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RESEARCH ARTICLE

Auditory evoked potentials of the plainfin midshipman fish (*Porichthys notatus*): implications for directional hearing

Andrew D. Brown[1,2,*], Ruiyu Zeng[3] and Joseph A. Sisneros[2,3,4]

ABSTRACT

The plainfin midshipman (*Porichthys notatus*) is an acoustically communicative teleost fish. Here, we evaluated auditory evoked potentials (AEPs) in reproductive female midshipman exposed to tones at or near dominant frequencies of the male midshipman advertisement call. An initial series of experiments characterized AEPs at behaviorally relevant suprathreshold sound levels (130–140 dB SPL re. 1 µPa). AEPs decreased in magnitude with increasing stimulus frequency and featured a stereotyped component at twice the stimulus frequency. Recording electrode position was varied systematically and found to affect AEP magnitude and phase characteristics. Later experiments employed stimuli of a single frequency to evaluate contributions of the saccule to the AEP, with particular attention to the effects of sound source azimuth on AEP amplitude. Unilateral excision of saccular otoliths (sagittae) decreased AEP amplitude; unexpectedly, decreases differed for right versus left otolith excision. A final set of experiments manipulated the sound pressure-responsive swim bladder. Swim bladder excision further reduced the magnitude of AEP responses, effectively eliminating responses at the standard test intensity (130 dB SPL) in some animals. Higher-intensity stimulation yielded response minima at forward azimuths ipsilateral to the excised sagitta, but average cross-azimuth modulation generally remained slight. Collectively, the data underscore that electrode position is an essential variable to control in fish AEP studies and suggest that in female midshipman: (1) the saccule contributes to the AEP, but its directionality as indexed by the AEP is limited, (2) a left–right auditory asymmetry may exist and (3) the swim bladder provides gain in auditory sensitivity that may be important for advertisement call detection and phonotaxis.

KEY WORDS: Fish hearing, Acoustic communication, Swim bladder, Sound localization

INTRODUCTION

The plainfin midshipman (*Porichthys notatus*) is a marine teleost fish studied extensively for its unique bioacoustic ecology (reviewed in Bass and McKibben, 2003; Sisneros, 2009a). During the late spring–summer breeding season, dominant 'type I' male midshipman excavate nests under rocks in the intertidal zone along protected shorelines of the North American Pacific coast (e.g. DeMartini, 1988). From these nests, males emit multiharmonic advertisement calls by contracting hypertrophied sonic muscles attached to the swim bladder at a rate of approximately 80–100 Hz (Bass et al., 1999). The propagated 'hum' attracts gravid female midshipman from surrounding waters, which then enter the nests to spawn. The positive phonotaxic response of gravid females (not observed in type I males) is so robust that it can be elicited in a laboratory tank via loudspeaker playback of an 80–100 Hz pure tone (McKibben and Bass, 1998), and midshipman have served as a model in several recent studies concerned with sound source localization by fishes (Zeddies et al., 2010, 2012; Coffin et al., 2014).

A number of auditory specializations work to augment hearing sensitivity in female midshipman. Seasonal increases in steroid hormones during the spring–summer breeding season are associated with improved frequency coding in primary auditory saccular afferents (Sisneros et al., 2004) and lower evoked potential thresholds of hair cells in the saccule, the primary auditory end organ of midshipman (Sisneros, 2009b). These changes are accompanied by an increase in auditory hair cell number within the saccular sensory macula (Coffin et al., 2012). Additionally, the swim bladder of the female midshipman exhibits rostral extensions ('horns') that terminate near the otic capsule (Bass and Marchaterre, 1989; Mohr et al., 2017). Such extensions, also present in certain other teleost species, are hypothesized to impart sound pressure sensitivity via mechanical coupling of the swim bladder and inner ear, akin to that provided by Weberian ossicles in otophysan species (e.g. Coombs and Popper, 1979; Ramcharitar et al., 2006), which is supported by recent saccular potential measurements (Colleye et al. 2019). We note that 'type II' male midshipman, an alternative male morph not considered further in the present report, also possess rostral swim bladder extensions and exhibit seasonally enhanced auditory sensitivity (see Mohr et al., 2017; Bhandiwad et al., 2017).

While hearing in the midshipman has been studied extensively, the mechanistic basis of sound source localization in midshipman and in teleosts more generally remains poorly understood (reviewed in Hawkins and Popper, 2018). In several teleost species including midshipman, the saccular otoliths (sagittae) are oriented oblique to the rostrocaudal midline, with their long axes angled approximately ±40 deg in azimuth (cf. Fay and Edds-Walton, 1997; Lu et al., 1998). These orientations are predicted to create two broadly tuned channels of 'left' (−40 deg) and 'right' (+40 deg) sensitivity, as the effective stimulus to an otolith and associated sensory macula should be maximal when the incident particle motion vector is parallel to its long axis (Rogers et al., 1988; Lychakov and Rebane, 2005). In support of this prediction, spatial receptive fields (cross-azimuth response functions) of auditory afferents of the oyster toadfish (*Opsanus tau*) (Fay and Edds-Walton, 1997) and sleeper goby (*Dormitator latifrons*) (Lu et al., 1998) have been shown to cluster around the ±40 deg azimuthal axes of the sagittae. Additional data suggest 'sharpening' of directional tuning in downstream neurons (in *O. tau*; Edds-Walton

[1]Department of Speech and Hearing Sciences, University of Washington, Seattle, WA 98105, USA. [2]Virginia Merrill Bloedel Hearing Research Center, University of Washington, Seattle, WA 98195, USA. [3]Department of Psychology, University of Washington, Seattle, WA 98195, USA. [4]Department of Biology, University of Washington, Seattle, WA 98195, USA.

*Author for correspondence (andrewdb@uw.edu)

A.D.B., 0000-0003-0838-2894

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and Fay, 2003). Bilateral convergence of left- and right-ear inputs, suggestive of binaural interaction as in other vertebrate taxa (reviewed in Grothe et al., 2010), may also occur (in *O. tau*; Edds-Walton and Fay, 2009), though the form or functional consequence of such interaction remains unclear.

In the sleeper goby, measurements of auditory evoked potential (AEP) thresholds by Lu and Xu (2002) indicated relatively homogeneous responses across source azimuth. However, removal of the right sagitta, ostensibly eliminating input from the right saccular macula (the effect of left sagitta removal was not evaluated), was found to cause a significant increase in AEP thresholds, i.e. decreased sensitivity, for rightward azimuths (+30 and +60 deg). This result was qualitatively consistent with the bias predicted from sagittal orientation (Lu et al., 1998; Lu and Xu, 2002), and is notable in that it suggests that gross hemispheric bias in spatial sensitivity is evident even in ensemble responses measured through the skull. While the AEP technique has been widely applied to measure auditory sensitivity (audiograms) in fishes (Kenyon et al., 1998; Ladich and Fay, 2013), and also to assay effects of certain peripheral manipulations including surgical deflation of the swim bladder (e.g. Yan et al., 2000; Yan, 2004), the investigation of Lu and Xu (2002) is the only study we are aware of that has exploited the method to study directional hearing. No AEP measurements have been reported in midshipman, to our knowledge.

Here, we report data from a series of experiments in female midshipman employing the AEP technique. Midshipman audiograms have been previously characterized using other physiological methods (e.g. Sisneros and Bass, 2005; Sisneros, 2009a,b); our goal was to characterize AEP responses at suprathreshold sound levels approximating those observed near nesting sites of vocalizing type I males. Initial experiments focused on characterization of midshipman AEPs at frequencies similar to the typical first and second harmonics of the male advertisement call and across recording electrode location, a variable that has been inconsistently addressed in AEP studies (see Lu and Xu, 2002; Hill, 2005; Maruska and Sisneros, 2016). Subsequent experiments evaluated AEPs following several manipulations. In one series of experiments, fish orientation was adjusted with respect to a static particle motion vector (1) with all end organs intact or (2) after unilateral sagitta excision. In a later series of experiments, directional measurements were completed after both unilateral sagitta and swim bladder excision. While the swim bladder was once believed to function as an omnidirectional receiver that might preclude localization (von Frisch and Dijkgraaf, 1935), empirical measurements (Barimo and Fine, 1998) suggest that the swim bladder can also exhibit intrinsic directionality (in *O. tau*), and behavioral measurements indicate that it is important for sound source localization in midshipman (Coffin et al., 2014).

