

## RESEARCH ARTICLE

# Pressure and particle motion detection thresholds in fish: a re-examination of salient auditory cues in teleosts

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### SUMMARY

The auditory evoked potential technique has been used for the past 30 years to evaluate the hearing ability of fish. The resulting audiograms are typically presented in terms of sound pressure (dB re. 1  $\mu$ Pa) with the particle motion (dB re. 1  $\text{m s}^{-2}$ ) component largely ignored until recently. When audiograms have been presented in terms of particle acceleration, one of two approaches has been used for stimulus characterisation: measuring the pressure gradient between two hydrophones or using accelerometers. With rare exceptions these values are presented from experiments using a speaker as the stimulus, thus making it impossible to truly separate the contribution of direct particle motion and pressure detection in the response. Here, we compared the particle acceleration and pressure auditory thresholds of three species of fish with differing hearing specialisations, goldfish (*Carassius auratus*, weberian ossicles), bigeye (*Pempheris adspersus*, ligamentous hearing specialisation) and a third species with no swim bladder, the common triplefin (*Forsterygian lappillum*), using three different methods of determining particle acceleration. In terms of particle acceleration, all three fish species have similar hearing thresholds, but when expressed as pressure thresholds goldfish are the most sensitive, followed by bigeye, with triplefin the least sensitive. It is suggested here that all fish have a similar ability to detect the particle motion component of the sound field and it is their ability to transduce the pressure component of the sound field to the inner ear *via* ancillary hearing structures that provides the differences in hearing ability. Therefore, care is needed in stimuli presentation and measurement when determining hearing ability of fish and when interpreting comparative hearing abilities between species.

Key words: auditory evoked potentials, fish, particle acceleration, sound pressure, ancillary hearing structures, shaker table.

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### INTRODUCTION

Sound detection is a basal vertebrate capability, first evolving in the aquatic environment (Popper and Fay, 1993; Popper and Fay, 1997). An underwater acoustic stimulus has two components, particle motion and sound pressure, both of which potentially provide important information to fish. Particle motion is in part generated by hydrodynamic flow near the acoustic stimulus source and in part by the oscillations associated with the sound pressure waves as they propagate from the acoustic source as a cyclic compression and rarefaction of water molecules (Rogers and Cox, 1988; Higgs et al., 2006). The ‘near-field’ is typically defined as the area close to the source where particle motion is dominant and the ‘far-field’ is essentially defined as the region outside the near-field where the sound pressure to velocity ratio approximates a plane wave value and the majority of the acoustic information is dominated by the propagating pressure wave (Rogers and Cox, 1988; Higgs et al., 2006; Montgomery et al., 2006; Maruska et al., 2007). The inner ear of fish is directly sensitive to the particle movement of an acoustic field as a result of whole-body accelerations (Rogers and Cox, 1988; Montgomery et al., 2006). However, sound pressure can only be detected by fish from pressure-induced oscillations of the walls of an air pocket, such as the swim bladder, that then are transduced into mechanical stimuli appropriate to hair cells of the ear (Higgs et al., 2006; Montgomery et al., 2006).

Detection of the pressure component of sound waves typically results in increased sensitivity and/or bandwidth of hearing (Popper and Fay, 1997; Fay and Popper, 2000; Higgs et al., 2006). Pressure information can be detected *via* either otophysic connections between a gas bladder and ear (Popper and Fay, 1993) or laterophysic connections where the swim bladder is in close association with the lateral line (Webb, 1998; Smith et al., 2003). It has been hypothesised (Popper and Fay, 1997; Fay and Popper, 2000) that the evolution of enhanced hearing sensitivity and/or bandwidth was driven by a selective advantage toward detecting higher frequency stimuli, primarily through conversion of acoustic pressure to a displacement stimulus, in the presence of predominantly low frequency background noise. However, the precise contribution of auditory specialisations to hearing ability has been somewhat clouded by the difficulty in separating the role of acoustic pressure from that of particle acceleration in auditory detection.

The electrophysiology technique of auditory evoked potentials (AEPs) has been used extensively to measure mammalian auditory thresholds (Hall, 2006), and was adapted for work in fish almost 30 years ago (e.g. Corwin et al., 1982; Kenyon et al., 1998) as a means to estimate acoustic capabilities using compound evoked responses rather than thresholds of individual auditory afferents. To date, the majority of studies utilising AEPs to investigate fish hearing have delivered stimuli through a speaker, either underwater or in air, for a multitude of species with differing hearing capabilities

(Kenyon et al., 1998; Mann et al., 1998; Higgs et al., 2003; Higgs et al., 2004; Maruska et al., 2007; Horodysky et al., 2008; Ladich and Wysocki, 2009; Mann et al., 2009; Wysocki et al., 2009; Wright et al., 2011). All these studies show a range of hearing thresholds across species, with the goldfish, *Carassius auratus*, typically being the most sensitive (Kenyon et al., 1998). While previous work has presented thresholds largely in terms of pressure, more recent studies have started to also estimate the particle acceleration thresholds from these types of experiments using one of two methods: pressure differences between two hydrophones (Euler equation) (Mann, 2006; Zeddies et al., 2010; Zeddies et al., 2012), and accelerometers (Wysocki et al., 2009; Zeddies et al., 2012). While presentation of acceleration thresholds is a useful advance, the assumptions behind the two estimation techniques have not been fully characterised nor has any comparison been made between AEP values obtained by using a speaker stimulus, which contains both particle and pressure information, and those using a shaker table, which presents a motion stimulus independent of pressure.

