning; underwater paddle pecking) of measuring audiograms from fish can take up to weeks or even months to complete [11,22]. The ABR recording method offers a rather rapid acquisition (i.e., within days) of data which enables researchers to conduct a broader scale of comparative study on fish hearing. The non-invasive recording approach also has the advantage of repeated recordings from the same subject after receiving experimental treatment, e.g., withdrawal of gas from gas-holding structure [19-21] or noise exposure [23]. The smallest size of fish recorded so far is 18 mm [24] and therefore the technique offers a practical way of investigating ontogenetic changes of hearing ability of fish which is impossible through the psychophysical methods or other electrophysiological methods. The additional advantage of the ABR method is that it records overall responses to acoustic stimuli along the ascending auditory neuronal pathways [25] which certainly gives better resolution that those obtained by microphonics or single unit recordings. It is important to point out, however, that the ABR technique does have its limitations. Since it records the overall acoustically evoked responses from many neuronal generators along the auditory pathways, how each generator contributes to the overall hearing is not clear. This discrepancy of understanding has to wait until detailed mapping of auditory pathways of fish as well as recording from experimental lesions to specific neuro-generators are achieved. In addition, it has been well documented that audiograms generated from electrophysiological recordings tend to have higher thresholds than those obtained by behavioral methods [17,26]. The same acoustic presentation system used to acquire ABR audiograms should be modified to obtain behavioral audiograms in order to compare possible existence of differences of two types of audiograms obtained.

Feedback from many colleagues who adopted the ABR system that was developed in my laboratory [17] indicates vibration of recording electrodes in response to low frequency (less than 200 Hz) acoustic stimuli tends to cause artifacts which are incorporated into averaged responses. Our original report [17] does not offer detailed description on the fabrication of the electrode. The following figure (Figure 10) gives a detailed description of the electrode.
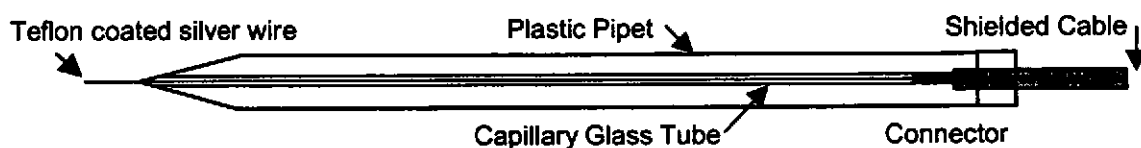

Figure 10.- Schematic diagram of the configuration of recording and reference electrodes

The key point of electrode fabrication is to provide rigidity to the electrode. The first step is to encase Telfon–coated silver wire with capillary glass tube. It is then housed inside a 10-cm long plastic pipet. The glass pipet can also be used. However, plastic pipet is easier to saw off with appropriate length as required by the setup. Both ends of the electrode are sealed with Epoxy glue to the pipet to provide firm contact with the housing unit to prevent any in sync movement with the acoustic signals. The whole electrode is clamped firmly to the micromanipuator. During the recording, the tip of electrodes have to be pushed firmly against the skin of the skull of fish to avoid any in sync vibration with acoustic signals, in particular at low frequency range. A simple way to detect whether vibrational artifacts are picked up through the electrodes is to run a test run of ABR recording with a preserved fish under highest sound pressure that can be generated from the system. A dead fish should not generate any physiological evoked potentials and the averaged response should show only a flattened wavy line representing noises from the system. When two tests, replicated from preserved fish, are overlaid both traces should not be identical due to random noise process. In addition, 2 or 3 attenuation levels of ABR should also be

generated and no difference of these waves should be noticed because dead fish should have no response to different sound pressure levels.