MATERIALS AND METHODS

Ethics statement

All animals were collected under a Washington State Scientific Collection Permit issued annually by the Washington State Department of Fish and Wildlife. All experimental procedures complied with a protocol approved by the University of Washington Institutional Animal Care and Use Committee.

Animals

Adult plainfin midshipman fish, *Porichthys notatus* Girard 1854, were collected by hand during low tide from the rocky shoreline of northeastern Hood Canal, WA, USA. Fish were placed in temporary holding tanks filled with aerated seawater obtained at the collection site and transported to Friday Harbor Laboratories on San Juan Island, WA, USA. Fish were transferred to long-term indoor and outdoor holding tanks filled with seawater generally ranging from 12 to 15°C as maintained by a gravity-fed flow-through system. All fish used for the reported measurements (n=46) were females (periodic measurements used for system setup, troubleshooting and verification included a mixture of female, type I male and type II male fish). Standard length varied from 131 to 201 mm (mean±s.d. 165±16 mm); body mass varied from 24.37 to 77.00 g (mean±s.d. 47.74±13.32 g). At the conclusion of each experiment, animals were euthanized via overdose of benzocaine sulfate, and the gonadosomatic index (GSI) was quantified. GSI ranged from 2.56 to 28.96 (mean±s.d. 13.48±8.01). All females were collected from nesting sites in the intertidal zone and were considered to be reproductively active.

Experimental apparatus

Testing was conducted in a custom-built PVC tank enclosure. The enclosure was designed with the intent to (1) create a simple sound field within which it would be possible to control the orientation of test animals relative to a reproducible particle motion vector at selected frequencies and (2) enable stereo control of the sound field (the second application is not addressed further in the present study). The shell of the tank was a PVC tee junction with 20.3 cm internal diameter and 8 mm wall thickness. Matching endcaps were affixed to the lateral terminations of the tee, for a total lengthwise dimension of 73 cm. A hole was cut in the center of each endcap and UW-30 loudspeakers (Electro-Voice, Fairport, NY, USA) were mounted inside. A wooden stand held the open-ended central compartment of the tee upright. This stand was mounted on a custom pneumatic floor stand. At the center of the tank, an animal holder was suspended on pivoting nylon screws: the holder was constructed from three concentric plastic rings with weakly elastic mesh stretched across the innermost ring, enabling tri-axial positioning of test animals (Fig. 1A; see below). The horizontal compartment of the tank was filled with seawater. An aquarium pump was used to perfuse aerated seawater over the gills of test animals during testing. The pump was located outside the tank (connected to a DC power supply that was itself placed under a Faraday cage) and circulated aerated water via the top of the PVC tee to the animal via silicone tubing. Water temperature was maintained at 15±1°C (mean±s.d.; as monitored by a digital probe) by passing the pump's return line through a bucket of ice water as necessary.

Procedures

Animals were anesthetized by immersion in a seawater bath of 0.025% ethyl-*p*-aminobenzoate (benzocaine). Once opercular movement ceased, fish were removed from the bath, placed on a damp sponge, and immobilized via an intramuscular injection of cisatracurium besylate (3 mg kg^{-1}). In later experiments, fish then underwent surgical manipulation and removal of the saccular otoliths (sagittae) or swim bladder (see below). To access the inner ear and sagittae, a scalpel was used to expose the dorsal surface of the skull overlying the hindbrain. The adjacent sagittae were easily visualized through the translucent skull, appearing as white bands on either side of the hindbrain. A pick and fine rongeurs were used to open the skull overlying the left or right sagitta, and needle forceps were used to extract the sagitta from the otic capsule. The otic capsule was then back-filled with chilled teleost Ringer's solution to ensure that no air bubbles were present. To close the craniotomy, a small piece of Parafilm was placed on the exposed

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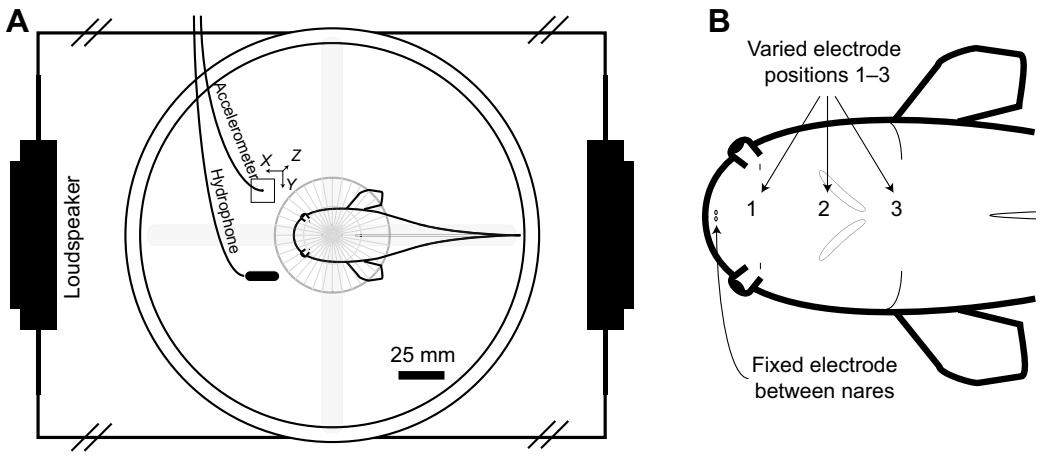

Fig. 1. Experimental setup. (A) The animal was suspended in an adjustable apparatus inside a tank consisting of a PVC tee with underwater loudspeakers mounted on either end. The sound field was characterized using a mini-hydrophone and triaxial accelerometer; in later experiments, the animal's bearing relative to this sound field was varied systematically. (B) The electrode montage used in auditory evoked potential (AEP) recordings included an electrode placed between the nares, three different recording electrodes – (1) between the eyes, (2) over the otic capsule and (3) along the caudal boundary of the opercula – and a ground electrode placed in the water below the fish (not shown).

skull and sealed to the surrounding tissue with cyanoacrylate. In control animals (for sagitta removal experiments), the dorsal surface of the skull was exposed (sham surgery), such that time and handling from anesthetic induction to testing were similar across groups.

Swim bladder manipulations, which were conducted in tandem with removal of the left sagitta (see Results), began by opening the left wall of the peritoneal cavity with microdissection scissors. Overlying tissue was removed to visualize the swim bladder. The swim bladder was then excised with a pair of forceps, typically requiring blunt dissection from surrounding fascia. The incision was sutured closed and sealed with cyanoacrylate. In one experiment (in which the otoliths were not manipulated), the swim bladder was intubated with a 25-gauge hypodermic needle connected to a length of thin (~0.5 mm internal diameter) tubing terminating at a 10 ml syringe. The syringe was then used to deflate the swim bladder, confirmed visually and by tactile inspection with a rounded periosteal elevator. Positive and negative pressure was then applied to the syringe, causing inflation and deflation and confirming that the syringe was effectively coupled to the swim bladder. The needle was glued in place with a drop of cyanoacrylate, and the peritoneal incision was closed around the tubing. The syringe was then used to dynamically re-inflate the swim bladder during successive AEP recordings (see below).

Following surgery (or, if no surgical manipulations were required, after anesthetic induction), shielded needle electrodes (27-gauge, SurePoint, Rochester Electro-Medical, Lutz, FL, USA) were inserted subcutaneously along the cranial midline. In all experiments, an electrode was placed between the nares. In initial experiments, which did not include surgical manipulation of the otic capsule and wherein electrode position was a parameter of interest, three separate recording electrodes were placed at three different locations: (1) between the eyes, (2) between stereotyped stitches of mid-cranial neuromasts centered above the otic capsule and (3) between the caudal boundary of the opercula (nominal positions 1–3, see Fig. 1B). The electrodes were placed simultaneously, prior to any recording, to ensure that electrode position would not be disturbed once the fish was positioned in the testing apparatus. In later experiments, the recording electrode was always placed at position 1 unless otherwise noted. In all cases, a ground electrode was placed in the water below the fish. Finally, the fish was placed inside the holder, cradled by the suspended mesh, and intubated with the outflow of the aquarium pump to flush aerated seawater across the gills. The fish was held in place for ≥10 min prior to recording to allow time for recovery from benzocaine (J.A.S., unpublished observations).