Shaker tables can mimic the particle motion component of the sound field and thus represent a more specific method for measuring particle motion thresholds than having a dual-source method such as a speaker. To our knowledge there have only been two studies that have used the AEP technique in association with a shaker table as the stimulus and those were conducted on a squid (Mooney et al., 2010) and two species of bamboo sharks, *Chiloscyllium plagiosum* and *Chiloscyllium punctatum* (Casper and Mann, 2007). Shaker table technology was first pioneered for use in fish to describe and measure the axis of particle motion of the sacculus otolith in the goldfish (Fay, 1984). Since then, shaker table technology has been used for other species (Edds-Walton and Fay, 1998; Lu et al., 1998; Weeg et al., 2002; Edds-Walton and Fay, 2003; Edds-Walton and Fay, 2005; Lu et al., 2010), not only to measure the axis of particle motion but also to describe how auditory signals are encoded in individual afferent fibres of the auditory nerve. Therefore, the aim of the present study was to: (1) describe the use of the AEP technique in association with a shaker table to measure particle acceleration thresholds in three species of fish with different hearing structures – triplefin (*Forsterygian lappillum*, Hardy 1989), which has no swim bladder (Montgomery et al., 2006); *Carassius auratus* (Linnaeus 1758), which like all cyprinids has Weberian ossicles (Von Frisch, 1938); and the New Zealand bigeye (*Pempheris adspersa*, Griffin 1927), which has a hearing specialisation connecting the swim bladder to the inner ear (Radford et al., 2011); (2) compare the above-mentioned different ways for determining particle acceleration thresholds (shaker table, pressure differences between two hydrophones, and accelerometers); and (3) compare particle acceleration thresholds to pressure thresholds to better separate each aspect of underwater sound for acoustic detection.

## MATERIALS AND METHODS

### Fish care

Bigeyes and triplefins were caught by SCUBA divers using hand nets and maintained in a flow-through seawater system (ambient temperature and salinity) at the Leigh Marine Laboratory, Leigh, New Zealand. Goldfish were purchased from a local supplier and maintained in a freshwater aquarium. All fish were fed three times a week. Thirty-six animals were used in these experiments: 18 (6 of each species) for *in vivo* AEP measurements in response to a speaker stimulus (delivering both pressure and particle acceleration stimuli) and 18 for *in vivo* AEP measurements in response to a particle acceleration stimulus alone.

On experimental days, three fish were moved from their holding aquaria to a 40 l bucket filled with either seawater or freshwater depending on the species tested. Animals were anaesthetised for each experiment in a bath of 2-phenoxyethanol ( $0.004 \text{ mol l}^{-1}$ ) to reduce movement when manipulating the fish in the fish holder. During testing, fish were respiration with a more dilute 2-phenoxyethanol solution ( $0.002 \text{ mol l}^{-1}$ ) to maintain anaesthesia throughout the recording process. Initial experiments showed that there was no effect of anaesthetic on fish hearing thresholds.

### AEPs

Auditory abilities of triplefin, goldfish and bigeye were determined using AEPs. For AEP testing, anaesthetised fish were completely submerged underwater in a PVC (0.5 mm thick) tank, 1.11 m long with a diameter of 0.25 m. The anaesthetised fish were positioned laterally upon a piece of clay on a Perspex slide attached perpendicular to a plastic pipette (fish holder). A piece of stocking was firmly positioned around the fish's body as a restraint. No muscle relaxants were needed for these experiments; however, a dilute mixture of anaesthetic ( $0.002 \text{ mol l}^{-1}$  2-phenoxyethanol) was dripped through the mouth and over the gills during the experiment to maintain the anaesthetised state. A micromanipulator was used to position the fish holder in the tank, with the surface of the fish at a depth of approximately 8 cm. An underwater speaker (University Sound UW-30, Columbus, OH, USA) was placed near the opposite end of the tank, approximately 0.75 m from the fish. Auditory stimuli were produced by a sound module (Tucker-Davis Technologies, TDT, Gainesville, FL, USA) operated by a computer running SigGen (version 4.4.1) and BioSig (version 4.4.1) software. The TDT apparatus linked to the underwater speaker delivered tone bursts (10 ms duration with a 2 ms rise–fall time gated through a Hanning window) with frequencies of 100, 200, 400, 600 and 800 Hz. The presentation order of the frequencies was conducted randomly. Sound levels were increased in 5 dB increments for each frequency until a stereotypical AEP was seen, and then continued for at least another 10 dB to examine suprathreshold responses. An average of 400 responses (200 from stimuli presented at 90 deg and 200 from stimuli presented at 270 deg to cancel stimulus artefacts) was taken for each SPL at each frequency.

