THE INNER EAR MORPHOLOGY AND HEARING ABILITIES OF TWAITE SHAD AND A SELECTION OF MARINE AND FRESHWATER FISH

J.M. Lovell[1,2] [1]Faculty of Science, [2]Plymouth Electron Microscopy Centre, University of Plymouth, Drake Circus, Plymouth PL4 8AA j.lovell@plymouth.ac.uk

1 INTRODUCTION

The twaite shad (*Alosa fallax* Lacepede), is an anadromous member of the herring family Clupeidae, and is listed in Appendix II of the Bern Convention and Annex IVa of the EC Habitats Directive. Due to these listings, anthropogenic activities (e.g. civil construction) that have the potential to pollute habitats exploited by endangered species such as *A. fallax* need to be assessed and the impact on marine life determined so mitigation controls can be put in place. For the appropriate information to be available for use in determining potential acoustic impact zones etc, an understanding of the audiological capabilities of the effected species is required. It is well known that the clupeids have specialist hearing abilities and detect a wider range of acoustic frequencies compared to less sensitive hearing generalists such as the salmon (*Salmo salar*), which possesses narrow frequency detection capabilities (Hawkins & Johnstone,1978). Other hearing specialists include the cyprinids (carps and catfishes); these fish possess a direct mechanical coupling formed from explicitly adapted anterior vertebrae that link the swim bladder to the inner ear, known as Weberian ossicles (Watson, 1939). For the clupeids to acquire maximum sensitivity to sound pressure, auditory specialisation has resulted in the adaptation of the swim bladder to enter the skull by means of cranial openings and terminating in two bulbs connected to the auditory apparatus (Blaxter, Denton & Gray, 1981). It is known that the directional response of the fish ear is a function of hair cell polarity and the orientation of the epithelium in space (Fay & Edds-Walton, 1997; Lu & Popper, 2001; Edds-Walton & Fay, 2002), allowing for fishes to locate the source of a sound in both the horizontal and vertical planes. Azimuths of peak sensitivity lie parallel to the plane of the otolith and sensory epithelium (Enger *et al*., 1973; Sand & Hawkins, 1973; Fay & Edds-Walton, 1997); excitation occurs when stereocilia are bent toward the kinocilium during the passage of a wave front, resulting in the cell becoming depolarized relative to its resting potential (Farris *et al*., 2006). Inhibition occurs when the bundle is deflected in the opposite direction, resulting in the hyperpolarization of the cell (Flock & Duvall, 1965). The ability to detect and accurately localise the source of a sound is of considerable biological importance to all fish species, especially during predator prey interactions and for navigation purposes (Myrberg, 1981). In order to demonstrate differences in hearing ability between specialist and generalist fish species, auditory sensitivity is tested using the Auditory Brainstem Response (ABR) approach in fish from the orders Atheriniformes, Clupeiformes and Salmoniformes, and the results presented along with polarisation patterns from the saccular epithelium in fish from the orders Clupeiform, Salmoniform and Perciform.

2 MATERIALS AND METHODS

2.1 PREPARATION OF THE SACCULE PRIOR TO SEM EXAMINATION AND AEP METHODOLOGY

In all histological preparations, the fish were dispatched using the conventional protocol approved by the Marine Biological Association of the United Kingdom, or by the University of Illinois Institutional Animal Care Committee (IACUC) 15.11.04 (protocol # 04271). 3 Specimens of paddlefish (*Polyodon spathula*), 6 bass (*Dicentrarchus labrax*), 12 brown trout (*Salmo trutta*) and one twaite shad (*Alosa fallax*) were anesthetised using MS-222 in a water bath and dispatched. The relatively high numbers of *D. labrax* and *S. trutta* specimens are owing to these fish being used in a

concurrent experiment not described here. Figure 1 shows the position of the ear and brain along with other main internal structures and organs found in most bony fish. The brain and inner ear have been removed for processing (see Lovell *et al*., 2005 for results).