AEP recordings

Stimuli were pure tones of 100 ms (early experiments) or 500 ms (later experiments) duration, in either case including cosine on- and off-ramps to reduce transient distortion. Stimuli were synthesized at a sampling frequency of 44,100 Hz using a USB D/A converter (Fireface UCX, RME, Frankfurt, Germany) and routed to the UW-30 speakers via a power amplifier (Crown D75-A, Harman, Northridge, CA, USA). Stimulus sound pressure level (SPL) was calibrated at each test frequency using a mini-hydrophone (Type 8103, Bruel & Kjaer, Nærum, Denmark) positioned adjacent to the animal's head. Although stimuli were calibrated according to SPL, particle acceleration was also measured during all recordings using a triaxial accelerometer waterproofed with epoxy and encased in syntactic foam to achieve neutral buoyancy [model VW356A12 with signal conditioner 482A16, PCB Piezotronics, Depew, NY, USA; triaxial accelerometer sensitivity at 100 Hz: 10.42 mV ms^{-2} (x-axis), 9.65 mV ms^{-2} (y-axis), 10.14 mV ms^{-2} (z-axis); see also Bhandiwad et al., 2017]. The accelerometer was also positioned adjacent to the animal's head (see Fig. 1A). In experiments concerned with directional sensitivity, the particle acceleration vector was confirmed to be parallel with the longitudinal axis of the tank at a test frequency of 85 Hz; all directional sensitivity experiments were conducted at this frequency. The animal's position relative to the static particle acceleration vector was readily and precisely adjustable by rotating the azimuthal bearing of the animal holder. During such adjustments, the animal's head was centered within the holder such that rotation was about a point centered between the sagittae (per visual inspection). To assess variation of SPL about this point (at the 85 Hz test frequency), the sound field within a 10 cm square region at the tank center was measured with 25 mm resolution.

Recording electrode leads were shielded external to their subcutaneous contacts via factory shielding or, near their

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terminations, nail polish. Electrodes were connected to a headstage cable and differential amplifier (BMA-200, CWE Inc., Ardmore, PA, USA). The signal was amplified (10,000–20,000×) and routed to a USB-connected A/D converter (Fireface UCX) that sampled the amplified signal at 44,100 Hz. For each condition (test frequency or azimuth), at least 200 (up to 1000) tone sweeps were presented to facilitate signal averaging and extraction of the small-amplitude AEP signal from background noise sources. Tones were presented with alternating polarity to (1) reduce possible stimulus artefacts (Kenyon et al., 1998) and (2) average out receptor (non-neuronal) potentials. The effect of stimulus polarity was observed to vary across recording electrode site, and thus became a variable of interest (see Results). All signal generation, presentation and acquisition was controlled via MATLAB (MathWorks, Natick, MA, USA) using custom-written scripts that interfaced with a compiled version of the 'Playrec' Port Audio utility (https://github.com/PlayrecForMatlab/playrec). Accumulating average AEP waveforms were visualized online to monitor the status of the preparation; all traces were stored for offline analysis. Specific analyses are described in associated subsections of the Results.

Statistical analysis

Data were analyzed in MATLAB using standard signal processing techniques (averaging, filtering, Fourier transformation, etc.). Distilled AEP metrics such as response amplitude were submitted to standard parametric statistical testing (repeated-measures or two-way ANOVA and paired and independent samples *t*-tests) (SPSS Statistics, IBM Corporation, Armonk, NY, USA). As these are the first midshipman AEP measurements we are aware of, *a priori* power analyses were not conducted. In cases of non-sphericity or non-homogeneous variance across groups, appropriate adjustments to degrees of freedom were applied as detailed in the Results, e.g. Greenhouse–Geisser and Welch–Satterthwaite adjustment methods. All reported *t*-tests were two-tailed.

RESULTS

Basic AEP characterization and influences of recording electrode position

Initial AEP recordings were completed with animals suspended in a PVC enclosure (Fig. 1A) using three different recording electrode positions (Fig. 1B) at test frequencies of 100 and 200 Hz (n=11 animals) at a sound level of 130 dB SPL (rms re. 1 µPa). Recordings were also completed at 300 Hz at 140 dB SPL in some animals. The 300 Hz responses were small in amplitude, could not be measured in all animals (at 140 dB SPL), and are not considered further in the present report. An example recording for a single animal at a test frequency of 200 Hz is given in Fig. 2. Fig. 2A plots the acoustic stimulus in terms of observed sound pressure (upper panel), the quantity by which stimuli were calibrated, and also particle acceleration (middle and lower panels). Long-term rms magnitude of particle acceleration across measurements was 0.0435 m s^{-2} (or −28 dB re. 1 m s^{-2}) at 200 Hz and 0.0375 m s^{-2} (or −28.5 dB re. 1 m s^{-2}) at 100 Hz for a fixed nominal dB SPL of 130 dB re. 1 µPa. Note that the nominal stimulus duration was 100 ms, extended somewhat in practice by signal reflections (reverberation) within the tank. Fig. 2B (upper panel) shows example evoked potential waveforms for electrode position 2 (over the otic capsule).

Tonal stimuli were presented with alternating acoustic polarity (see Materials and Methods). Traces in the upper panel of Fig. 2B are plotted separately for 'positive' (red) and 'negative' (blue) polarities along with the traditional cross-polarity averaged AEP (black). The difference between positive and negative polarity waveforms, which indexes the extent to which responses were stimulus polarity dependent, is given by the thin gray trace. The amplitude spectrum of the AEP waveform is given in the lower panel of Fig. 2B.

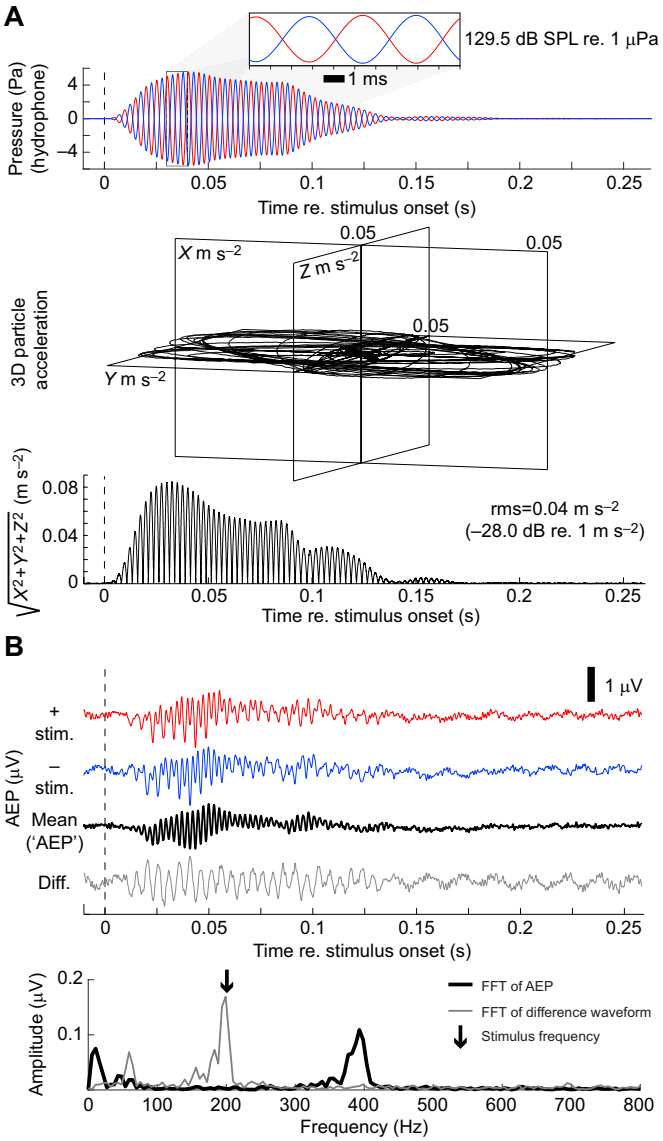

Fig. 2. Example stimuli and AEP recordings. (A) In initial experiments, stimuli consisted of windowed 100 ms tone bursts (200 Hz shown) presented at a nominal level of 130 dB SPL re. 1 µPa (rms) with alternating polarity [see Materials and Methods; inset shows waveform detail for + (red) and – (blue) polarities]. Effective stimulus duration was extended somewhat by reverberation within the test tank. Sound pressure (upper panel) and particle acceleration (middle and lower panels) were both quantified. The 3D trajectory of particle acceleration (middle panel) was dominated by a longitudinal (X) component, parallel with the long axis of the tank and perpendicular to the speaker face, but lateral (Y) and vertical (Z) components were also present. The resultant magnitude (X, Y and Z components combined) defines the total particle acceleration stimulus (lower panel). (B) Elicited auditory potentials (electrode position 2; see Fig. 1B) exhibited a characteristic double-frequency response. The polarity of the response depended on the polarity of the stimulus, resulting in a notable difference between + (red) and – (blue) potentials, easily visualized in the difference between the two (gray) (upper panel). The dominant frequency of the averaged potential was ∼400 Hz, twice the stimulus frequency (lower panel). FFT, fast Fourier transform.

