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Dipole hearing measurements in elasmobranch fishes

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Summary

The hearing thresholds of the horn shark *Heterodontus francisci* and the white-spotted bamboo shark *Chiloscyllium plagiosum* were measured using auditory evoked potentials (AEP) in response to a dipole sound stimulus. The audiograms were similar between the two species with lower frequencies yielding lower particle acceleration thresholds. The particle acceleration audiograms showed more sensitive hearing at low frequencies than previous elasmobranch audiograms, except for the lemon shark *Negaprion brevirsotris*. Auditory evoked potential signals were also recorded while the dipole stimulus was moved to different locations above the head and body. The strongest AEP signals were recorded from the area around the parietal fossa, supporting previous experiments that suggested this region is important for elasmobranch hearing. This is the first time that hearing experiments have been conducted using a dipole stimulus with elasmobranchs, which more closely mimics the natural sounds of swimming prey.

Key words: dipole, auditory evoked potentials, elasmobranch, hearing, *Heterodontis francisci*, *Chiloscyllium plagiosum*.

Introduction

To date, all hearing measurements (Kritzler and Wood, 1961; Olla, 1962; Banner, 1967; Nelson, 1967; Kelly and Nelson, 1975; Casper et al., 2003; Casper and Mann, 2006), as well as field attraction experiments (Nelson and Gruber, 1963; Richard, 1968; Myrberg et al., 1969; Nelson et al., 1969; Myrberg et al., 1972; Myrberg, 1978) of elasmobranchs have used a monopole sound source (i.e. underwater speaker) as the mode of acoustic stimulus. However, several authors (Kalmijn, 1988; Myrberg, 2001) have suggested that a dipole sound source would be the more appropriate stimulus for measuring elasmobranch hearing as it more closely represents a biological action (Bass and Clark, 2003) that these fishes could be listening for in the natural environment (i.e. a swimming fish). A dipole stimulus is directional along the axis of motion and attenuates as a function of $1/r^3$ (r=radial distance from sound source) in the near field, whereas a monopole radiates sound out in all directions equally, and attenuates as a function of $1/r^2$ in the near field. This is important when considering the field attraction experiments in which many species of sharks have been attracted to large underwater speakers (monopoles) producing 'stronger-than-natural' stimuli (Kalmijn, 1988). Depending on the frequency and intensity, these monopole stimuli could travel potentially hundreds of meters in the far field and still be detectable by sharks.

Dipole stimuli have been used to measure responses of the lateral line in bony fishes (Harris and Van Bergeijk, 1962; Denton and Gray, 1983; Karlsen and Sand, 1987; Coombs et al., 1989; Coombs, 1994; Abboud and Coombs, 2000; Kirsch et al., 2002) and elasmobranchs (Bleckmann et al., 1987; Bleckmann et al., 1989; Maruska and Tricas, 2004). The dipole stimulus has not become as commonly used in hearing experiments as the monopole stimulus (e.g. Coombs, 1994; Coombs and Fay, 1997; Braun and Coombs, 2000; Fay et al., 2002) even though it provides a more biologically relevant stimulus. The dipole stimulus is usually a small metal or plastic ball attached to a rigid post that is driven by a mechanical shaker. It vibrates along one axis and therefore is highly directional compared to a monopole source.

A variation of a dipole stimulus was used to measure the vibration sensitivity of the parietal fossa in sharks (Fay et al., 1974). The parietal fossa is a subdermal area of loose connective tissue dorsal to the inner ear. It has been proposed that this structure could provide a direct pathway for sound transmission to the macula neglecta endorgan of the inner ear (Tester et al., 1972; Fay et al., 1974; Corwin, 1977; Corwin, 1981). In these experiments a vibrating rod was used to stimulate the surface of the head while recording microphonic potentials from the ear. Fay et al. found that vibrations on the parietal fossa produced stronger responses from the ramus neglectus nerve of the macula neglecta than from other areas around the head (Fay et al., 1974).