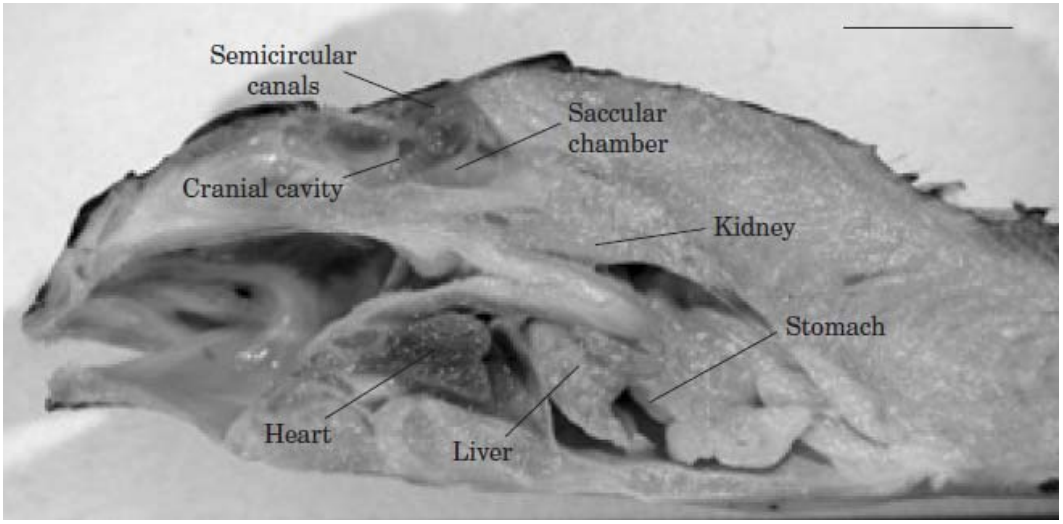

Figure 1. Cross-sectionthrough a 97mm FL Perciform *Taurulus bubalis*, showing the internal organs and cranial cavity (the brain, saccule and lagena of the inner ear have been removed from the specimen). Bar = 10 mm. (from Lovell *et al*., 2005).

The cranium containing the inner ears from *P. spathula*, *D. labrax, S. trutta* and *A. fallax* were trimmed to small blocks and immersed in chilled fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer with 3.5% sodium chloride), and delivered to the Plymouth EM unit within 72 h post removal. The Scanning Electron Microscope (SEM) preparation methodology employed in this study was based on techniques used by Lovell *et al*. (2005 b). The processed SEM specimens were investigated and photographed using a JEOL JSM 5600 scanning electron microscope, operated at 15 kV and a 15 mm working distance. The Transmission Electron Microscope (TEM) preparation methodology employed in this study was based on techniques used by Lovell *et al*. (2005a). Fully processed images were taken using a JEOL 1200 EX II Transmission Electron Microscope, and the images captured using an SIS Mega view III.

In order to concisely identify the frequency and intensity of sounds audible to hearing specialist and generalist fish species, 7 specimens of *A. fallax*, of mixed sex and ranging in size from 330 mm (fork length) (350 g) to 420 mm (570 g), 12 specimens of sand smelt (*Atherina presbyter*) with mean lengths of 65 mm (9.7 g), and 4 specimens of Atlantic salmon (*Salmo salar*) with mean lengths of 150 mm (no mass taken) were stimulated with sounds ranging in the frequency domain between 30 Hz to 2000 Hz (ultrasonic frequencies were not tested). Similar water temperatures were maintained in both the holding tanks and test tank, and when not under experimental protocols the fish were kept in natural daylight but shaded from direct sunlight. The procedure used to acquire the evoked potentials was approved by the United Kingdom Home Office. AEP measurements were taken in the electro-physiological far field using two cutaneous electrodes placed firmly against the skin above the ear and medulla and connected to a differential amplifier by 1 m lengths of screened coaxial cable with an external diameter of 1.5 mm. The development of the shielded electrodes allows for recordings to be made outside of the usual controlled laboratory conditions required for ABR type electrophysiology, benefiting the portability of the system and allowing for measurements to be made in challenging environments such as an aquaculture facility or research vessel (see Lovell *et al*., 2005 b. for more detail on the electrodes and protocols used). The fish were placed in a 0.5 mm gauge plastic or wire mesh cradle lined with “carp sacking” and the mesh adjusted so the cradle did not actually contact the fish, but prevented gross movement of the head and tail. A clip was fastened to the top of the cradle and a retort stand clamp fitted with ball joint electrode manipulator arms and the aerated water supply pipe was attached. A reservoir of oxygenated water was positioned 1 m above the experimental tank and water was gravity fed at an

