

## RESEARCH ARTICLE

# Auditory evoked potentials of utricular hair cells in the plainfin midshipman, *Porichthys notatus*

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## ABSTRACT

The plainfin midshipman, *Porichthys notatus*, is a soniferous marine teleost fish that generates acoustic signals for intraspecific social communication. Nocturnally active males and females rely on their auditory sense to detect and locate vocally active conspecifics during social behaviors. Previous work showed that the midshipman inner ear sacculle and lagena are highly adapted to detect and encode socially relevant acoustic stimuli, but the auditory sensitivity and function of the midshipman utricle remain largely unknown. Here, we characterized the auditory evoked potentials from hair cells in the utricle of non-reproductive type I males and tested the hypothesis that the midshipman utricle is sensitive to behaviorally relevant acoustic stimuli. Hair cell potentials were recorded from the rostral, medial and caudal regions of the utricle in response to pure tone stimuli presented by an underwater speaker. We show that the utricle is highly sensitive to particle motion stimuli produced by an underwater speaker positioned in the horizontal plane. Utricular potentials were recorded across a broad range of frequencies with lowest particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) thresholds occurring at 105 Hz (lowest frequency tested; mean threshold  $-32 \text{ dB re. } 1 \text{ m s}^{-2}$ ) and highest thresholds at 605–1005 Hz (mean threshold range  $-5$  to  $-4 \text{ dB re. } 1 \text{ m s}^{-2}$ ). The high gain and broadband frequency sensitivity of the utricle suggest that it likely serves a primary auditory function and is well suited to detect conspecific vocalizations including broadband agonistic signals and the multiharmonic advertisement calls produced by reproductive type I males.

**KEY WORDS:** Utricle, Tuning, Acoustic communication, Fish hearing

## INTRODUCTION

Soniferous teleost fishes rely on the auditory inner ear and lateral line to detect and encode behaviorally relevant, social acoustic signals (Bass and Ladich, 2008; Kelley and Bass, 2010; Ladich, 2004; Radford and Mensinger, 2014; Tricas and Webb, 2016). For these fishes, the otolithic end organs of the inner ear (sacculle, utricle and lagena) function as biological accelerometers that detect linear acceleration and respond to the fish's direct displacement by local particle motion (Fay, 1984; Platt and Popper, 1981; Popper and Fay, 1993; Schulz-Mirbach et al., 2018). Investigations into the auditory functions of the otolithic end organs have primarily focused on the

sacculle, which is considered to be the main organ of hearing in most fishes (Popper and Fay, 1993). Less is known about the functions of the lagena and utricle, but, in general, limited studies suggest that the lagena serves primarily an auditory function (Fay and Olsho, 1979; Lu et al., 2003; Meyer et al., 2004; Sand, 1974; Vetter et al., 2019) while the utricle may serve both an auditory and vestibular function (Boyle et al., 2001, 2018; Lu et al., 2004; Maruska and Mensinger, 2015; Riley and Moorman, 2000). Recently, Maruska and Mensinger (2015) showed in the soniferous oyster toadfish (*Opsanus tau*) that the utricle and its afferents are capable of detecting and encoding social acoustic signals, and that the toadfish inner ear utricle can serve both an auditory and vestibular function.