Stainless steel subdermal electrodes (Rochester Electromedical Inc., Tampa, FL, USA) were used to collect AEPs. The recording electrode was positioned dorsally, just anterior to the operculum, whilst the reference electrode was placed dorsally in the nasal region, with a ground electrode positioned under the body of the fish. Each electrode was insulated with nail varnish, except the tip, and was positioned using a micromanipulator.

### Shaker evoked potential measurements

To test the effects of accelerations alone, a custom-built moving coil shaker (LDS V780T minishaker) system was used to provide sinusoidal horizontal stimulation (Fig. 1), similar to tones presented in the tank. This motion stimulus was free of pressure and interference phenomena found in the tank set-up, therefore primarily providing an acceleration stimulus. The anaesthetised animals were held in place on top of a bed of clay by a stocking pinned into the clay. A dilute mixture of anaesthetic ( $0.002 \text{ mol l}^{-1}$  2-phenoxyethanol) was dripped through the mouth and over the gills during the experiment to maintain the anaesthetised state, as above. Sinusoidal particle accelerations were generated by a sound module (TDT) operated by a computer running SigGen (version 4.4.1) and BioSig (version 4.4.1) software. The TDT apparatus linked to the shaker table delivered sinusoidal acceleration bursts (10 ms duration with a 2 ms rise–fall time gated through a

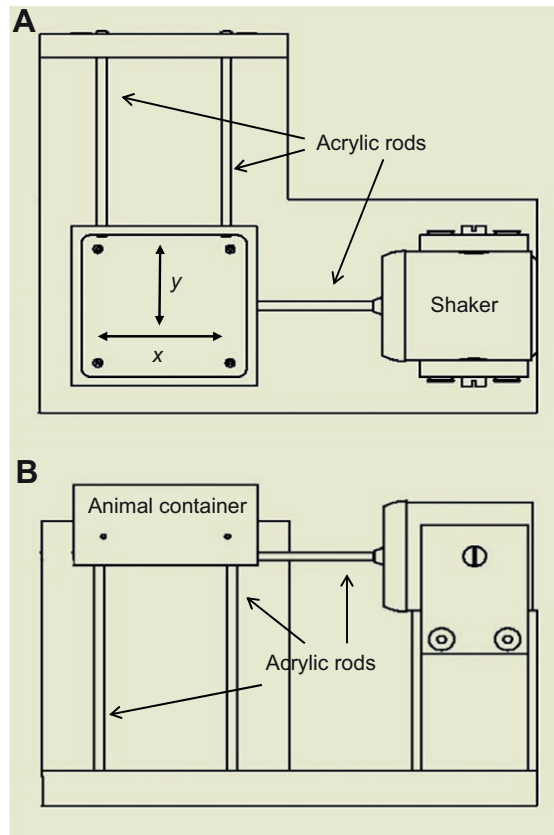


Fig. 1. Plan (A) and elevation (B) diagrams of the shaker table design. The shaker was connected to the animal container by an acrylic rod (10 mm diameter). The animal container was held up by four acrylic rods and the axis of stimulation was maintained by two acrylic rods.  $x$ ,  $x$ -axis of stimulation;  $y$ ,  $y$ -axis of stimulation.

Hanning window) with frequencies of 100, 200, 400, 600 and 800 Hz. An average of 400 responses (200 from stimuli presented at 90 deg and 200 from stimuli presented at 270 deg to cancel stimulus artefacts) was taken for each SPL at each frequency in both the  $x$ - and  $y$ -axis stimulus direction (Fig. 1). AEPs were measured in the same way as for the speaker stimulus (see above).

#### Sound and particle calibrations

Sound pressure and particle accelerations in the tank and particle accelerations in relation to the shaker table were calibrated in absence of the fish. Sound pressure calibration was carried out daily using an HTI-96-MIN hydrophone (calibration sensitivity  $-164.5$  dB re.  $1 \text{ V } \mu\text{Pa}^{-1}$ ; High Tech Inc., www.hightechincusa.com/) placed in the position of the fish holder. An oscilloscope was used to measure the sound pressure level (SPL) at each frequency, which was then attenuated through BioSig to output the desired decibel levels.

Particle accelerations for speaker-induced stimuli were calculated in two ways. The first used two underwater hydrophones to obtain pressure differences at all dB levels and frequencies (modified from Mann, 2006). Tones of 3 s duration were played and the output from the hydrophones was sent to a spectrum analyser (Model 217 SR760, Stanford Research Systems, Sunnyvale, CA, USA). The root mean square voltage ( $V_{\text{r.m.s.}}$ ) for the entire response was averaged and divided by the hydrophone calibration ( $174.5 \text{ V } \mu\text{Pa}^{-1}$ ). The corrected pressure difference between the two hydrophones was divided by the distance between the hydrophones (3 cm). The pressure

difference was then divided by the density of freshwater (used in the experimental tank) as given by the Euler equation (Mann, 2006):

$$\mathbf{a} = -(p_{\text{grad}} / \rho), \quad (1)$$

where  $\mathbf{a}$  represents the particle acceleration ( $\text{m s}^{-2}$ ),  $p_{\text{grad}}$  is the pressure gradient ( $\text{Pa m}^{-1}$ ) and  $\rho$  is the density of the medium ( $\text{kg m}^{-3}$ ), in our case freshwater. The second technique to measure acceleration used a calibrated Brüel and Kjær accelerometer (Deltatron 4524 cubic triaxial accelerometer,  $100 \text{ mV g}^{-1}$ ; Helsinki, Finland) that had been waterproofed and made neutrally buoyant by embedding it in a syntactic foam enclosure (Zeddies et al., 2012). The accelerometer was then connected to a three-channel conditioning amplifier (Deltatron 2693-A-OS3), with the output fed into an oscilloscope (Tektronix DPO 2014; Beaverton, OR, USA).