adjustable flow rate of between 5 to 30 millilitres per second (dependant on fish size and species) and directed toward the gills through a soft rubber mouth tube with a diameter of 4 to 12 mm. The fish was stationed 500 mm below the water surface in a heavy gauge steel tank with a volume of 1 m^3 and filled with water from the holding tanks. In each of the audiological tests, the stimulus sound was presented using two submerged transducers generating a phase dependant region between them dominated by either sound pressure or particle velocity. The projectors were driven directly from an audio amplifier, and the stimulus tones (presented to the fish in alternate phase) were measured using a Bruel and Kjaer Type 8104 calibrated Hydrophone, and the signal amplified using a Bruel and Kjaer Type 2365 Charge Amplifier.

3 RESULTS

3.1 ELECTRON MICROSCOPY

The TEM section in Figure 2.a shows a crosswise section through the afferent hair cell from the saccular macula from, *P. spathula* buttressed by supporting cells. The anterior kinocilium and shorter stereocilia can be seen anchored to the cuticular plate at the top of the cell. The cell body has a mean length and width of 23 µm and 9.3 µm respectively, and contains a single nucleus 6 µm in diameter. Figure 2.b presents a hair cell from the saccule of *D. labrax*; the mean cell body dimensions are 11.2 µm in length, 4.7 µm in width and a nucleus 2.3 µm in diameter. As can be seen by comparing the scale bar units, the Acipenseriform hair cells are larger than the Perciform by a factor of two.

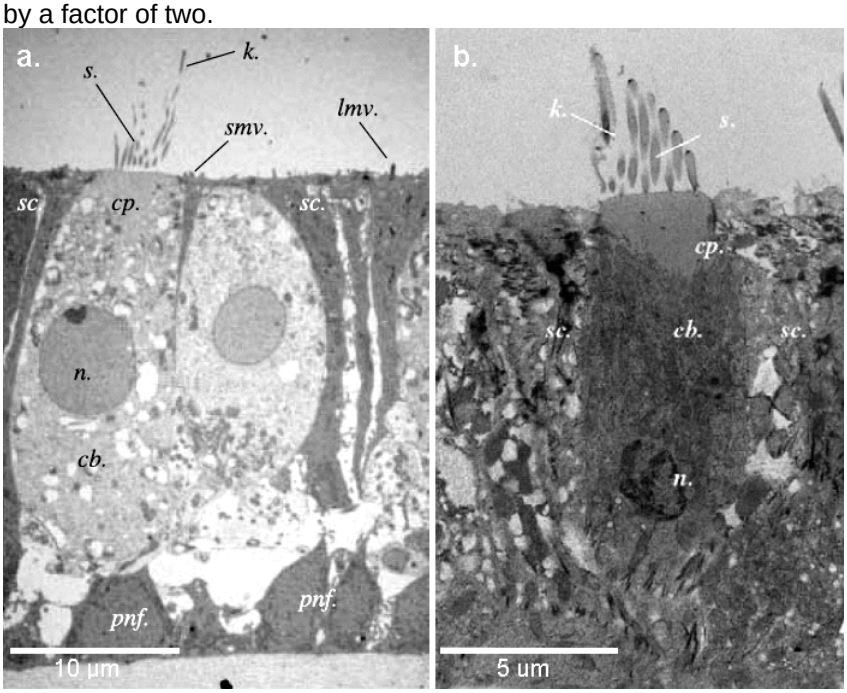

Figure 2.a TEM cross-section through the saccular hair cells and innervating nerve fibres from *P. spathula*. (**b**) saccular hair cell from *D. labrax*. *cb,* cell body; *cp,* cuticular plate; k, kinocilia; *lmv*, long microvilli; *n*, nucleus; *pnf*, peripheral nerve fibres; *sc*, support cells; *s*, stereocilia.