Some of the most extensively studied species of soniferous fishes are found in the Family Batrachoididae (toadfishes and midshipman fish). The plainfin midshipman (*Porichthys notatus*, Girard 1854) is a well-suited species to investigate mechanisms of acoustic communication because they have evolved a number of adaptations related to their physiology, endocrinology, morphology and behavior that help mediate intraspecific acoustic communication during social behaviors (Bass and McKibben, 2003; Coffin et al., 2012; Feng and Bass, 2017; Forlano et al., 2016; Mohr et al., 2017; Sisneros et al., 2004a). Plainfin midshipman are a nocturnally active marine fish that produce a relatively simple repertoire of acoustic signals during social and reproductive behaviors that include 'grunts', 'growls' and 'hums' (Bass et al., 1999; Sisneros, 2009a). Grunts are short-duration, broadband signals produced during aggressive and defensive interactions by all midshipman sexual phenotypes (females and males: type I and II) (Brantley and Bass, 1994; Ibara et al., 1983). Growls are long-duration, broadband agonistic signals produced only by type I nesting males during the breeding season in the context of territory and nest defense (Bass et al., 1999; Sisneros, 2009a), while hums are long-duration, multiharmonic advertisement signals produced only by breeding type I males to attract gravid females to nest sites for spawning (Bass and McKibben, 2003; Brantley and Bass, 1994; Forlano et al., 2016). Nocturnally active females rely on their auditory sense to detect and locate 'mate calling' males during the late-spring and summer reproductive season. Thus, the bioacoustic ecology and reproductive success of the plainfin midshipman depends on the production and reception of social acoustic signals.

The auditory sensitivity of the midshipman sacculle and lagena is known to be well-suited to detect conspecific vocalizations during the breeding season in all three sexual phenotypes (females and males: type I and II) (Bhandiwad et al., 2017; Rohmann and Bass, 2011; Sisneros, 2009b; Vetter et al., 2019). Previous studies showed that reproductive state-dependent changes occur in saccular sensitivity of females and males (type I and II) such that reproductive animals are highly tuned to detect and encode conspecific vocalizations (Bhandiwad et al., 2017; Rohmann and Bass, 2011; Sisneros, 2009b; Sisneros and Bass, 2003). In addition, recent work by Vetter et al. (2019) showed that the auditory

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sensitivity of the lagena is also well suited to detect and encode conspecific vocalizations, especially when close to the sound source. Although the auditory sensitivity of the saccule and lagena are both well established in the midshipman, the sensitivity and function of the utricle remains largely unknown.

The objective of this study was to characterize auditory evoked potentials from hair cells in the utricle of the plainfin midshipman and to test the hypothesis that the utricle is sensitive to behaviorally relevant acoustic stimuli. We focused on the utricular potentials of type I non-reproductive males to better understand the response characteristics of the utricle to auditory stimuli and to determine whether the utricle serves an auditory function. In addition, we compared the auditory evoked response characteristics of the utricle with those of the saccule and lagena from our previous studies and interpret our findings as they relate to the detection and reception of conspecific acoustic communication signals.

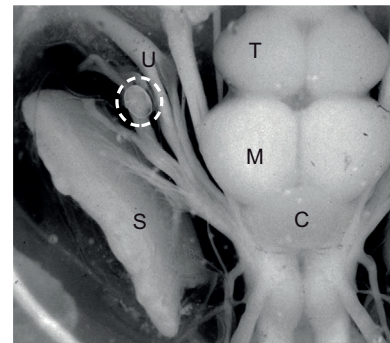
## MATERIALS AND METHODS

### Animal collection and husbandry

Non-reproductive adult plainfin midshipman fish, *Porichthys notatus* Girard 1854, were collected via otter trawls (*R/V John H. Martin*, Moss Landing Marine Laboratories) in Monterey Bay near Moss Landing, CA, USA, at depths ranging from 85 to 100 m during the non-reproductive midshipman season. Fish were then transported to the University of Washington where they were housed in 35 l recirculating saltwater tanks that were maintained at  $13\pm 2^\circ\text{C}$  and kept on a 9 h:15 h light:dark photoperiod. Before each physiology experiment, standard length (SL; cm) and body mass (BM; g) were recorded. Following each physiology experiment, sex via visual inspection of the gonads and gonadosomatic index [GSI, defined here as  $100 \times \text{gonad mass} / (\text{BM} - \text{gonad mass})$ ] according to Tomkins and Simmons (2002) were determined. The GSI and standard length of the fish used in the present study were consistent with those reported for type I male midshipman in previous physiology studies (Rohmann and Bass, 2011; Sisneros, 2007; Vetter et al., 2019). All utricular potential recordings were performed within 23 days following trawl collection to minimize any effects of captivity on auditory sensitivity. All experimental procedures conformed to NIH guidelines for animal care and use of animals and were approved by the University of Washington Institutional Animal Care and Use Committee.