The shaker table was calibrated using three single-axis Brüel and Kjær accelerometers (4507B-002 Deltatron accelerometer,  $1000 \text{ mV g}^{-1}$ ) connected to a conditioning amplifier (Deltatron 2693-A-OS3) with the output measured on an oscilloscope (Tektronix DPO 2014). The  $x$ -axis was the dominant stimulus direction and was always of the order of 10–12 dB greater than the  $y$ - and  $z$ -axes. For all three particle acceleration methods, accelerations were calculated for the  $x$ -,  $y$ - and  $z$ -planes and the acceleration magnitude [calculated as  $\sqrt{(x^2+y^2+z^2)}$ ] is reported in the current study.

#### Data analysis

Auditory threshold was defined as the lowest level at which a clear response could be detected with AEP and was obtained visually for both the sound pressure and particle acceleration stimuli. Visual detection has been shown to produce comparable results to the use of statistical approaches (Mann et al., 2001; Egner and Mann, 2005). Data were tested for normality and homogeneity before parametric analyses were performed. To test the hypothesis that the  $x$ - and  $y$ -axis stimulus directions (Fig. 1) on the shaker table produced similar audiograms, two-way repeated measures ANOVA was used (factors: axis and frequency). To test the hypothesis that hearing sensitivities differed across the frequency range and between the three different ways in which the acceleration stimulus was measured [shaker table, two hydrophones in the tank (Euler) and accelerometer on the fish holder in the tank], two-way repeated measures ANOVA was used (factors: method and frequency). Where significant differences were found, Tukey's HSD *post hoc* tests were conducted. For all tests, the significance level was  $\alpha=0.05$ .

## RESULTS

### Stimulation axis

All three fish species (triplefin, goldfish and bigeye) tested on the shaker table, whether in the  $x$ - or  $y$ -axis direction, showed a typical AEP response (Fig. 2). All fish showed the same pattern in terms of sensitivity, being significantly (triplefin  $F_{4,20}=87.95$ ,  $P<0.001$ ; goldfish,  $F_{4,20}=42.65$ ,  $P<0.001$ ; and bigeye,  $F_{4,20}=127.94$ ,  $P<0.001$ ) more sensitive at the low frequencies of 100 and 200 Hz, which were similar ( $-48$  to  $-55$  dB re.  $1 \text{ m s}^{-2}$ ) (Fig. 3). Above 200 Hz all fish showed significant decreases in their auditory sensitivity. Hearing thresholds for all fish species were similar at 400, 600 and 800 Hz, ranging from  $-30$  to  $-15$  dB re.  $1 \text{ m s}^{-2}$ . More importantly, the hearing thresholds for the three fish species were similar (triplefin,  $F_{1,20}=0.72$ ,  $P=0.43$ ; goldfish,  $F_{1,20}=4.72$ ,  $P=0.06$ ; and bigeye,  $F_{1,20}=3.46$ ,  $P=0.12$ ) between the  $x$ - and  $y$ -axis of particle motion stimulation (Fig. 3).

### Pressure versus particle motion

When combining the two axes to get the overall magnitude of the shaker table response, all three fish species showed similar