The view of the saccular macula from the left ear of *D. labrax* (Figure 3.a) has been annotated to show the overall orientation of the hair cells found in each quadrant, as viewed perpendicular to the macula surface. The polarisation of the ciliary bundles is depicted by the white arrows, and reveals that *D. labrax* possesses a standard orientation pattern in common with other hearing generalist fish such as *S. trutta* (Figure 3.b). As can be seen in Figure 3.c, the hair cells on the saccular macula in *A. falax* are also divided into four oppositely oriented groups rather than the usual two found in the saccule of specialist ostariophysian fish. It should be noted that the usual tadpole shape is not

present in the saccule of *A. fallax*. The arrows marked D and R (dorsal and rostral) represent the orientation of the macula with respect to the fish and apply to each of the Figures.

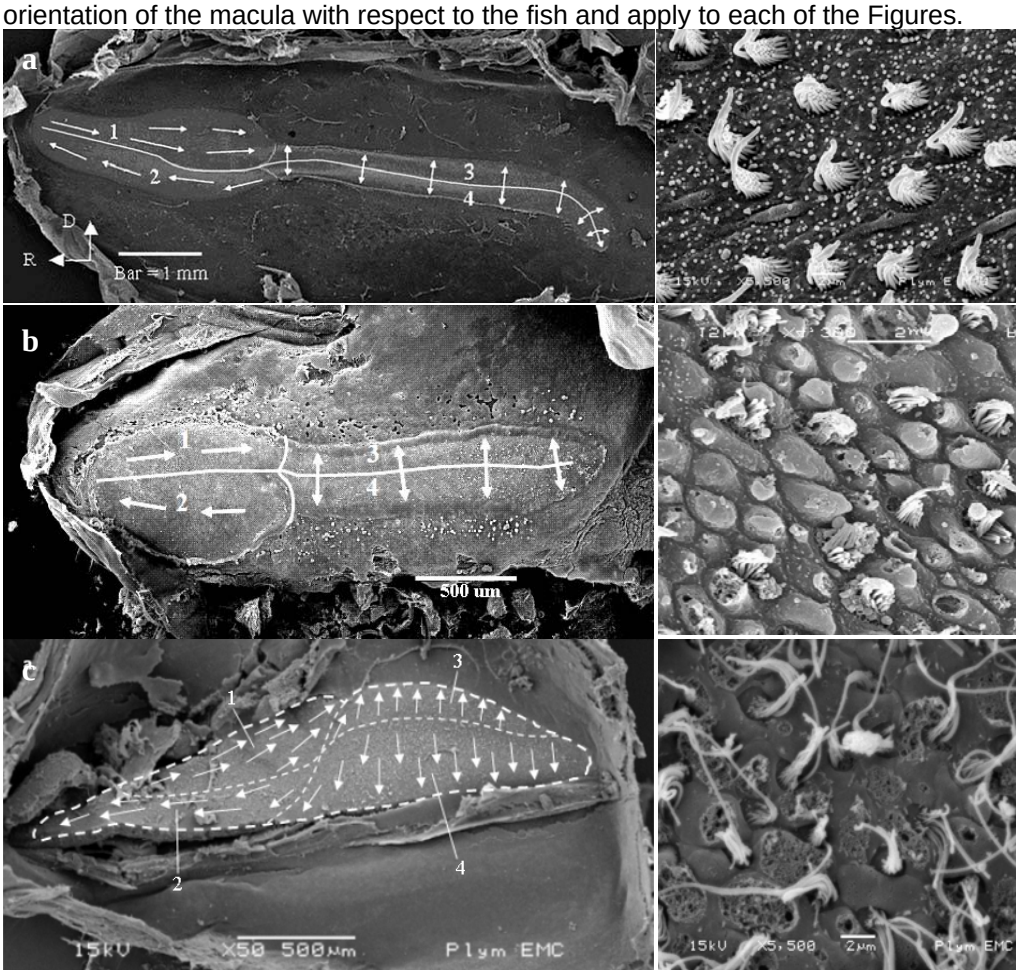

Figure 3.a. Orientation of ciliary bundles on the left ear saccular epithelium of *Dicentrarchus labrax*, orientated horizontally (1 and 2), and vertically (3 and 4) in diametrically opposed quadrants; **b.