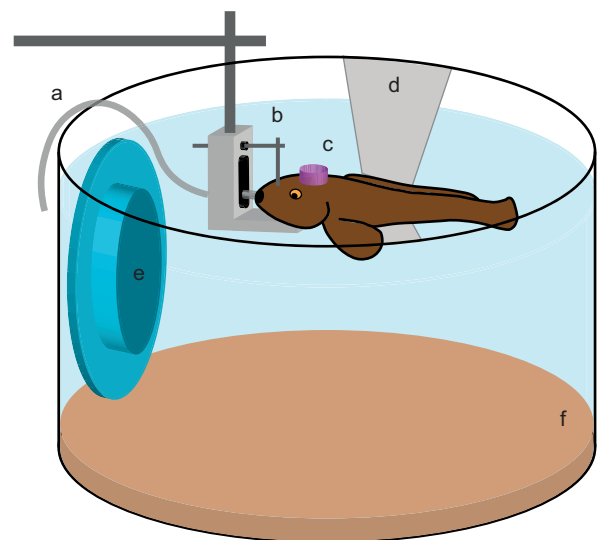
### Utricular potential measurements

The methodology for recording utricular hair cell potentials was similar to the technique used in previous studies that measured auditory evoked potentials from hair cells in the midshipman saccule and lagena (Alderks and Sisneros, 2011; Bhandiwad et al., 2017; Colley et al., 2019; Sisneros, 2007, 2009a; Vetter et al., 2019). Briefly, midshipman were first anesthetized by immersion in a 0.025% ethyl *p*-aminobenzoate (benzocaine) buffered saltwater bath and then given an intramuscular injection of cisatracurium besylate ( $\sim 3 \text{ mg kg}^{-1}$  of BM) and bupivacaine HCl ( $\sim 1 \text{ mg kg}^{-1}$  of BM) for immobilization and analgesia, respectively. Next, a craniotomy was performed on the dorsal surface of the skull to expose the right and left utricles, and then the cranial cavity and inner ear were filled with chilled teleost Ringer solution. Note that the position of the utricle is lateral to the caudal part of the telencephalon (forebrain) and the otolith (lapillus) lies in the horizontal plane in relation to the midshipman brain and head. In addition, the utricle is oriented in a plane that is approximately orthogonal to the plane of saccule orientation (approximately vertical) (see Fig. 1, but also see the following references for other



**Fig. 1. Dorsal view of brain and inner ear of the plainfin midshipman.** Dashed circle highlights the utricle (U). T, telencephalon; M, midbrain; C, cerebellum; S, saccule. Image from a type I male midshipman (standard length, SL 20.4 cm; body mass, BM 99.7 g). Note that the lapillus (utricle otolith) is positioned in the horizontal plane relative to the brain/head of the fish and is approximately orthogonal to the sagitta (saccular otolith), which has been slightly deflected laterally in the photo in order to better view the utricle and the auditory afferents of the saccule and utricle.

visual descriptions of the utricle and its orientation: Cohen and Winn, 1967; McKibben and Bass, 1999; Sisneros, 2009a,b). To prevent saltwater contamination of the inner ear during experimental testing, a hydrophobic barrier of approximately 4–5 cm height by 1 cm thickness made of denture adhesive cream (Fixodent, Proctor and Gamble Company, Cincinnati, OH, USA) was constructed around the craniotomy (Fig. 2c). The fish was then suspended using acoustically transparent film (Fig. 2d), which allowed the fish to be lowered below the water line in the center of the experimental tank (40 cm diameter, 20 cm water depth). The fish's head was then positioned using a custom-built acrylic head holder (Fig. 2b) such that the utricle and inner ear cavity were 4 cm below the water's surface. Once the fish was secured, a small silicone tube was then inserted in the buccal cavity of the fish so that chilled saltwater ( $13\text{--}15^\circ\text{C}$ ) could be perfused over the fish's gills throughout the experiment (Fig. 2a). For all experiments, the experimental testing