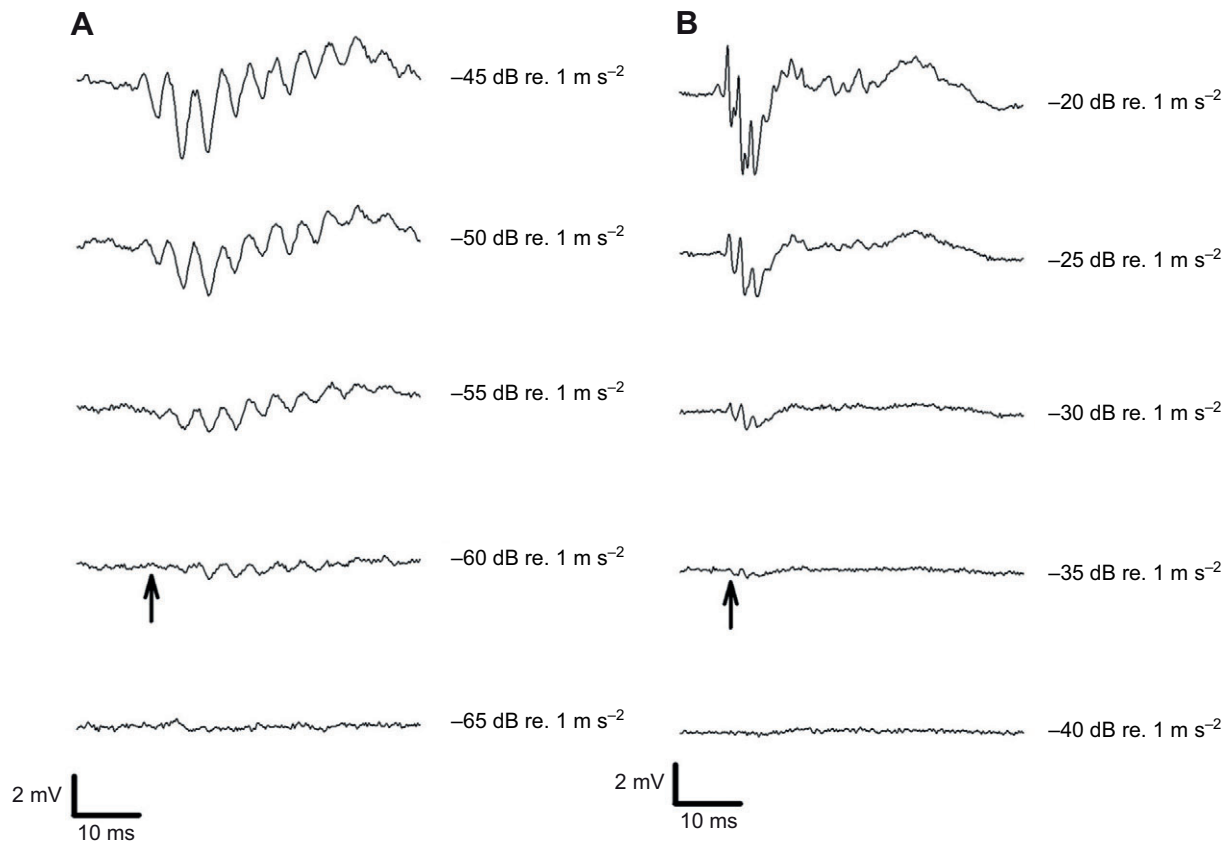


Fig. 2. Auditory evoked potential (AEP) response of *Forsterygian lappillum* to (A) 100 Hz and (B) 400 Hz shaker table stimulus. The traces for goldfish and bigeye were identical in form to those of triplefin, so only the latter are shown here. All intensities are expressed as dB re.  $1 \text{ m s}^{-2}$ . The arrow indicates the start of the response. Auditory threshold or the lowest sound pressure level (SPL) to show a definitive response occurred at  $-60 \text{ dB}$  for 100 Hz and at  $-35 \text{ dB}$  for 600 Hz in this example.

( $F_{2,40}=3.89$ ,  $P=0.06$ ) audiograms in terms of particle motion (Fig. 4A). Again, there were differences between frequencies ( $F_{4,40}=139.58$ ,  $P<0.001$ ), with significantly more sensitivity at 100 and 200 Hz than at 400, 600 and 800 Hz, but there were no differences between species. However, when comparing pressure-derived audiograms there were significant differences ( $F_{2,40}=102.89$ ,  $P<0.001$ ) between the three species (Fig. 4B). The goldfish was the most sensitive, followed by the bigeye and then the triplefin. For the pressure-derived audiograms there was also a significant interaction between the fish species and frequency ( $F_{8,40}=9.89$ ,  $P<0.001$ ). Triplefin were significantly less sensitive than both the goldfish (by 30–60 dB re.  $1 \mu\text{Pa}$ ) and bigeye (by 15–25 dB re.  $1 \mu\text{Pa}$ ) across all frequencies. The bigeye and the goldfish had a similar pressure-derived hearing threshold at 100 Hz.

#### Comparisons between particle motion calculation methods

There were significant differences (triplefin  $F_{2,40}=107.95$ ,  $P<0.001$ ; goldfish,  $F_{2,40}=67.82$ ,  $P<0.001$ ; and bigeye,  $F_{2,40}=78.95$ ,  $P<0.001$ ) in the audiograms between the three different methods of determining particle acceleration sensitivity of the three fish species (Fig. 5). For the triplefin there was an approximately 21 dB re.  $1 \text{ m s}^{-2}$  decrease in the sensitivity estimates between the accelerations calculated using the Euler equation and the accelerometer in the tank, and the auditory threshold for particle acceleration determined by the shaker table was positioned between the two (Fig. 4A). For the goldfish and bigeye the difference in auditory thresholds determined by the Euler equation in the tank and the shaker table were not as large; 4 dB re.  $1 \text{ m s}^{-2}$  and between 4 and 8 dB re.  $1 \text{ m s}^{-2}$ ,

respectively (Fig. 4B,C). The difference in particle acceleration auditory threshold determined by the accelerometer in the tank for goldfish was as much as 60 dB less than that determined by the Euler equation in the tank and the shaker table, and the largest difference in particle acceleration thresholds for bigeye was 50 dB.