** Orientation of hair cells on the saccule from the brown trout (*Salmo trutta*) showing that both *D. labrax* and *S. trutta* have standard orientation ciliary bundles in common with many hearing generalists **c.** The macula of the saccule from *A. falax*, also divided into four quadrants. The images in the right column show hair cells from each corresponding macula.

3.2 ELECTROPHYSIOLOGY

Figure 4 illustrates auditory evoked potentials from *A. fallax* in response to tone bursts at frequencies of 50, 75, 100, 250, 500, 750, 1000, 1500 and 2000 Hz. Each of the waveform sets were recorded from stepped amplitudes from a particular frequency and overlaid, revealing a latency change in response to the attenuation of the sound. Above threshold AEP waveforms in both Figures are presented with a blue colour coding, whilst below threshold recordings are red. The onset latency of the centre or largest sinusoid of the ABR response varied with frequency, ranging from 20 ms after stimulus onset at 50 Hz to 1 ms at 500 Hz. The stimulus sound was attenuated in steps of between 10, 5 and 2 dB steps until no response could be measured.

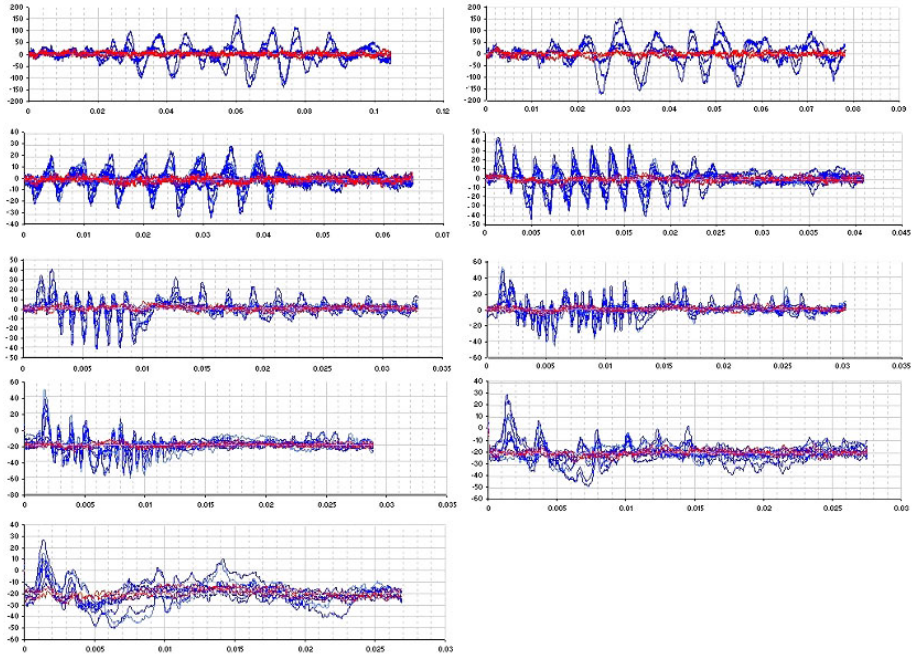

Figure 4. Overlaid auditory evoked potentials from *A. fallax* in response to tone bursts at frequencies of 50, 75, 100, 250, 500, 750, 1000, 1500 and 2000 Hz