The following experiments were designed to measure the responses of two shark species, the horn shark *Heterodontus francisci* and the white spotted bamboo shark *Chiloscyllium plagiosum*, to dipole sound stimuli. *H. francisci* hearing

thresholds have been measured previously with a monopole underwater speaker (Kelly and Nelson, 1975), whereas *C. plagiosum* is from a family of elasmobranchs (Hemiscylliidae) that have never had their hearing tested. These shark species were chosen because of their demersal life style, making them ideal for experiments in which they must remain motionless for long periods of time. Hearing tests were conducted using the auditory evoked potential method (AEP), a neurophysiological method of recording evoked potentials from the brain in response to acoustic stimuli (Corwin et al., 1982; Kenyon et al., 1998). This method has been used to measure hearing thresholds in the little skate *Raja erinacea*, the nurse shark *Ginglymostoma cirratum*, and the yellow stingray *Urobatis jamaicensis* (Casper et al., 2003; Casper and Mann, 2006). The first goal was to measure the audiogram of each species using the dipole shaker fixed in one location. The second goal of our experiments was to measure spatial sensitivity of the sound stimulus by moving the dipole to several locations above the head and measuring the level of the evoked response. Since the dipole is directional, it allows mapping of responses over a fine spatial scale.

Materials and methods

Three *Heterodontus francisci* Girard (63–74 cm total length) and five *Chiloscyllium plagiosum* Bennett (65–78 cm total length) were maintained in aquaria on a 12 h:12 h light:dark cycle and were fed squid. Hearing experiments were conducted in a sound isolation booth (2.44 m×2.44 m×2.23 m) in a large, fiberglass tank (1.96 m×0.95 m×0.60 m) with a water depth of 0.5 m. The tank was placed on top of a wooden pallet separated from the floor of the booth by four vibration-isolation mounts (Tech Products Corporation, Dayton, OH, USA, model 52512). Experimental procedures followed guidelines for the care and use of animals approved by the Institutional Animal Care and Use Committee at University of South Florida, protocol no. 2118.

Each subject was placed in stiff plastic mesh holders (2.54 cm×2.54 cm holes). These holders were tightened with tie wraps that were tight enough to keep the shark from moving, but did not affect breathing. The shark was suspended by an elastic cord hooked through the mesh at the head and tail and looped across an aluminum bar held above the tank by two aluminum A-frames. The A-frames were not directly connected to the tank. The sharks were suspended 20 cm below the surface of the water (Fig. 1). The mechanical shaker (Brüel and Kjaer mini-shaker type 4810) was attached to another aluminum bar which was suspended independently from the experimental tank by PVC pipes attached to the walls of the booth. The setup was designed so that the shaker could be moved in an *x*–*y* plane above the tank. A stainless steel tube (27 cm long×0.4 cm diameter) that was threaded at one end and had a PVC ball (1.3 cm diameter) glued to the other end was screwed into the shaker to provide the dipole stimulus (Fig. 1).

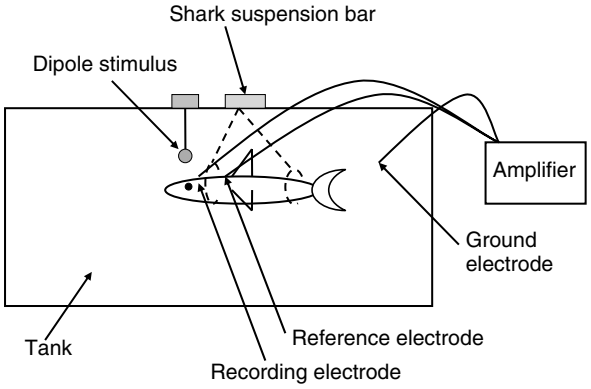

Fig. 1. Diagram of the dipole hearing setup. Drawing not to scale.

Wire electrodes (12 mm length, 28 gauge low-profile needle electrode; Rochester Electro-Medical, Inc., Tampa, FL, USA) were placed subdermally 1 cm posterior to the endolymphatic pores (recording electrode), in the dorsal musculature near the dorsal fin (reference electrode), and free in the water (ground electrode; Fig. 1). The electrodes were connected to a pre-amplifier (TDT HS4), which was then connected by a fiber-optic cable to a TDT (Tucker-Davis Technologies, Gainesville, FL, USA) evoked potential workstation with BioSig software.