Myogenic noises can distort averaged brainwaves. To avoid this artifact some form of neuromuscular junction blocker has to be administered to the fish prior to the tests. A very widely used chemical is gallamine triethiodide, an antagonist for muscarinic acetylcholine receptors. The dosage, however, has to be empirically determined due to great latitude of responses to this chemical in different species. Among some 30+ species of fish examined so far in my laboratory, only one species, the Little skate (Leucoraja erinacea) fails to respond to this neuromuscular junction blocker. However, d-tubocurarine chloride (T2379, Sigma Chemicals) is found to be able to sedate the animals [Caspor B. and Yan HY, unpublished data]. Gallamine trithiodide exerts its blocking effect on all teleosts tested but fails on Little skate, the only elasmobranch examined. This observation indicates that there are possible different types of neurotransmitters or receptors to the neurotransmitters between elasmobranchs and teleosts.

Tight coupling of the posterior element of the Weberian ossicles to the gasbladder in goldfish is very obvious under the view of radiograph [20]. The removal of gas from the gasbladder essentially renders the function of Weberian ossicles obsolete. The significant elevation of hearing thresholds (Figure 3) after gas removal supports the long held hypothesis [3-4] that mechanically coupled gasbladder does assist in fish hearing. This change is also evident by a simple examination of the changes of amplitude of evoked brainwaves before and after gas removal. For example, a goldfish shows a peak-to-peak to potential of about 2.35 μV in response to a signal of 300 Hz tone burst at 132 dB. After the deflation, the amplitude drops to 0.3 μV with the same stimulus [20]. The elevated thresholds return to its baseline values 7 days later after deflated gasbladders are allowed to refill [20]. These data further validate the role of coupling between inner ear and gasbladder in enhancing overall hearing of goldfish (Figure 3).

Most of fish houses their inner ear inside the skull to protect this vital sensory organ. The layout of inner ears in gouramis is an exception to the norm. The otic capsule that contains the three hearing endoragns is encased in a membrane-like thin bony structure and protrudes into suprabranchial chamber. The advantage of such an anatomical arrangement can be interpreted as to maximize the exposure of inner ear to the compressible gas held inside the chamber to enhance their hearing. Significant elevation of thresholds to three gouramis examined after removal of gas from the suprabranchial chamber and refilling of gas inside the chamber brings thresholds back to baseline level support the hypothesis that gas held inside the chamber facilitates and enhances overall hearing of gouramis (Figures 4, 5, 6)[19].

The direct coupling of otic gasbladder to the saccule of mormyrids prompts von Frisch to suggest it may play a role in hearing enhancement [4]. The deflation of both sides of otic gasbladder leads to significant elevation of hearing threshold (Figure 7) as well as reduction of evoked potentials (Figure 8). Thus, the hypothesis first raised by von Frisch that the tightly coupled otic gasbladders can transmit the sound pressure component of the acoustic signals into the inner ear to enhance overall hearing ability in mormyrids is confirmed. However, the finding that deflation of only side of otic gasbladder does not result in any significant change of hearing threshold is somewhat intriguing. Our hypothesis originally predicts that deflation of only one side of otic gasbladder should lead to an elevated threshold in between those of baseline and deflation of both otic gasbladders. Interestingly clinical work has demonstrated monaural stimulation of human subjects resulted in an acoustically evoked brainwave that is similar in shape but with reduced amplitude when compared to bilaterally-stimulated [27]. A similar finding is observed in mormyrid work [21] when comparing trace B (one otic gasbladder deflated) with trace A (otic gasbladder intact fish) of Figure 8. Even with deflation of one otic gasbladder, the saccule should be still functional, albeit with less sensitivity. The acoustically evoked brainwave is the summation of neuronal activities from various neurogenerators at different sites in the ascending auditory pathways [25]. In humans, the first bilateral representation of acoustic stimuli occurs at the olivary complex where it receives inputs from both ipsilateral and contralateral cochlear nuclei [25,28]. Therefore, as long