When two replicate waveforms showed dissimilar polarities (the ABR trace at each intensity of a tone burst is repeated to ensure consistency), the response was considered as being below threshold (cf. Kenyon *et al*., 1998). All threshold responses were measured in this way and each audiogram was produced using sequential AEP waveform data, with stimulus intensity successively reduced in steps of between 4 dB to 0.5 dB at threshold.

Figure 4 presents sequentially arranged AEP waveforms (and replicate) in response to a 500 Hz tone burst presented in alternating phase. The AEP waveforms evoked by the tone bursts typically consisted of a series of eight rapid negative peaks that follow a DC potential (similar to the mammalian summating potential) upon which the neural or receptor potentials are superimposed. This presents as a negative DC shift in Figure 5, typically lasting as long as the stimulus duration.

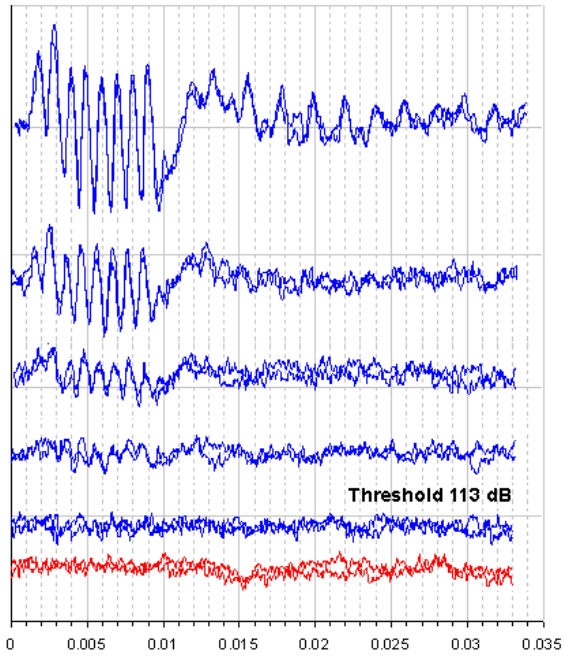

Figure 5. Auditory evoked potentials from *A. fallax* in response to a 500 Hz tone burst attenuated in 10, 5 and 2 dB steps (threshold at 113 dB re. 1 µPa).

The individual audiograms acquired from the population of *A. fallax* were combined to create the mean audiogram presented in Figure 6 (open squares). This Figure also presents the average audiogram for the generalist sand smelt (*Atherina presbyter*) in response to tone bursts from 100 Hz to 1000 Hz with open diamonds designating thresholds obtained in a sound field dominated by sound pressure and the closed diamonds designating particle velocity. The lowest thresholds from *A. presbyter* were obtained from a 200 Hz tone burst presented at 109 dB (re. 1 µPa) (pressure dominated sound field) and 98 dB (re. 1 µPa) (particle Velocity dominated sound field). The audiogram for *S. salar* generated with the transducers operated in phase and generating a region between them with high particle velocity resembles (within 10 dB at 100 Hz) the behavioural audiogram by Hawkins and Johnstone, (1978), which is also presented in Figure 6 (closed red triangles).