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Two main observations can be made about the AEPs shown. Firstly, as has been described in many other teleosts, the averaged AEP of the midshipman exhibited a prominent 'double-frequency' response, here at ∼400 Hz given the 200 Hz stimulus frequency (Fig. 2B), consistent with contributions of opposed-polarity groups of hair cells within the saccular macula (e.g. Zotterman, 1943; Furukawa and Ishii, 1967; Sisneros, 2007; Coffin et al., 2012). Secondly, phase characteristics of the waveforms elicited by positive- and negative-polarity stimuli did notably differ, with the result that there was a relatively large 'residual' difference potential, with a dominant frequency equal to the stimulus frequency (Fig. 2B). The magnitude of the difference waveform appeared to vary across recording electrode positions, which differed in their spatial relationship to the otic capsule (and thus the sensory hair cells).

Influence of recording electrode position on phase dependence of AEPs

Differences in response amplitude across the three recording electrode positions evaluated were expected on the basis of (1) their spatial relationship to the presumed generators of the AEP (e.g. the VIII nerve) and (2) previous evidence that electrode position can influence AEP response amplitude (e.g. Lu and Xu, 2002). In our measurements, response amplitude was generally highest at position 2, centered over the otic capsule, but it was not significantly higher here than at either positions 1 or 3. A repeated-measures ANOVA (with Greenhouse–Geisser degrees of freedom adjustment to account for non-sphericity) on polarity-averaged AEP amplitude with factors of stimulus frequency (100 Hz, 200 Hz) and electrode position (1–3) demonstrated a significant main effect of stimulus frequency ($F_{1,10}$=53.28, P<0.001), consistent with notably higher response amplitudes at 100 Hz than at 200 Hz, but no main effect of electrode position ($F_{1.44,14.43}$=1.30, P=0.291) or frequency×electrode position interaction ($F_{1.41,14.08}$=1.30, P=0.288).

However, the influence of stimulus polarity on response phase (and thus the difference waveform amplitude) was strongly dependent on recording electrode location. Responses elicited by positive and negative polarities were generally homophasic at position 1, with little difference potential in many animals (e.g. Fig. 3A, upper AEP trace), but notable difference potentials at electrode positions 2 and 3 in most animals (Fig. 3A, middle and lower AEP traces). A repeated-measures ANOVA on AEP difference amplitudes with factors of stimulus frequency (100 Hz, 200 Hz) and electrode position (1–3) demonstrated a significant main effect of frequency (as for polarity-averaged amplitudes) ($F_{1,10}$=24.89, P=0.001), but also a significant main effect of electrode position ($F_{1.61,16.11}$=8.95, P=0.004) and a significant frequency×electrode position interaction ($F_{1.64,16.43}$=8.61, P=0.004). Follow-up paired-samples t-tests indicated larger difference amplitudes for both positions 2 and 3 than for position 1 at 100 Hz (position 2 versus 1, t_{10}=3.744, P=0.004; position 3

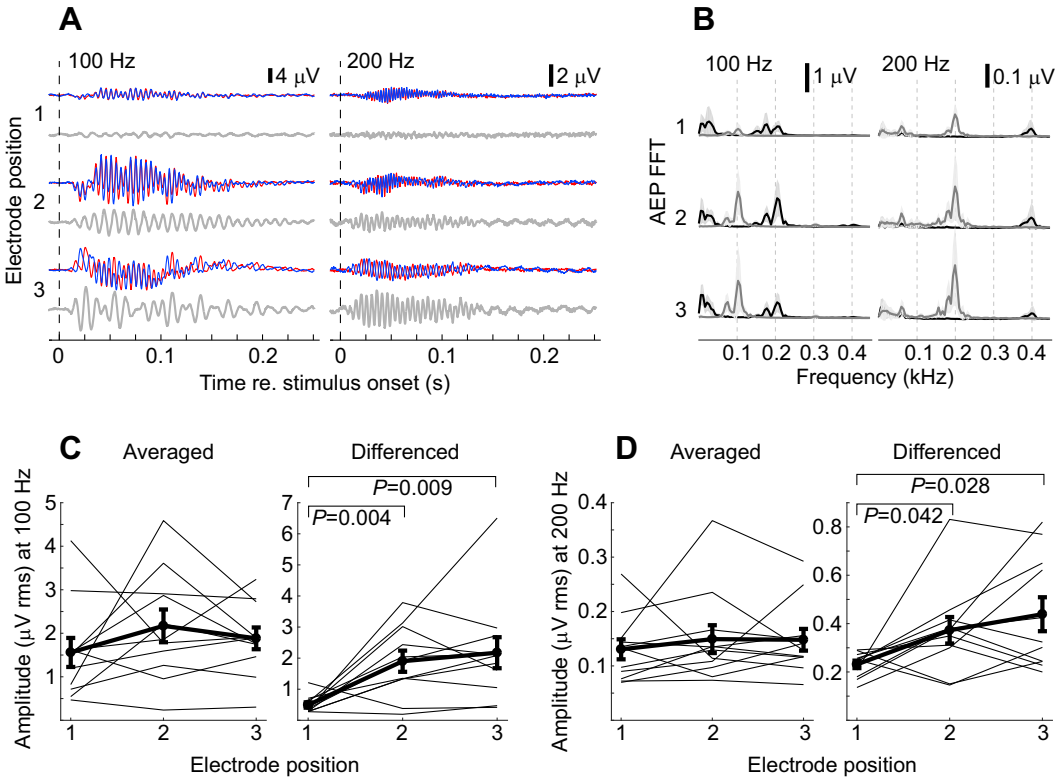

Fig. 3. Effects of electrode position on midshipman AEP waveform. (A) Example AEP waveforms elicited by + phase (red) and − phase (blue) stimuli at stimulus frequencies of 100 Hz (left) and 200 Hz (right). Each row shows example waveforms from electrode positions 1, 2 and 3 in a single animal. The difference waveform (+ minus −) is given by the thin gray trace (see Materials and Methods). (B) Mean amplitude spectra (across 11 fish) obtained via FFT for the polarity-averaged AEP waveform (black) and the difference waveform (gray) at each electrode position (1–3 as labeled). The dominant frequency of the AEP was generally twice the stimulus frequency, while that of the difference waveform matched the stimulus frequency (see Results). Shading indicates ±1 s.d. (C,D) The amplitude and phase dependence of AEP waveforms elicited at 100 Hz (C) and 200 Hz (D) varied across electrode position. Polarity-averaged AEP amplitude (left in C and D) tended to be higher at more caudal recording sites, but variability across animals was high and differences were not significant. However, AEP waveforms recorded at position 1 were less dependent on stimulus phase than those at positions 2 or 3, yielding smaller difference waveform amplitudes (right in C and D) for electrode position 1 (P-values given for paired-samples t-tests; see Results).