#### DISCUSSION

As sound propagates through water, regions of rarefaction and compression are generated by local particle motion and pressure fluctuations (Rogers and Cox, 1988). Hair cells within the fish's inner ear transduce the particle motion through the deflection of their kinocilia (Popper and Fay, 2011). By having a higher density object, such as the otolith in fish (Chapman and Sand, 1974) or statolith in crustaceans (Budelmann, 1992), the movement of the higher density objects relative to the hair cell sensors generates differential motion and deflection of the kinocillia (Popper and Fay, 2011). Sound pressure detection, however, requires compressible components, such as the swim bladder of fish, to act as pressure to particle motion transducers (Fay and Popper, 1974; Sand and Karlsen, 2000). When trying to define hearing ability in fishes, therefore, it is crucial to understand and accurately measure both pressure and particle motion to determine which stimuli are contributing to the observed response.

To our knowledge, this is the first study that has used the AEP technique to compare speaker- and shaker table-derived particle acceleration thresholds of teleost fishes. Overall, there is no difference in particle acceleration sensitivity between the three species of fish. However, there are large differences in their ability

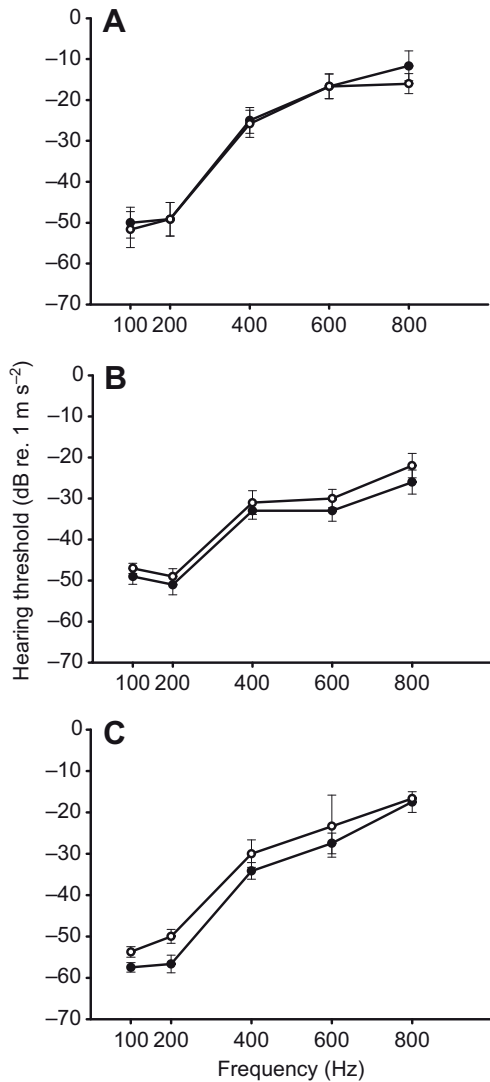


Fig. 3. Mean ( $\pm 1$  s.e.m.) particle acceleration thresholds (dB re.  $1 \text{ m s}^{-2}$ ) measured in both the  $x$ -axis (filled circles) and  $y$ -axis (open circles) on the shaker table for (A) *F. lapillum*, (B) *Carassius auratus* and (C) *Pempheris adspersa*.

to detect pressure; the goldfish was the most sensitive, followed by the bigeye and then the triplefin (Fig. 4B). These results suggest that particle motion sensitivity may be essentially similar across the Teleostei (although of course many more species must be assessed) and it is their ability to convey pressure sensitivity to the inner ear *via* ancillary structures that generates the difference in hearing ability between species. The argument has been made that diversity in hair cell structure may drive differences in hearing ability (Popper and Fay, 2011), and there are certainly differences in ion channel dynamics between different types of auditory hair cells in fish (Coffin et al., 2004), but to date there has been little progress in characterising how these structural differences drive changes in response dynamics at the level of sensory input to the brain. Expanding our comparative approach to more species would better address this question but on the basis of the present data it is reasonable to conclude that selection has primarily acted at the level of ancillary structures to influence hearing in teleosts, as suggested elsewhere (Popper and Fay, 2011), with basic particle motion detection conserved across groups.

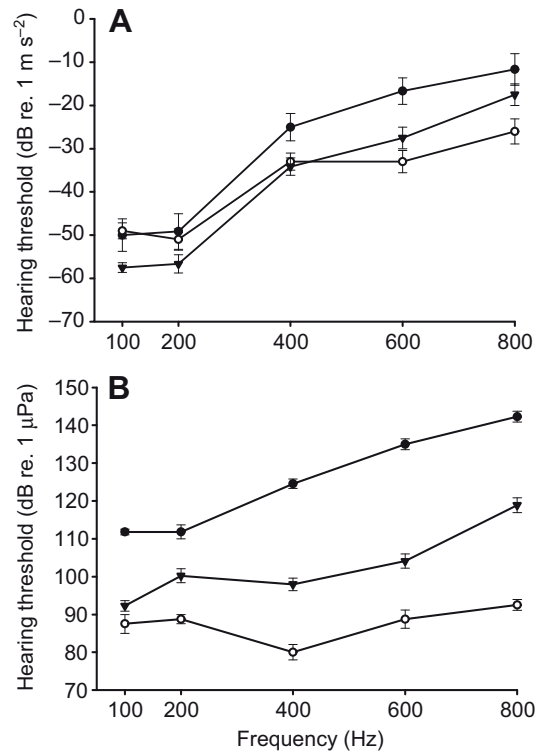


Fig. 4. Mean ( $\pm 1$  s.e.m.) particle acceleration-derived (A,  $x$ - and  $y$ -axis combined; dB re.  $1 \text{ m s}^{-2}$ ) and pressure-derived (B, dB re.  $1 \mu\text{Pa}$ ) AEP thresholds for three species of fish. Filled circles, *F. lapillum*; open circles, *C. auratus*; and filled triangles, *P. adspersa*. Note the different scales on the  $y$ -axis for the two plots.