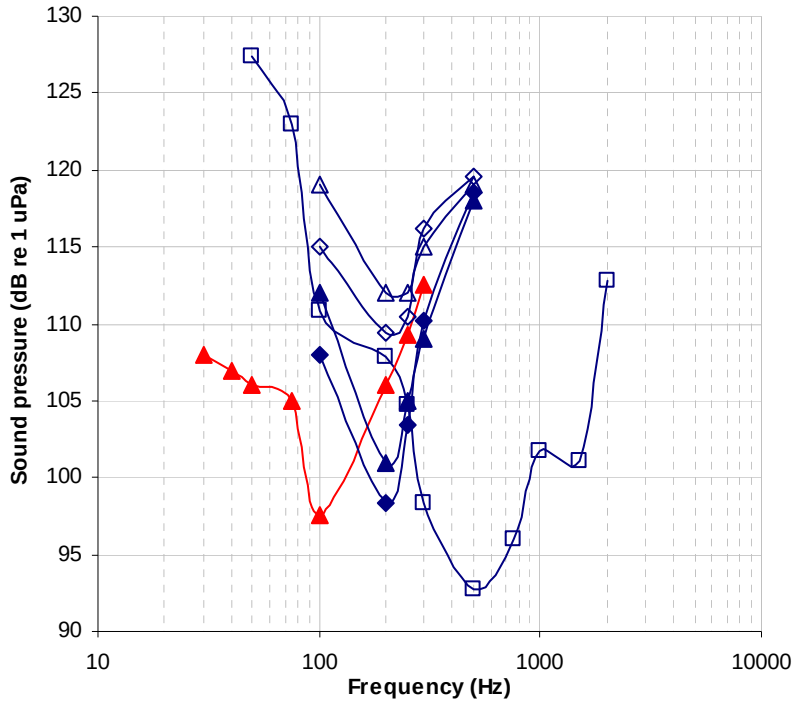

Figure 6. Mean audiograms for *A. fallax* in response to pressure (open squares), *A. presbyter* in response to pressure (open diamonds) and particle velocity (closed diamonds), audiogram for *S. salar* in response to sound pressure (open triangles), particle velocity (closed triangles) and the behavioural *S. salar* audiogram from Hawkins and Johnstone (1978) (red closed triangles).

4 DISCUSSION

4.1 ELECTRON MICROSCOPY

Morphological information is of benefit in the study of aquatic animal hearing for environmental monitoring purposes as SEM data can be used to confirm if raised hearing thresholds (determined using electrophysiological or behavioural techniques), are a direct result of damage to the inner ear ultrastructure. The internal structure of the hair cells from each of the inner ear end organ from the cartilaginous *P. spathula* are similar to the hair cells found in the vestibular organs of many higher vertebrates; however, the dimensions of the hair cells are almost double those found in the end organs of teleost fish, which average out at around 13 µm in length, 6 µm in width, with a nucleus 3.5 µm in diameter (calculated using data from Saidel *et al*., 1995; Lovell *et al*., 2005 b). According to Lovell *et al*., 2005 b), the size of the cell has little influence on hearing thresholds, as the audiogram for *P. spathula* is not unlike many other hearing generalists, in both sensitivity and audible frequency range.

The polarisation of hair cells on the macula of the saccule from *D. labrax* and *S. trutta* both follow the standard orientation pattern defined by Platt & Popper (1981), where the macula is divided into two anterior (ostium) and two posterior (cauda) sections. Hair cells are orientated rostrocaudally in the ostium and dorsoventrally in the cauda divisions. As Figure 3.c shows, the hair cells on the saccular macula from the hearing specialist *A. falax* are also divided into four oppositely oriented quadrants, rather than the two found in the saccule of the specialist ostariophysan fish (Popper & Platt, 1983). The overall tadpole like shape of the macula is similar in both *D. labrax* and *S. trutta*,

and the ostio-caudal differentiation is easily observed in these fish; however, in *A. fallax*, it is considerably less obvious.

4.2 ELECTROPHYSIOLOGY

The AEP generated audiogram for the hearing specialist *A. fallax* closely follows the behavioural audiogram for the American shad (*A. sapidissima*) by Mann *et al*. (1997), established using a classical conditioning technique to determine hearing thresholds. The mean audiogram for *A. fallax* was 1.3 dB (re. 1 µPa) above the behavioural thresholds from *A. sapidissima* and 4.5 dB (re. 1 µPa) higher (at 500 Hz) than ABR thresholds from the spotlined sardine (*S. melanostictus*) by Akamatsu *et al*. (2003). The lowest hearing threshold from *A. fallax* was 113.8 dB (re. 1 µPa), evoked by a 500 Hz tone burst. Fish in the family Alosinae (shads) are able to detect ultrasound up to 180 kHz (Mann *et al*., 2001), though frequencies above 2000 Hz were not tested on *A. fallax*.