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versus 1, t_{10}=3.232, P=0.009) and a similar pattern at 200 Hz (position 2 versus 1, t_{10}=2.326, P=0.042; position 3 versus 1, t_{10}=2.561, P=0.028). (Note: obtained P-values can be evaluated against a conservative Bonferroni-corrected familywise criterion P-value of 0.0167, corrected for three possible comparisons per frequency.) Electrode position 1 was used for all subsequent recordings; implications of electrode position effects in our AEP measurements and in general are considered further in the Discussion.

Contribution of the saccule to midshipman AEPs – directional modulation

As described above, test animals were suspended in an adjustable holder that enabled reproducible positioning with respect to the longitudinal axis of the test tank (Fig. 1A; see Materials and Methods) up to azimuthal limits of ±60 deg. Although the 3D trajectory of particle acceleration measured at the center of the tank varied somewhat across frequency (with notable lateral and vertical components at some frequencies, e.g. 200 Hz, as shown in Fig. 2A), it was found to be parallel to the longitudinal axis of the tank at a test frequency of 85 Hz (Fig. 4A). Measured sound pressure near the tank center was also homogeneous at 85 Hz, varying <1 dB SPL over a 10 cm square region (Fig. 4B). By presenting 85 Hz stimuli (also within the typical range of the fundamental frequency of the male midshipman advertisement call), it was thus possible to study directional modulation of AEPs as the fish was rotated with respect to a static and well-defined sound field.

Baseline measurements were completed in a set of control animals (n=9) at a test level of 130 dB SPL (rms re. 1 µPa). During testing, AEPs were measured sequentially as animals were rotated in azimuth in counterbalanced order (Fig. 4C,D). Animals were first rotated from left (−60 deg) to right (+60 deg) in 20 deg increments, then from right (+50 deg) to left (−50 deg) in 20 deg increments (measurements were initially also attempted at ±70 deg, but later discontinued because of concerns about deformation of the animal suspension apparatus). The intent of this approach was to reveal any obvious order effects, i.e. the shape of the cross-azimuth function should have been similar regardless of azimuth testing order, and a departure from this

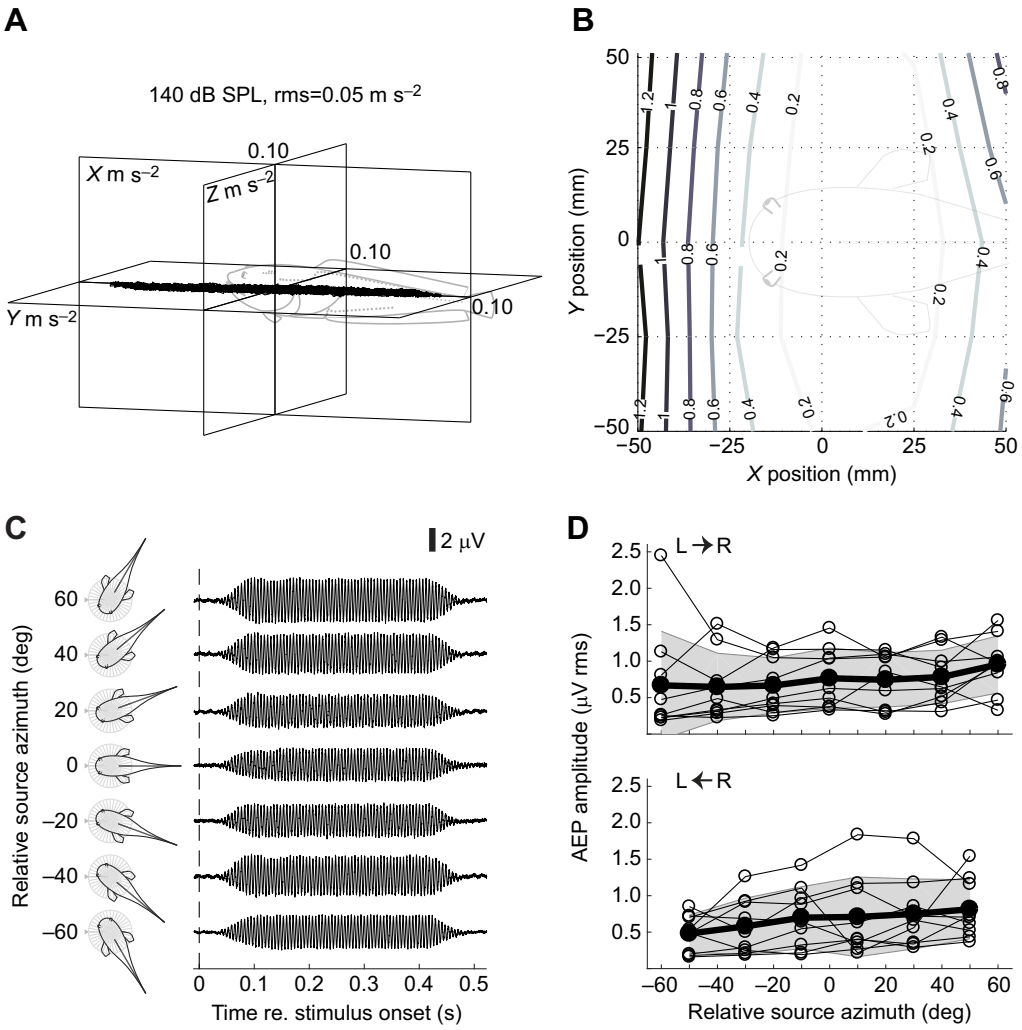

Fig. 4. Evaluating directional modulation of AEPs. (A) The 3D trajectory of particle acceleration at the tank center was almost exclusively longitudinal (dominated by *X* component) at 85 Hz. This axis defines the '0' position relative to the fish. (B) SPL at the tank center was homogeneous at 85 Hz (contour lines=0.2 dB re. tank center). (C) Method for controlling stimulus directionality. The fish was rotated about a point centered over the otic capsule. Example AEP waveforms are shown for a subset of sampled positions. (D) Average waveform amplitude (rms) across the azimuths –60 to +60 deg; subpanels show amplitudes obtained while varying the animal's position either left to right (top) or right to left (bottom) in 20 deg increments (see Results). The filled circles give the mean across animals (n=9) and the gray shading indicates ±1 s.d.

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would suggest changes in the condition of the preparation or other confounding effects.

While systematic variation of responses across azimuth in the control condition was not expected *a priori* (cf. Lu and Xu, 2002), some variation was noted. Average response amplitude was generally of the order of 0.5–1.5 µV rms, with values increasing slightly for rightward azimuths. Such bias was apparent for both right-to-left and left-to-right test order; the average amplitude of right hemifield responses (azimuths 10 to 60 deg, mean 0.93 µV rms) was found to be greater than that of left hemifield responses (azimuths −10 to −60 deg, mean 0.72 µV rms) (t_8=3.045, P=0.016). The reasons for such bias were not immediately clear, but greater responses on average in the right hemifield were broadly consistent with data later obtained following saccular manipulations, which suggested larger responses from the right than from the left saccule (see below).

In order to evaluate the hypothesis that the oblique orientation of the saccule relative to midline yields gross hemispheric bias in female midshipman auditory sensitivity (cf. Lu and Xu, 2002), groups of animals underwent unilateral surgical excision of the sagittae (see Materials and Methods). Following surgery, AEPs were measured using the same procedures as applied to control animals. Cross-azimuth response functions, given as the rms amplitude of the AEP waveform, are shown in Fig. 5. Note that data are again shown for right to left and left to right test order (subpanels), although they were combined across azimuths for statistical evaluation.

The first group of animals underwent right sagitta excision (Fig. 5B) as previously evaluated in the sleeper goby (Lu and Xu, 2002). A two-way repeated-measures ANOVA of stimulus hemifield-averaged AEP amplitude with a between-groups factor of sagitta condition (control, right sagitta removed) and a within-subjects factor of stimulus hemifield [left (azimuths −60 to −10 deg) versus right (azimuths +10 to +60 deg)] demonstrated significantly lower overall AEP amplitude in the right sagitta-removed group (main effect of group, $F_{1,16}$=8.13, P=0.012), was expected given the elimination of a major auditory input, as well as a significant hemifield×group interaction ($F_{1,16}$=10.70, P=0.005); the main effect of hemifield was not significant. The interaction of stimulus hemifield and sagitta condition appeared to be attributable to a relatively greater effect of sagitta removal for right-hemifield azimuths, i.e. the hemifield ipsilateral to the removed sagitta (independent samples t-test for control versus right sagitta-removed amplitude, t_{16}=3.510, P=0.003) than for left-hemifield azimuths (t_{16}=1.609, P=0.127). A greater loss of response amplitude for rightward than leftward azimuths following right sagitta excision is consistent with the hypothesis that obliquely oriented sagittae yield an ipsilateral bias in saccular tuning (cf. Lu and Xu, 2002), although in the present case the result appeared to depend on the baseline asymmetry in response amplitude across azimuth (greater right- than left-hemifield responses in control animals).