Hearing threshold measurements

These methods follow those described (Casper and Mann, 2006), except that they were performed in an audiology booth rather than outdoors. All sounds were pulsed tones that were 50 ms in duration and shaped with a Hanning window (25 ms rise and fall time). Sounds above 20 Hz were delivered with a 70 ms presentation period (14 s^{-1}), and 20 Hz sounds had a 1000 ms presentation period (1 s^{-1}). Test frequencies ranged from 20 Hz–2000 Hz (20, 50, 100, 200, 300, 400, 800, 1000, 2000 Hz). Sounds were attenuated in 6 dB steps beginning at the highest level that could be generated at each frequency (Fig. 2A). The AEP waveforms were digitized at 25 kHz and averaged between 100–1000 times. More averages were needed as the signal moved closer to the threshold in order to pull the signal out of the noise floor.

A 2048-point Fast Fourier Transform (FFT) was used to analyze the AEP signals in the frequency domain (Fig. 2B). The entire 70 ms window was FFT-transformed, because for many of the lower frequencies that were tested the AEP signal took up the entire window. This was done at every frequency for the analysis to remain consistent. An AEP was determined to be present if the recorded signal showed a doubling of the sound frequency (e.g. a 400 Hz peak when the signal played was 200 Hz) with a peak at least 3 dB above the noise floor. The noise floor is estimated from the AEP power spectrum with a window of 100 Hz around the doubling frequency (i.e. 50 Hz on each side of the peak). This frequency doubling occurs in all low frequency fish AEP testing (Mann et al., 2001; Egner and Mann, 2005; Casper and Mann, 2006).

Following all hearing tests the fish was removed and

replaced with a pressure/velocity probe (Uniaxial Pressure/Velocity Probe, Applied Physical Sciences Corporation, Groton, CT, USA) that was positioned where the head of the fish had been. The probe contained a velocity geophone (sensitivity, 9.36 mV cm^{-1} s^{-1}; bandwidth, 10 Hz–1 kHz) and a hydrophone (sensitivity, –186.1 dB re. 1 V/μPa; bandwidth, 10 Hz–2 kHz), which could simultaneously record sound pressure and particle velocity (Fig. 2C). Calibration with the geophone was performed in all orientations [0° horizontal (*x*-axis), 90° horizontal (*y*-axis), and vertical (*z*-axis)] and all calibrations were computed as the root mean square (RMS) for the magnitude of the three axes combined. The hydrophone was omni-directional and therefore did not need to be measured along different axes. Many researchers have suggested that the inner ear of fishes act as accelerometers and therefore detect acoustic particle acceleration (Kalmijn, 1988; Fay and Edds-Walton, 1997; Braun et al., 2002; Bass and McKibben, 2003). Therefore, all audiograms have hearing thresholds shown in units of particle acceleration (m s^{-2}). Particle velocity of tonal signals can be converted to acceleration with the following equation: acceleration=velocity(2π×frequency). The acceleration thresholds are also given as a function of the magnitude of the three (*x*, *y*, *z*) directions measured. Background noise was also measured and was consistently below 10^{-7} m s^{-2}.