Previous research on fish hearing employing the shaker table system has aimed to describe the axis of particle motion, i.e. to determine the direction of greatest sensitivity (Fay, 1984; Lu et al., 1996; Edds-Walton and Fay, 1998; Lu et al., 1998; Weeg et al., 2002; Edds-Walton and Fay, 2003). The present study designed a single axis shaker system (Fig. 1), where gross particle acceleration responses could be determined. The hearing thresholds of the three species tested showed similar results for the  $x$ - and  $y$ -axis of stimulation (Fig. 2), suggesting that all these fish have omnidirectional ears, which is further supported by previous anatomical research on the inner ear hair cell polarities (Lu and Popper, 1998). Goldfish hearing has been measured in other shaker table studies with similar resultant sensitivities (Fay, 1984; Casper and Mann, 2007). Fay used single-unit recordings at 140 Hz, with hearing thresholds ranging from  $7.74 \times 10^{-7} \text{ m s}^{-2}$  for the most sensitivity neurons to  $7.74 \times 10^1 \text{ m s}^{-2}$  for the least sensitive (Fay, 1984), while Casper and Mann using a similar approach to the current study measured thresholds of approximately  $1 \times 10^{-3} \text{ m s}^{-2}$  at 100 Hz (Casper and Mann, 2007). The data obtained in the present research for goldfish evoked potentials at 100 Hz of  $3.16 \times 10^{-3} \text{ m s}^{-2}$  fall within this range, showing the utility of the current approach and supporting the idea that the evoked potentials in the current study represent collective inputs from across the auditory epithelia.

The only other study to our knowledge using whole-field evoked potentials in fish in response to a shaker stimulus (Casper and Mann, 2007) found no difference in the gross hearing thresholds of two species of bamboo shark. In the same setup, however, goldfish had lower hearing thresholds than the sharks, except at 100 Hz, even though the swim bladder had been theoretically neutralised by the lack of sound pressure in the experiment. One of the possible

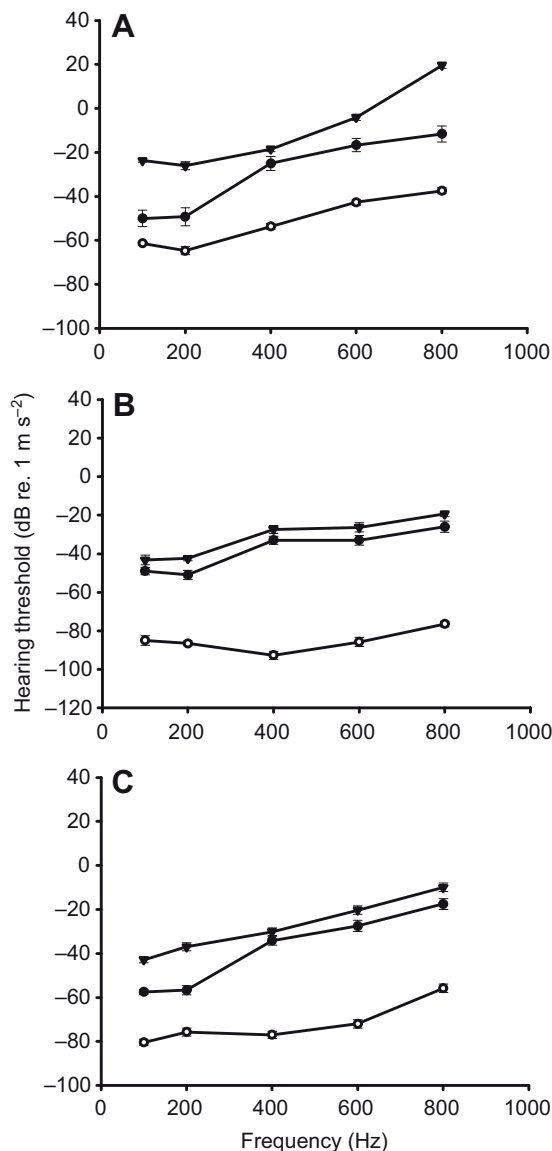


Fig. 5. Mean ( $\pm 1$  s.e.m.) particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) audiograms for three fish species; (A) *F. lapillum*, (B) *C. auratus* and (C) *P. adspersa*, calculated in three different ways. Filled circles, shaker table; open circles, accelerometer on the fish holder in the tank; and filled triangles, using the Euler equation with two hydrophones in the position of the fish's head in the tank.

explanations for the differences between goldfish and sharks relates to the differences in the composition of the otoliths of the goldfish compared with the shark's otoconia. The otoliths in teleosts are generally composed of a solid calcium carbonate matrix, where the shark otoconia are calcium carbonate, with exogenous siliceous material in a gelatinous matrix (Casper and Mann, 2007). It has been suggested that ears with otoliths of higher density are more sensitive to particle acceleration (Lychakov and Rebane, 2005); therefore, the goldfish otolith should result in greater sensitivity than the less dense shark otoconia. In the present study, the hearing sensitivity to particle acceleration was similar for each species, despite there being differences in their ancillary hearing specialisations. As all three of these fish species will have otoliths of approximately the same density, and pressure has been eliminated from the experimental set-up, the *a priori* prediction that all three

fish would have similar hearing thresholds to particle acceleration was accepted and is likely to apply to other teleosts as well.