For completeness, and to gain additional insight on a possible asymmetry in left versus right AEP response amplitude, we next completed the opposite manipulation – excision of the left sagitta – in a separate group of animals (Fig. 5C). Whereas we predicted reduced responses (relative to control) for leftward azimuths, i.e. the opposite result to that for right sagitta excision, we instead observed no average decrease in response (two-way ANOVA as for right sagitta excision: main effect of sagitta condition $F_{1,16}$=0.29, P=0.600). In fact, responses reached a minimum, on average, contralateral to the excised sagitta (i.e. at rightward azimuths, the same as for right sagitta excision, although the difference in right hemifield responses for left-removed and control animals was not significant, independent samples t-test, t_{16}=1.300, P=0.212). This result was unexpected, and suggested significant asymmetry in the AEP of reproductive female midshipman: whereas animals that underwent right sagitta excision exhibited significantly lower-amplitude AEPs with the greatest loss of response for rightward azimuths, removal of the left sagitta resulted in no average decrease in AEP response amplitude. The cross-azimuth mean AEP amplitude for the left sagitta-removed group (mean±s.d. 0.70±0.50 µV) was approximately twofold higher than that of the right sagitta-removed group (mean±s.d. 0.36±0.11 µV), although the difference did not reach statistical significance (independent samples t-test with degrees of freedom adjusted for unequal variance using Welch–Satterthwaite method, $t_{8.71}$=2.02, P=0.075).

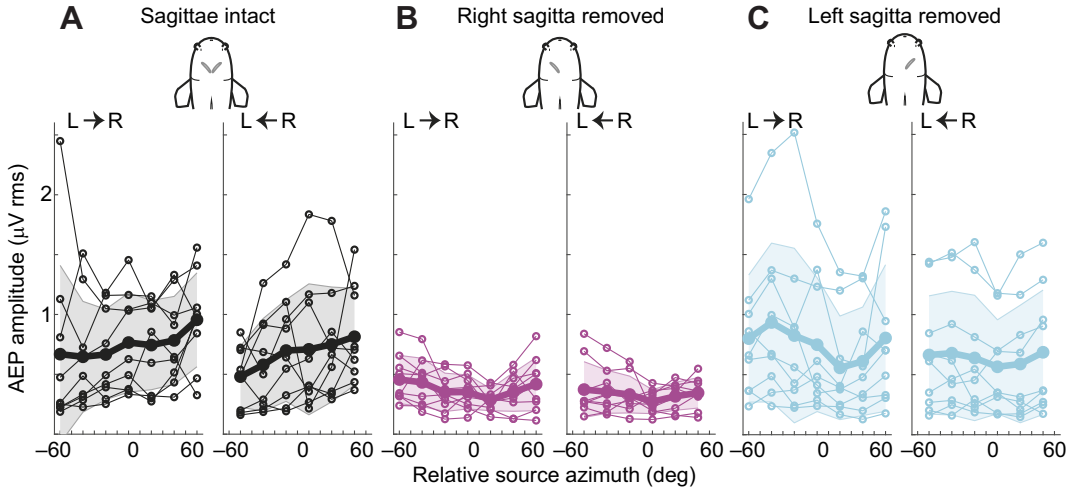

Fig. 5. Effect of saccular otolith removal on AEP amplitude as a function of azimuth. (A) Control data (sagittae intact), as in Fig. 4. The left panel gives data collected when adjusting the animal from left to right (azimuths −60 to +60 deg) while the right panel gives data collected when adjusting from right to left (azimuths −50 to +50 deg) in 20 deg increments (see Results). (B) As in A, but for a group of animals that underwent surgical excision of the right sagitta prior to testing (as in Lu and Xu, 2002). (C) As in A, but for a group of animals that underwent surgical excision of the left sagitta prior to testing. In all panels, open circles give data for individual animals, filled circles give the mean across animals (n=9) and shading indicates ±1 s.d.

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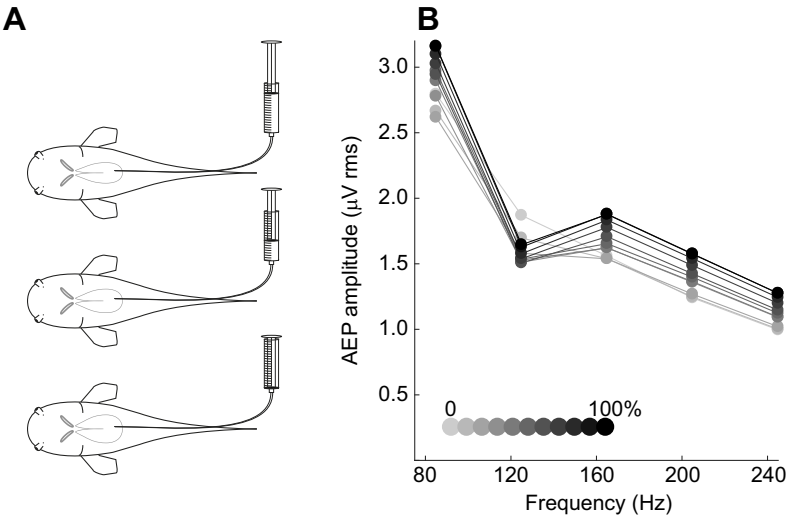

Fig. 6. Contribution of the swim bladder to the AEP. (A) The swim bladder of a single test animal was surgically intubated and deflated using a syringe. It was then possible to re-inflate the swim bladder in graduated steps while recording AEPs. (B) AEP response amplitude measured at five frequencies at a test level of 140 dB SPL. Increasing swim bladder inflation led to systematic increases in AEP response amplitude at most frequencies. Nominal inflation (%) is indicated via grayscale, from the baseline deflated state (light gray) to nominal full inflation (black).

The generally limited directional modulation of AEPs we observed following unilateral sagitta excision compared with observations by Lu and Xu (2002) in the sleeper goby led us to consider differences across the two studies. Whereas Lu and Xu (2002) measured AEP threshold, we measured responses at suprathreshold sound levels (approximating those observed near nesting sites where phonotaxis naturally occurs) using response magnitude as the dependent variable. Our method of directional stimulation was also different (see Discussion). A more basic difference was that the female midshipman, unlike the sleeper goby (or *O. tau*, see Introduction), may be sound pressure sensitive via the swim bladder (see Introduction). Such sensitivity could mask or otherwise interact with directionality intrinsic to the sagittae/saccules by providing response gain independent of the orientation of the sagittae. Thus, we next sought to understand influences of the swim bladder on midshipman AEPs, and subsequently evaluated AEP directional tuning after swim bladder excision.

Influence of the swim bladder on the midshipman AEP and directional tuning

We first sought to verify a contribution of the swim bladder to the AEP in midshipman using a novel within-animal design in which the inflation of the swim bladder was dynamically varied (Fig. 6A; see Materials and Methods). As shown in Fig. 6B, swim bladder re-inflation caused a steady increase in response amplitude at each of five test frequencies (except 120 Hz, where the change in response was interestingly non-monotonic). This proof-of-concept experiment points to interesting possibilities for future acoustic/auditory studies of swim bladder functionality, but in the present context served simply to demonstrate that influences of the swim bladder on auditory responses in female midshipman could indeed be measured by the AEP technique.