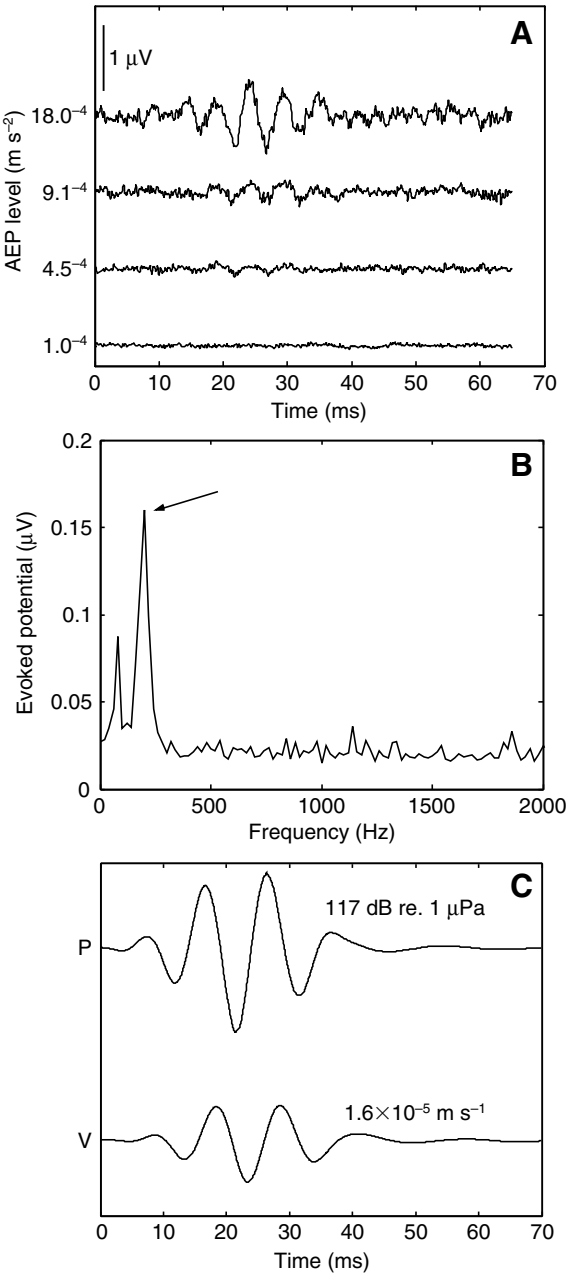

Fig. 2. (A) Auditory evoked potentials (AEPs) from a bamboo shark in response to a 100 Hz signal at four signal levels. As the signal is decreased in level (particle acceleration, m s^{-2}) the AEP signal also decreases until it is lost in the noise at 1.0^{-4} m s^{-2}. (B) 2048 fast fourier transform (FFT) of the same AEP for the bamboo shark in response to a 100 Hz sound. The arrow indicates the frequency doubling peak, which occurs at 200 Hz. A positive detection is when the peak (at twice the frequency played) is at least 3 dB above the noise floor. The noise floor is estimated from the AEP power spectrum with a window of 100 Hz around the doubling frequency. (C) Pressure and particle velocity raw signals as recorded from the pressure/velocity probe. This example of particle velocity has been recorded in the *z*-axis. P, pressure; V, velocity.

A repeated-measures ANOVA (SigmaStat) was used to compare threshold measurements between *H. francisci* and *C. plagiosum* to determine if the two species had similar hearing thresholds at each frequency.

Auditory cranial mapping

The experimental setup for cranial mapping was exactly the same as with the hearing threshold measurements detailed above. Auditory evoked potentials were recorded only at the highest sound levels for 50, 100 and 200 Hz. To determine the area of the head of the shark that produces the strongest AEP, the dipole stimulus which was still suspended above the shark, was moved to specific locations around the shark. These locations included, (1) 5 cm in front of the anterior end of the shark, (2) directly over the anterior end of the shark, (3) 2.5 cm posterior of the anterior end, (4) between the shark's eyes, (5) directly over the endolymphatic ducts, (6) 2.5 cm posterior of the endolymphatic ducts, (7) 5 cm posterior of the endolymphatic ducts, (8) 2.5 cm lateral of the endolymphatic ducts, (9) 5 cm lateral of the endolymphatic ducts, (10) 10 cm lateral of the endolymphatic ducts and (11) at the tail (Fig. 3). As the stimulus was moved over each location the AEP was obtained at the three frequencies. The AEPs were transformed using a 2048-point FFT to determine their voltage level.

Field measurements of ambient noise particle acceleration

The geophone/hydrophone apparatus was attached to a ring stand, which was driven into the sediment in Bayboro Harbor outside of the University of South Florida, College of Marine Science, in 1.2 m deep water. This location was chosen since it is a relatively quiet location with little boat activity to provide a baseline for a 'quiet' environment that sharks have been observed to inhabit. Ten recordings of ambient noise were