Popper and Fay have argued that although there are significant interspecific differences in the ear structure, at the receptor cell level (i.e. hair cells) the basic function of the ear and auditory system are similar among vertebrate groups (Popper and Fay, 1997). Therefore, as part of the present study we directly tested this hypothesis and propose that it is the fish's ability to convert sound pressure into particle motion that provides the basis for differences in hearing abilities between species. The triplefin has no swim bladder so theoretically has no capability to detect a pressure stimulus, and this was represented in their pressure hearing sensitivity by their being the least sensitive of the three species examined. The two other species tested in the present study have ancillary hearing structures; Weberian ossicles for the goldfish (Von Frisch, 1938; Fay and Popper, 1974) and an otolaterophysic connection for the bigeye (Radford et al., 2011). It has been shown that as the Weberian ossicles develop in the zebrafish (*Danio rerio*) and connect the inner ear to the swim bladder, the hearing range increases from 1000 to 4000 Hz and the fish become more sensitive to sound (Higgs et al., 2002). Severing the specialised hearing connection in the bigeye increases their hearing sensitivity by 15–20 dB re.  $1 \mu\text{Pa}$  (Radford et al., 2011). Our current study in conjunction with this previous research provides further evidence that it is the presence and the ability of the ancillary hearing structures to convey pressure sensitivity to the inner ear that differentiates the hearing ability of different species of fish.

Traditionally, studies on fish hearing employing the AEP technique have presented the sound stimulus using a speaker (underwater or air), which generates both particle motion and sound pressure within the tank environment. More recently, researchers have become increasingly interested in particle motion sensitivity because it is the primary way fish sense sound. The majority of the studies (Mann et al., 2007; Horodysky et al., 2008; Mann et al., 2009; Wysocki et al., 2009; Schulz-Mirbach et al., 2010) presenting particle acceleration thresholds have used the same experimental set-up as for determining pressure sensitivity. Particle acceleration values in response to a speaker stimulation – which transmits both pressure and particle motion simultaneously – have been calculated using the pressure differences between two hydrophones (Euler equation) (Mann et al., 2007; Horodysky et al., 2008; Mann et al., 2009) or more directly with accelerometers (Wysocki et al., 2009; Schulz-Mirbach et al., 2010). The present study showed that particle acceleration thresholds differed depending on the mechanism by which they were determined. Overall, measuring particle acceleration with an accelerometer in response to a speaker stimulus appeared to result in much lower estimates of sensitivity compared with the other two methods. This difference may be due to the measured stimulus levels not accurately reflecting the actual effective movement stimulus to the fish. In comparing the different methods of determining the acceleration response, the shaker table-assessed hearing thresholds consistently lie between the Euler- and accelerometer-based assessments made using the speaker stimulus. The Euler method consistently underestimates the hearing threshold, presumably due to over-estimating the stimulus strength, particularly in the case of the swimbladder-less triplefin. The accelerometer-based assessments provide significantly lower hearing thresholds, possibly due in part to an underestimation of the effective acceleration stimulus, but also to the confounding effect of the simultaneous presentation of a pressure stimulus in fish, like the goldfish and bigeye, with pressure reception hearing specialisations.

The problem with using an AEP experimental set-up with a speaker as a sound source is that it is impossible to separate the two

components of the sound field using a speaker. Additionally, the fish is positioned well within the theoretical near-field at the lower frequencies (100–200 Hz) using a speaker stimulus (Rogers and Cox, 1988), and AEP experiments are typically conducted in a small tank with undefined acoustic properties, so it is not really possible to separate pressure from particle effects in these systems. If the intent is to measure changes in hearing thresholds after some experimental manipulation (e.g. noise exposure), then AEP with a speaker stimulus can be an effective tool. If, however, the intent is to characterise hearing ability with a goal of understanding the detection of acoustic stimuli in a natural environment, then both speaker stimulation and shaker table AEPs would provide a more accurate estimation of hearing ability in aquatic animals and would allow proper estimation of both pressure and particle acceleration thresholds.

In conclusion, the present study provides the first evidence that there are differences between particle acceleration thresholds depending on the stimulation technique employed, i.e. speaker or shaker table. Researchers that try to define particle acceleration thresholds using a speaker stimulus should present their results with caution as it was shown here that there are large differences in threshold depending on how particle motion is measured. The only true way to define particle motion thresholds is to use a shaker table stimulus, which effectively eliminates any sound pressure stimuli. The results also suggest that teleosts may have similar hearing thresholds in response to particle motion and that selection has acted on accessory auditory structures rather than the auditory epithelia to drive differences in hearing ability across species.

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