To probe the contribution of the swim bladder to the AEP in a directional context, we completed a final series of experiments in which the swim bladder was excised in tandem with unilateral sagitta excision. The premise of this manipulation was that eliminating response gain provided by the swim bladder – the directional characteristic of which is likely to be broad and independent of saccular orientation (cf. Barimo and Fine, 1998) – could act to reveal directionality intrinsic to the intact saccule. Given the unexpected responses observed after left sagitta excision in the previous experiment (Fig. 5C), we aimed to further evaluate the effect of left sagitta excision. Thus, a final group of animals (n=8) underwent both left sagitta excision and swim bladder excision. Directional AEP modulation (at 85 Hz) was then evaluated.

Stimuli were initially presented at 130 dB SPL, the standard stimulus level used in prior experiments (Fig. 7A). As testing order was not previously found to influence responses, azimuths were tested in continuous order (slightly expediting testing), with animals adjusted from left to right (n=4) or right to left (n=4) (any unexpected order effects would have thus remained detectable). Compared with the earlier group of animals in which the left sagitta was excised but the swim bladder was intact, mean response amplitude was significantly lower (independent samples t-test, $t_{8.22}$=−2.343, P=0.046; degrees of freedom adjusted for unequal

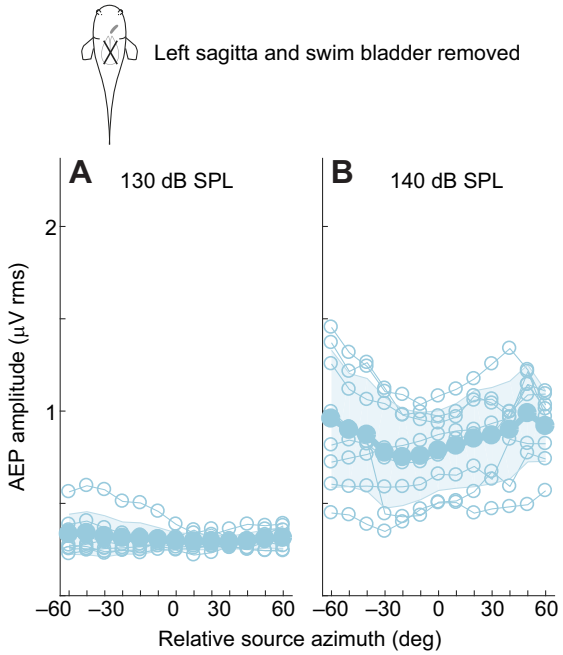

Fig. 7. Effect of simultaneous swim bladder and saccular otolith removal on AEP amplitude as a function of azimuth. (A) Response amplitude at 130 dB SPL (rms re. 1 µPa) at azimuths from –60 to 60 deg tested in 10 deg increments in animals with the left sagitta and swim bladder removed. In some animals, responses fell to the noise floor (see Results). (B) Response amplitudes in the same animals at 140 dB SPL. In each panel, open circles give data for individual animals, filled circles give the mean across animals (n=8) and shading indicates ±1 s.d.

variance). In some animals, responses fell to the noise floor (i.e. could not be distinguished from the background), as indexed via both the waveform and the fast Fourier transform (FFT) of the waveform, for which the stereotyped double-frequency response was no longer evident (data not shown). Although the loss of response at 130 dB SPL following swim bladder removal was an interesting result in itself, floor effects prevented evaluation of directional tuning, so testing was repeated in each animal at an amplitude of 140 dB SPL (Fig. 7B). Reproducible responses were elicited at this intensity in all cases. Response variation across azimuth was again rather slight, and no gross hemispheric difference was evident [right hemifield (azimuths 10 to 60 deg) versus left hemifield (azimuths −10 to −60 deg), t_7=1.31, P=0.233]. However, the response minimum occurred in the left hemifield in most animals, consistent with the hypothesis that left sagitta excision should exert a left hemifield deficit. A significant difference could be identified by comparing selected leftward minima (*post hoc*) against responses at the opposing rightward azimuth (e.g. −20 versus +20 deg, t_7=−8.34, P<0.001), but responses at many opposing azimuths (including −40 and +40 deg) were similar. In sum, although swim bladder excision may have served to eliminate a source of response gain that was unrelated to sagitta-derived saccular tuning, and responses in the sagitta- and swim bladder-excised group appeared somewhat less variable than those in the sagitta-excised (swim bladder-intact) group, it must be concluded that variation of stimulus azimuth exerted consistently little influence on the amplitude of the midshipman AEP.

DISCUSSION

The AEP technique has provided insight on hearing ability of dozens of fish species (Ladich and Fay, 2013). Although explicit comparisons with behavioral data have indicated that AEP measurements should be interpreted cautiously (e.g. Sisneros et al., 2016), the AEP method has also provided unique insight on auditory contributions of specific structures, including the swim bladder (Yan et al., 2000; Yan, 2004) and saccular otoliths (Lu and Xu, 2002). Here, we report AEP measurements in the plainfin midshipman, an important model species in auditory neuroscience and bioacoustics.

Influences of electrode position on AEP measurements

One parameter that has received relatively little attention in measurements of fish AEPs is that of electrode position (‘montage’). As electrode position defines the spatial relationship between recording contacts and the auditory structures that generate the AEP, it is intuitively an important variable to control. However, whereas standard electrode montages are well defined and routinely applied in measurements of mammalian AEPs (e.g. the human auditory brainstem response; Mason and Herrmann, 1998), survey of the fish AEP literature indicates that applied montages can vary significantly, even within species. Given the heterogeneity of auditory anatomy and also cranial morphology in fishes, it may not be feasible to identify a useful ‘standard’ montage. In this case, parametric variation of electrode position for the species under study could provide a means of understanding and accounting for its influence. Our measurements in the plainfin midshipman suggest that recording electrode location only weakly influences response amplitude (cf. Lu and Xu, 2002; Maruska and Sisneros, 2016) but significantly influences the phase dependence of the evoked response, suggestive of shifts in the neural and sensory structures evaluated. While the recording location nearest the otic capsule (nominal position 2) provided a slightly higher-amplitude response, it was also sensitive to the polarity of the stimulus, consistent with an influence of receptor (hair cell) rather than neural discharge. Recordings with a more rostral montage (position 1) were generally less sensitive to stimulus phase, suggesting better isolation of neural (versus sensory) sources (cf. Kenyon et al., 1998). The gradient of difference waveform amplitude ran counter to that which would have been expected for stimulus artefact (as electrode position 1 was nearest to the active loudspeaker); additionally, test recordings on our AEP system in dead fish confirmed the absence of responses, i.e. stimulus artefacts were not observed.

As we sought to evaluate the influence of saccular otolith and swim bladder manipulations on the central (neural) response to the stimulus – and required surgical access to the otic capsule – we completed directional sensitivity measurements using electrode position 1. Future fish AEP investigations should consider the selection of recording electrode montage in the context of the particular questions (and auditory structures) under study. Multichannel recording systems are also widely available and could readily be employed to efficiently measure responses at multiple sites simultaneously (an approach commonly employed in mammalian studies).

Weak directional modulation of AEPs following sagitta excision

Our AEP measurements failed to demonstrate strong modulation of AEP amplitude by sound source azimuth. We note that studies of directional hearing in fish have most commonly employed a head-fixed preparation wherein the animal is rigidly attached to a vibrating apparatus. Our animals were instead freely suspended in a calibrated sound field, ostensibly a more ecological preparation (see also Maruska and Mensinger, 2015), but a variation that could yield stimulation/responses different from those elicited with the head fixed. In the present data, the clearest example of directional bias was derived by comparing responses in animals with the right sagitta excised with those in control (intact) animals, whereby a significant loss of response amplitude was evident in the right hemifield only (ipsilateral to the missing sagitta). This condition mirrored the measurements of Lu and Xu (2002) in the sleeper goby, in which removal of the right sagitta led to a significant elevation of AEP threshold (decrease in sensitivity) in the right, but not left, hemifield (a difference of the order of 6–7 dB re. control at +30 and +60 deg). As we measured suprathreshold modulation of response amplitude rather than thresholds, our results are not directly comparable with those of Lu and Xu (2002). Notably, our control measurements included a baseline asymmetry, with greater responses for rightward than for leftward azimuths (see below), and this asymmetry contributed to the difference evident after right sagitta excision. Moreover, the opposite manipulation – excision of the left sagitta – had no significant influence on overall response amplitude, and certainly did not lead to lower response amplitudes for leftward azimuths as predicted according to the opposite orientation of the left (re. right) sagitta. Responses in this condition were particularly variable across animals. One factor that could have contributed to such variability was electrode depth. In addition to influences of contact location on the cranial surface, recording amplitude has been shown to increase with electrode depth (Lu and Xu, 2002). The skull of the midshipman is thin, and despite careful efforts to achieve equal electrode depth in all fish, it is possible that contacts could have occasionally been positioned deeper than intended. However, such random variation should have occurred for both right and left sagitta removal experiments, so would be unlikely to lead to the systematic left–right asymmetry observed. We would also predict response gain to be affected roughly equally

across azimuth, preserving any intrinsic cross-azimuth variation that would have been observed at some other electrode depth. That is, while variation in electrode depth could have shifted overall response gain, directional tuning, if present, would have been expected to persist.