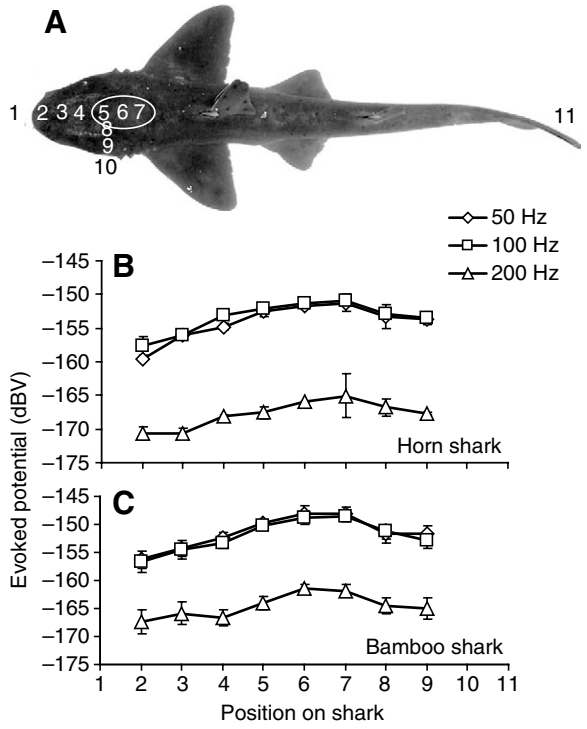

Fig. 3. (A) Overhead view of a horn shark depicting the different locations that were stimulated by the dipole stimulus. (1) 5 cm in front of the anterior end of the shark, (2) right over the anterior end of the shark, (3) 2.5 cm posterior of the anterior end, (4) between the shark's eyes, (5) directly over the endolymphatic ducts, (6) 2.5 cm posterior of the endolymphatic duct pores, (7) 5 cm posterior of the endolymphatic duct pores, (8) 2.5 cm lateral of the endolymphatic duct pores, (9) 5 cm lateral of the endolymphatic duct pores, (10) 10 cm lateral of the endolymphatic duct pores, and (11) at the tail. The oval surrounding locations 5, 6 and 7 indicates the areas that yielded the strongest evoked potential from the dipole stimulus. Positions 5 and 6 are the location of the parietal fossa. (B,C) Evoked potential levels (mean ± s.d.) recorded from the horn shark and bamboo shark, respectively, at each location for 50, 100 and 200 Hz. Note that the closer the level obtained (in dBV) was to 0, the stronger the evoked potential that was recorded. 200 Hz yielded a weaker evoked potential in both species than 50 and 100 Hz, as it is the upper range of hearing in these species. Position numbers correspond to those in A.

obtained for periods of 10 s at a sample rate of 50 kHz and were analyzed using a 2048-point FFT in MATLAB.

Results

Auditory evoked potential levels decreased with decreasing signal level and showed a doubling of the frequency of the test signal (Fig. 2). Hearing thresholds were determined for both species of sharks and are plotted as audiograms (Fig. 4). There was no significant inter-individual difference in the hearing threshold for both species or between the overall audiograms of the two species (P>0.05). Both species had their most sensitive hearing at 20 Hz with increasing thresholds as the

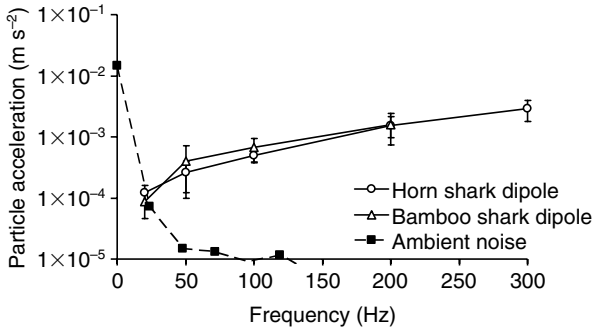

Fig. 4. Dipole audiograms of the horn shark *Heterodontus francisci* (N=3) and the white-spotted bamboo shark *Chiloscyllium plagiosum* (N=5). Values are means ± s.e.m. Ambient noise levels found in a quiet, tidal-dominated shallow harbor are plotted for comparison (broken line). Broad-band background noise in the test tank was consistently below 10^{-7} m s^{-2}.

frequency increased. The highest frequencies that could be detected were 200 Hz for *C. plagiosum* and 300 Hz for *H. francisci.* The ambient noise measurements measured in Bayboro Harbor were also plotted relative to these audiograms. Ambient noise levels were greatest at low frequencies and decreased with increasing frequency.