According to Hawkins and Johnstone (1978), *S. salar* has generalist hearing abilities and only responds to low frequency tones (below 380 Hz), with particle velocity rather than sound pressure proving the relevant stimulus. This is in agreement with the results of this study, though ABR thresholds at 100 Hz are around 15 dB higher than published behavioural thresholds. In normally hearing humans, Gorga *et al*. (1988) found that ABR thresholds were higher than behavioural thresholds for all frequencies tested, especially at the lower frequencies where intersubject variability was greatest (again in agreement with this study). Similar results to *S. salar* were obtained from *A. presbyter* in both the frequency and sensitivity domains, thus revealing that this fish also possesses generalist hearing abilities.

In order to suppress any transients generated by loud tone bursts (especially when testing generalist fish hearing), the ABR traces were recorded and averaged in response to alternating phase tone bursts, in line with the majority of ABR type audiological tests of fish hearing (cf. Kenyon *et al*., 1998 and Horodysky *et al*., 2008). In many ABR tests, the resultant AEP waveform has twice the number of sinusoidal peaks than the stimulus sound; a phenomenon that is reasonably consistent and with little variation across all fish orders tested using the ABR technique. Figure 7 shows 5 separate pairs of waveforms overlaid in the AEP response; each potential is associated with the 500 Hz tone burst (lower waveforms) presented in alternating phase and at decreasing stimulus intensities to threshold.

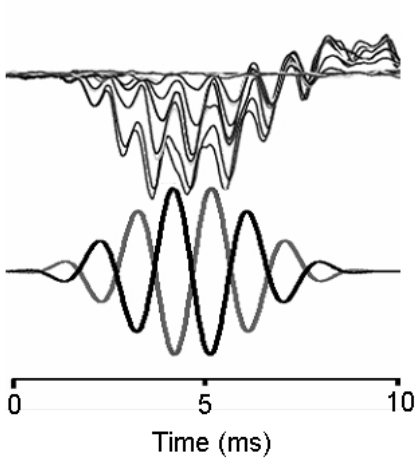

Figure 7. Recording of the 500 Hz tone burst showing the position of each sinusoid when using alternating phase tone bursts, and above, the AEP response from *A. fallax* showing a corresponding doubling in response frequency. Note: the position of the tone burst has been moved forward by approximately 2 ms to line up with the AEP response.

Furukawa and Ishii (1967) report that in *C. auratus*, the frequency doubling phenomenon from a single phase continuous tonal signal could be as a result of the polarisation/hyperpolarisation of hair

cells from opposing regions of macula, orientated along the sound propagation axis. Another possible reason for AEP frequency doubling is asymmetrical Frequency Following Responses (FFRs) or non-neural asymmetrical receptor potentials, similar to mammalian cochlear microphonics (CMs). Complete phase cancellation is not achievable due to the asymmetrical response; however, in this work, the doubling of the AEP is unlikely to be rectification because the phenomenon is not present when stimulating with a single phase tone burst. It is clear, when the alternating phase tone bursts are presented together (lower waveforms in Figure 7), that the apparent mismatch between the number of cycles in the stimulus sound and the AEP response actually correlates well with the position of the stimulus sinusoids when presented in alternating (flip) phase. It is therefore more likely that neural activation occurs only on the rarefaction phase of the stimulus transmission and is analogous to the response of the mammalian auditory system to opposite phase tone bursts (cf. Krishnan, 2007). Thus, the sum of the AEP waveforms will show an apparent doubling, though this is simply the superposition of the two response waveforms where activity from the alternate phase tone burst will shift by 180 degrees from the driving phase.

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