The AEP indexes the ensemble response of the auditory system, which in teleosts may include the saccular, lagenar and utricular end organs. Neither the lagena nor the utricle was manipulated in our experiments, thus recorded responses could have reflected bilateral contributions from both. The directional characteristics of the lagena and utricle have not been evaluated in midshipman. In this sense, while our data and those of Lu and Xu (2002) indicate that directional hearing might be indexed to a limited extent via the AEP, other approaches will likely be required to provide deeper insight into the contributions of specific end organs.

In the broader context of directional hearing in fishes, although it is tempting to posit that the opposed orientations of the saggitae/saccules and resultant biases in sensitivity could provide a basis for sound source localization, the parsimony of this account, particularly as a general solution for teleosts, is complicated by several factors. Firstly, within all studied teleost species including those discussed hereto, hair cell stereociliary bundle polarity, which defines the axis of best sensitivity at the level of the receptor, varies across the saccular macula (Popper and Lu, 2000; Coffin et al., 2012). Thus, many receptors (and their associated afferents) respond best to particle motion that is not parallel to the long axis of the adjacent sagitta, a result already evident in the heterogeneity of saccular afferent spatial tuning functions (e.g. Fay and Edds-Walton, 1997). Moreover, azimuth-selective auditory utricular afferents have been characterized in the oyster toadfish specifically, suggesting that even if hemispheric bias may arise via the opposed sagittae, contributions from other end organs may be important (Maruska and Mensinger, 2015). Additionally, while data from Batrichoididae (midshipman and toadfish) offer some of the clearest evidence of teleost behavioral sound source localization (reviewed in Fay, 2011), classic data from goldfish (Moulton and Dixon, 1967) and cod (Schuijf and Buwalda, 1975), which possess relatively more parallel otoliths, indicate that these species are nonetheless able to discriminate sound location. Finally, and certainly of direct relevance in the present context, data from female midshipman suggest that the pressure-receptive swim bladder is necessary for sound source localization in this species (Coffin et al., 2014) and, by extension, that the spatially opposed saccular (and other otolithic) end organs are by themselves insufficient (see below).

Asymmetry of right versus left saccular manipulations

We observed asymmetry in measured AEPs both (1) in control animals, with greater responses for rightward than for leftward azimuths, and (2) in animals that underwent unilateral sagitta excision, with higher-amplitude responses for the left sagitta-removed (right remaining) group than for the right sagitta-removed (left remaining) group. Apart from the possibility that unintended variation in electrode depth yielded greater response amplitude in left sagitta-removed animals, we cannot satisfactorily explain the observed asymmetry. Several findings in the literature bear mentioning in this context. Asymmetries in fish auditory (Lychakov and Rebane, 2005; Lychakov et al., 2008) and sound production (Rice and Bass, 2009) systems have been of interest to many investigators. A ‘right ear advantage’ (defined using various assays) has been demonstrated in several fish species, including certain flatfishes (Bothidae and Citharidae; Lychakov et al., 2008), as well as marine mammals (specifically in the context of conspecific call sensitivity; Boye et al., 2005) and even humans (Chung et al., 1983). One highly systematic and intensively studied example of auditory asymmetry – also a foundational example in the topic of directional hearing – is the asymmetric facial ruff of the barn owl (*Tyto alba*), which imparts different directional sensitivity to the left and right ears (and, of note, is not common to all owls, i.e. is a species-specific adaptation; Volman and Konishi, 1990). Further investigation is warranted to determine in which respects and to what extent the midshipman auditory system may be asymmetric. Recent unpublished data (J.A.S. and P. M. Forlano) has shown asymmetric immunolabeling patterns elicited by leftward (−45 deg) versus rightward (+45 deg) stimulation: rightward stimulations appear to elicit bilateral hindbrain (secondary octaval nucleus) activation (as indexed by c-Fos labeling), while leftward stimulation elicits only unilateral activation. As female midshipman hearing is also seasonally dynamic, it may be worth evaluating asymmetry in reproductive versus non-reproductive specimens specifically. Investigations in the two male morphs (type I and type II) could also prove informative.

Auditory contributions of the swim bladder – implications for directional hearing

Teleosts comprise more than 30,000 species, and hearing ability varies widely among the very small subset that have been studied experimentally (e.g. Ladich and Fay, 2013). While two fundamentally different modes of hearing (inertial acceleration and sound pressure reception) may still be identified, caveats against the reductionist dichotomy of hearing ‘specialists’ and ‘generalists’ (Popper and Fay, 2011) are underscored in the case of midshipman, wherein swim bladder–inner ear coupling varies both inter- and intra-sexually (Mohr et al., 2017). The AEP technique has been previously applied to assay contributions of the swim bladder in several species, with higher AEP thresholds following swim bladder deflation demonstrated in some species (such as the goldfish, *Carassius auratus*, a well-studied otophysan), but not others (such as *O. tau*) (Yan et al., 2000). To directly evaluate the contribution of the swim bladder to the AEP in midshipman, we manipulated swim bladder inflation dynamically within a single specimen via surgical intubation (Fig. 6) while repeatedly measuring the AEP at several different frequencies. This preparation (see Materials and Methods) points to interesting possibilities for future studies of swim bladder acoustic/auditory functionality, but in the present study served chiefly to demonstrate that contributions of the swim bladder could be measured in female midshipman via the AEP paradigm we employed (cf. Colleye et al. 2019, describing saccular potential measurements after swim bladder excision).

Further supporting a role for the swim bladder in midshipman hearing, response amplitudes were found to be significantly lower in fish that underwent both unilateral sagitta and swim bladder excision than in animals that underwent sagitta excision only. In some animals that underwent swim bladder and sagitta excision, responses fell to the noise floor at a standard test intensity of 130 dB SPL (but could be elicited at 140 dB SPL), whereas responses were elicited in all animals in which only the sagitta was removed at 130 dB SPL. This observation suggests that the elimination of phonotaxis following swim bladder deflation could relate simply to reduced detectability of the stimulus, versus more elaborate explanations, e.g. invoking the ‘phase model’ of directional hearing (Schuijf and Buwalda, 1975; Coffin et al., 2014). The general form of directional modulation did appear to be somewhat different for sagitta-removed animals versus sagitta+swim bladder-removed animals (tested at a higher intensity), but directional

modulation generally remained subtle. If the swim bladder acts as a truly omnidirectional receiver (cf. von Frisch and Dijkgraaf, 1935) it should add gain to responses at all azimuths. Barimo and Fine (1998) demonstrated that the acoustic radiation pattern of the toadfish swim bladder during sound production was notably directional. Future studies might evaluate whether the complex geometry of the swim bladder in fish such as female midshipman gives rise to receptive directionality, which could then interact with directionality intrinsic to the otoliths. Such experiments could further elucidate whether and in what fashion the swim bladder may contribute to directional hearing in fish.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.D.B., J.A.S.; Methodology: A.D.B., R.Z., J.A.S.; Software: A.D.B.; Validation: A.D.B.; Formal analysis: A.D.B.; Investigation: A.D.B., R.Z., J.A.S.; Resources: A.D.B., J.A.S.; Data curation: A.D.B.; Writing - original draft: A.D.B., J.A.S.; Writing - review & editing: A.D.B., R.Z., J.A.S.; Visualization: A.D.B.; Supervision: A.D.B., J.A.S.; Project administration: A.D.B., J.A.S.; Funding acquisition: A.D.B., J.A.S.

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