Evoked potentials were also recorded as the dipole stimulus was moved across the body of the shark. In both species of shark, the strongest response was obtained when the dipole was located 5 cm posterior to the endolymphatic pores followed by an almost equally strong response at 2.5 cm posterior to the endolymphatic pores (Fig. 3A). As the stimulus was moved to anterior, posterior and lateral locations the response diminished (Fig. 3B,C). No responses were obtained when the dipole was located in front of the shark, the side of the shark or the caudal fin.

Discussion

The sharks in this study were most sensitive to the lowest frequencies tested. The only other auditory thresholds obtained from a dipole stimulus in fishes were for the mottled sculpin *Cottus bairdi* and the goldfish *Carrassius auratus* (Coombs, 1994). The shark hearing thresholds measured in this study were lower than *C. bairdi* and *C. auratus* thresholds at frequencies below 200 Hz (Fig. 5). Above 200 Hz, *C. auratus* was more sensitive than the two shark species and *C. bairdi*. Dipole hearing data are particularly relevant as it has been suggested that a dipole stimulus more closely represents the type of stimulus that fishes with no hearing specializations (i.e. swim bladder/ear connections or auditory bullae), including elasmobranchs, would detect in the environment (Kalmijn, 1988; Myrberg, Jr, 2001; Bass and Clark, 2003). However, it should be noted that there are very few, pure monopole or dipole sound stimuli that exist in nature. Most sounds are inherently more complex and take the form of multipole stimuli.

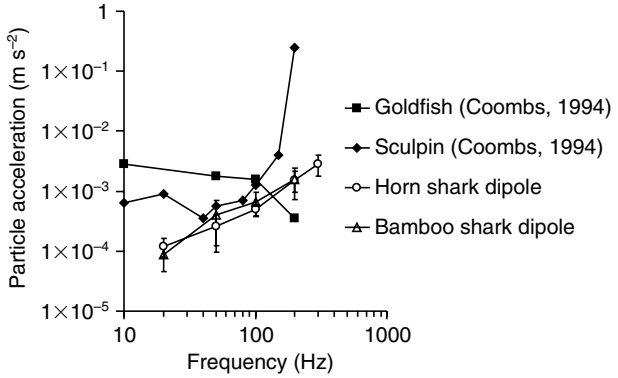

Fig. 5. Comparison of shark dipole particle acceleration audiograms (white symbols) with dipole audiograms from the goldfish *Carassius auratus* (black squares) and the mottled sculpin *Cottus bairdi* (black diamonds), which were obtained using classical conditioning (Coombs, 1994).

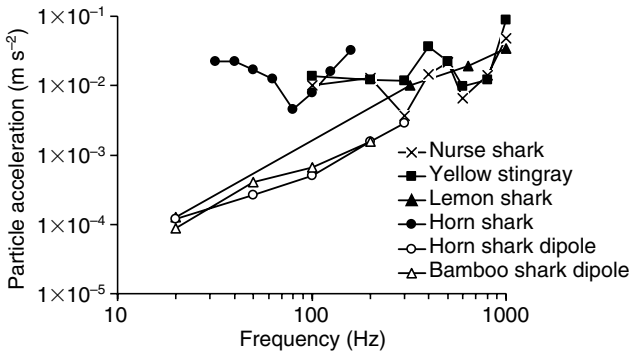

Fig. 6. Particle acceleration audiograms of all tested species of elasmobranchs. Values for the nurse shark *Ginglymostoma cirratum* and yellow stingray *Urobatis jamaicensis* (Casper and Mann, 2006), lemon shark *Negaprion brevirostris* (Banner, 1967) and horn shark *Heterodontus francisci* monopole (Kelly and Nelson, 1975) were modified to compare to the particle acceleration data obtained in the dipole experiment. These four species were all tested with a monopole sound stimulus. The nurse shark and yellow stingray audiograms were obtained with auditory evoked potentials in terms of particle acceleration. The lemon shark and horn shark were obtained using classical conditioning methods with measurements in terms of particle displacement, which was converted to particle accelerations in this figure.