as one ear is properly stimulated, neurogenerators on both ipsilateral and contralateral sides of the ascending pathways above the first cross over site (the olivary complex) are stimulated. Hence, the overall acoustically evoked waveform does not deviate greatly from the waveform generated in monaurally and binaurally stimulated subjects [27]. The auditory pathway of a mormyrid (Pollimyrus isidori) has been well mapped [29]. The bilateral projection pattern of saccular nerve fibers into dorsomedial zone of the descending octaval nucleus (dzD) suggests that auditory inputs from each ear may be combined early in auditory processing at this major first-order nucleus. It is suggested that the bilateral projection pattern of primary afferents into dzD may have evolved to integrate information from the two ears and to create a fused representation of the pressure component of the sound field [29]. Assuming a similar pathways in B. brachyistius then it is possible that B. brachyistius integrates acoustic inputs from both ipsi- and contralateral sides of ears at the dzD nucleus. This may explain why there is little difference in the acoustically evoked brainwave between otic gasbladder-intact and one-side deflated fish (Figure 8; trace A vs. trace B).

There is an unexpected finding in the organization of otic gasbladder in B. brachyistius. Histological sections reveal tight coupling between the otic gasbladder and the saccule as first suggested by von Frisch [4] and Stipetic [30]. However, about 20% in distance from the top of the otic gasbladder, a thin and slightly slanted septum (about 2 μm in thickness) separates the otic gasbladder into two unequal sized chambers. In light of this finding an interesting question to ask is what function of acoustic sensation is served by such a two-chamber amplification design? This intriguing question requires further study.

Gasbladders have been compared to pulsating underwater bubbles that are strongly resonant structures [31]. The gas inside the gasbladder is readily compressible and pulsates when exposed to sound, re-radiating the sound in all directions, including toward the ears [32]. van Bergijk [33] even argues that "a fish with a swim bladder is thus potentially sensitive to a spectrum of far-field sounds ranging in frequency from below 100 Hz to somewhere in the low kHz range since the swim bladder is a simple resonating device." The notion that gasbladder aids in underwater hearing is widely accepted and can be seen in many fish biology and ichthyology textbooks [34-35]. The deflation of gasbladder of oyster toadfish shows no significant change of thresholds [20] indicating that the accepted notion may not be completely correct. However, taken together the findings from goldfish, gouramis, mormyrid and compare it with the finding in oyster toadfish, it is clear that only gasbladder that has direct or mechanical coupling between gas-holding device and the inner ear can enhance hearing of fish.

It has been widely accepted that species having a particularly efficient mechanical coupling between the gas-filled chamber and the otolith organs, i.e., the hearing specialists, tend to have high sensitivity to sound pressure and may hear in a relatively wide frequency range [36-38]. The results of gas-holding device deflation experiments clearly demonstrate elevation of hearing thresholds after the treatment (Figures 3-7). However, removal of gas fails to reduce any change of hearing frequency range and it seems to contradict the long held belief of the role of coupled gas-holding device in extending the hearing frequency range. The observed fact points to a possibility that there exists distinct differences between the sensory hair cells of hearing generalist and hearing specialists. Further experiments are needed to elucidate this issue.

Fish hearing research, at least in terms of measurement audiograms, has long lagged behind those of mammalian and avian species despite more than half of vertebrate species are fish. The constraint of physical media, i.e., water is a major factor and lack of a rapid protocol of measurement is another technical limiting factor. The auditory brainstem response protocol reported and discussed here represents an improvement in the pace of acquiring baseline hearing ability data of fishes. The noninvasive nature of the technique also provides researcher greater latitude of manipulation in the treatments of subjects for various experimental purposes to address more questions relate to fundamental hearing ability of fishes.

The removal of gas from gas-holding device confirms its role in the enhancement of hearing, at least in terms of hearing threshold. However, it is also discovered that such a device in fact does not necessary contribute to the enhancement of hearing frequency. It remains an enigma as to how wider frequency range is achieved among hearing specialist species.

6. ACKNOWLEDGEMENTS

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