These audiograms were compared with ambient noise levels that were recorded in a shallow (1.2 m), tidally dominated body of water (Fig. 4). The ambient noise levels are well below the hearing thresholds at all frequencies except for 20 Hz for both species of sharks. This low frequency 'noise' is probably associated with wind and wave action, which were low at the time of recording. It has been suggested that elasmobranchs might orient to biological noise sources in this frequency range, including sounds produced by a wounded fish as well as normal swimming motions of fishes, using both the ear and lateral line (Nelson and Gruber, 1963; Banner, 1968; Banner, 1972; Myrberg, 2001). The ambient noise measurements suggest that it is possible that some low frequency, biologically produced sounds could be masked by ambient noise levels (20 Hz) depending on their distance from the sharks and the intensity of the sounds being produced. This could be even more prevalent during periods of high wind, rain or anthropogenically generated noise such as boat traffic. Biological sounds at higher frequencies, such as calls from soniferous fishes (typically >100 Hz), would apparently not be masked by ambient noise levels under similar conditions, and could be important sound cues for piscivorous elasmobranchs searching for prey.

The dipole hearing thresholds of *H. francisci* and *C. plagiosum* are most similar to those of *N. brevirostris* (Banner, 1967) (Fig. 6). The thresholds at 20 Hz and the shape of their audiograms are very similar. However, as the only low frequencies tested for *N. brevirostris* were 20 Hz and 320 Hz, the shape of the audiogram is only estimated relative to the dipole audiograms based on these two points. The *H. francisci* dipole audiogram measured in this study differs from the *H. francisci* monopole audiogram (Kelly and Nelson, 1975) (Fig. 6). The monopole audiogram shows much higher thresholds relative to the dipole audiogram, which could, in part, be due to the ambient noise levels present during these experiments. The ambient noise power spectrum in the *H. francisci* monopole experiment was similar in level to the audiogram at low frequencies (<100 Hz) suggesting that thresholds may have been masked at those low frequencies. By contrast, the dipole experiments reported here were conducted in a sound dampening chamber with a low noise floor, well below the thresholds that were obtained.

Comparing audiograms collected with auditory evoked potentials, there is a large difference in thresholds of *G. cirratum* and *U. jamaicensis* measured with a monopole source (Casper and Mann, 2006) *versus* the sharks measured with the dipole source (Fig. 6). The most probable explanation for the differences between these [and possibly Kelly and Nelson's horn shark monopole experiment (Kelly and Nelson, 1975)], even though the same physiological methods were used, is the endorgans that were being stimulated in each experiment. Previous experiments have shown that the macula neglecta and the sacculus are the primary endorgans for acoustic detection in the elasmobranch ear, with some responses obtained from part of the utricle (Lowenstein and Roberts, 1951). The saccular macula has hair cell polarizations in the anterior–posterior as well as dorsal–ventral directions in two species of skates (Lowenstein et al., 1964; Barber and Emerson, 1980), and a three-dimensional arrangement in *N. brevirostris* (Corwin, 1981). The utricular macula has hair cells polarized primarily in the anterior–posterior directions with some hair cell polarizations in the dorsal–ventral directions (Lowenstein et al., 1964; Barber and Emerson, 1980). The macula neglecta is located in the posterior semicircular canal. It is connected by the fenestrae ovalis membrane to the parietal fossa, an area of the head composed of loose connective tissue. It has been suggested that the parietal fossa is the likely pathway for sound

travel directly to the macula neglecta endorgan, which has hair cells polarized in the dorsal–ventral direction (Lowenstein and Roberts, 1951; Tester et al., 1972; Fay et al., 1974; Corwin, 1977; Corwin, 1978; Corwin, 1981; Bullock and Corwin, 1979; Barber et al., 1985). The macula neglecta does not have mass-loading otoconia like the other endorgans that are sensitive to particle acceleration, and is more similar in design to the ampullae of the semi-circular canals or the lateral line organs having a cupulae overlying the hair cells. These organs are stimulated by fluid flowing across them causing a movement of the cupulae relative to the hair cells. The lateral line free neuromasts of *Xenopus laevis* have been shown to be sensitive to particle velocity and yield a flat particle velocity response from approximately 0.1–80 Hz (Kroese et al., 1978). If the macula neglecta is velocity sensitive, we hypothesize that it would show a similar particle veleocity threshold response regardless of a change in frequency. When the particle acceleration thresholds of the shark dipole experiments are converted to particle velocities [acceleration/(2π×frequency)] the data show a flat response with changing frequencies (Fig. 7). Furthermore, when examining the other existing elasmobranch audiograms (Fig. 6) there is typically a relatively flat response in terms of acceleration, supporting acceleration detection using the otoconia when using a monopole stimulus. It is important to note that this hypothesis assumes that the summed neural response measured by AEPs does not show frequency filtering that may be produced by higher levels of the auditory system.

Since the dipole was located much closer to the head and/or ear of the sharks compared to the monopole (1 m *versus* <15 cm), it is probable that the macula neglecta received a stronger effective stimulus from the dipole, since stimulation of the macula neglecta would require relative movement between the parietal fossa and the rest of the shark skull. With the monopole located at 1 m from the shark's head, the vertical particle motion is equivalent over all parts of the head, and thus would not generate a strong stimulus through the parietal fossa.

Previous work has suggested that the parietal fossa is one of the pathways of sound (Lowenstein and Roberts, 1951; Tester et al., 1972; Fay et al., 1974; Corwin, 1977; Bullock and Corwin, 1979; Corwin, 1981). Two experiments found that placing a lead weight over the parietal fossa of a lemon shark reduced the acoustic-evoked activity in response to a speaker playing directed sounds over the head (Bullock and Corwin, 1979; Corwin, 1981). Another experiment (Fay et al., 1974) stimulated the surface of the head of a shark directly with a vibrating pole and found that the region of the parietal fossa yielded stronger voltage potentials from the macula neglecta than any of the surrounding areas of the head. In this study with *H. francisci* and *C. plagiosum*, the strongest evoked potentials were recorded when the dipole stimulus was located in the region above the parietal fossa and just posterior to the parietal fossa (Fig. 3). As the stimulus was moved away from this region the evoked potential voltage decreased, adding further evidence that the parietal fossa is a probable pathway for sound travel with a local stimulus.

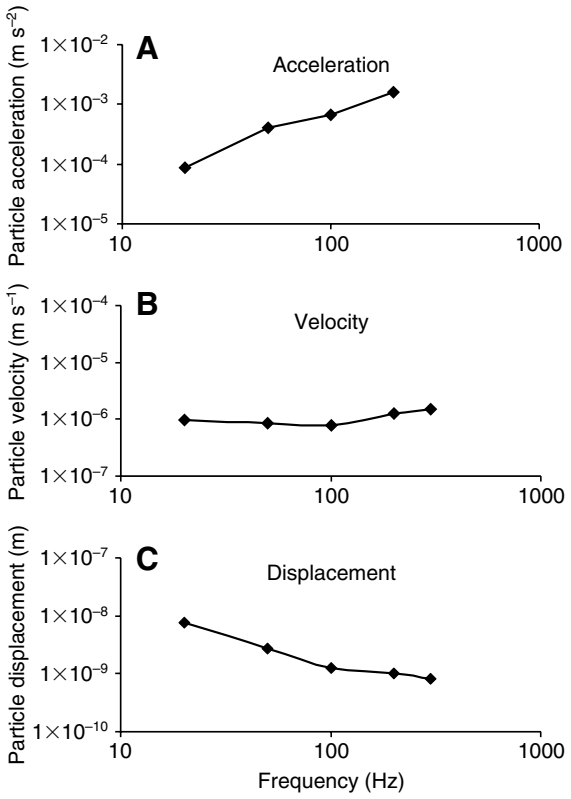

Fig. 7. Bamboo shark audiogram plotted in terms of (A) acceleration, (B) velocity and (C) displacement. These results support the proposal that the macula neglecta is a velocity detector, as there is a substantially flat response in terms of particle velocity irrespective of the change in frequency (B). For a velocity sensitive organ, if the thresholds are plotted in terms of acceleration (A) there is an increase in threshold with increase in frequency (approximately 6 dB per octave) and a decrease in threshold with increase in frequency when expressed in terms of particle displacement (C).

This dipole hearing experiment has provided the first audiograms obtained using a dipole stimulus for any elasmobranch. This is important as a dipole stimulus more closely represents biological sounds that fishes detect. Further evidence has also been provided suggesting that the parietal fossa region is a probable pathway for sound travel in elasmobranchs. If elasmobranchs orient to dipole stimuli, then they would probably be limited to near-field acoustic detection. This would severely limit the ability of elasmobranchs to track prey based on far-field acoustic stimuli.

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