**Fig. 2. Schematic representation of experimental physiology tank.** For each physiology experiment, a fish was affixed and suspended 4 cm below the water's surface with the otic capsule 10 cm perpendicular from the face of the underwater speaker (e). Labels are as follows: a, respiration tube; b, head holder; c, hydrophobic water dam; d, Parafilm sling; f, sediment. Physiology tank dimensions: 40 cm diameter, 20 cm water depth.

tank was maintained on a vibration-isolation table (TMC Vibration Control, Peabody, MA, USA), which was situated inside a sound attenuation chamber (Industrial Acoustics, New York, NY, USA). All additional experimental equipment was maintained outside of the sound attenuation chamber.

The auditory evoked potentials of hair cells in the utricle were recorded using glass microelectrodes filled with 3 mol l<sup>-1</sup> KCl (impedance: 2.0–8.0 MΩ) that were visually guided into the otic capsule and positioned along rostral, medial or caudal regions of the utricle. Auditory evoked hair cell potentials were recorded from both left and right utricles. The analog evoked potential signals were pre-amplified (10×; Model 5A, Getting Instruments, San Diego, CA, USA), bandpass filtered (0.07 to 3 kHz) and then amplified (10×) via a digital filter (model SR650, Stanford Research Systems, Sunnyvale, CA, USA). Signals were then sent to a lock-in amplifier (SR830, Stanford Research Systems), which yielded an output signal that was proportional to the relative amplitude of the utricular hair cells' response to the pure tone stimulus frequency that was locked to the reference frequency. The reference frequency of the lock-in amplifier was set to the second harmonic of the stimulus frequency, which corresponds to the greatest evoked potentials due to populations of oppositely oriented hair cells in the inner ear of teleost fishes (Cohen and Winn, 1967; Furukawa and Ishii, 1967; Lozier and Sisneros, 2019; Sisneros, 2007). All data were stored on a computer that used a custom-written MATLAB script, which acquired data and controlled stimulus timing. Each experimental recording session began with control trials that measured electrical background noise conditions (no auditory stimulus present), which was then followed by stimulus trials at the various tested frequencies and amplitudes.

### Acoustic stimulus and calibration

The methodology used for acoustic stimulus presentation and calibration was similar to that used in previously published work (Alderks and Sisneros, 2011; Bhandiwad et al., 2017; Colley et al., 2019; Sisneros, 2007, 2009b; Vetter et al., 2019). Acoustic stimuli were generated by a lock-in amplifier (SR830, Stanford Research Systems), which sent pure-tone signals to an audio amplifier (BG-1120, TOA Corporation, Hyogo, Japan) and then to an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA). As the midshipman utricle end organs reside primarily within the *x–y* plane (Cohen and Winn, 1967; McKibben and Bass, 1999; Sisneros, 2009a,b) and the hair cells of the utricle are oriented within the horizontal plane (Coffin et al., 2012), the underwater speaker was positioned upright within a custom-fabricated speaker mount on the bottom of the experimental tank (40 cm diameter, 20 cm water depth) with the speaker submerged 2 cm below the water's surface (Fig. 2). Another reason why we chose to position the underwater speaker in the horizontal plane is because we were unable to record auditory evoked utricular potentials when we positioned the speaker beneath the animal (vertical axis of sound projection). The vertically oriented speaker produced particle motion stimuli primary along the vertical (*z*) axis of the water column, which is orthogonal to the horizontal orientation of the utricle, and resulted in no measureable auditory evoked hair cell potentials (i.e. the utricular potential measurements were no different from those of recorded electrical background levels with no sound stimuli). Acoustic stimuli consisted of single 500 ms pure tones repeated 8 times at a rate of one every 1.5 s. Acoustic stimuli were randomly presented at the following frequencies: 105, 125, 185, 205, 285, 305, 405, 605, 705, 805, 905 and 1005 Hz. The tested frequencies were chosen because they encompass the

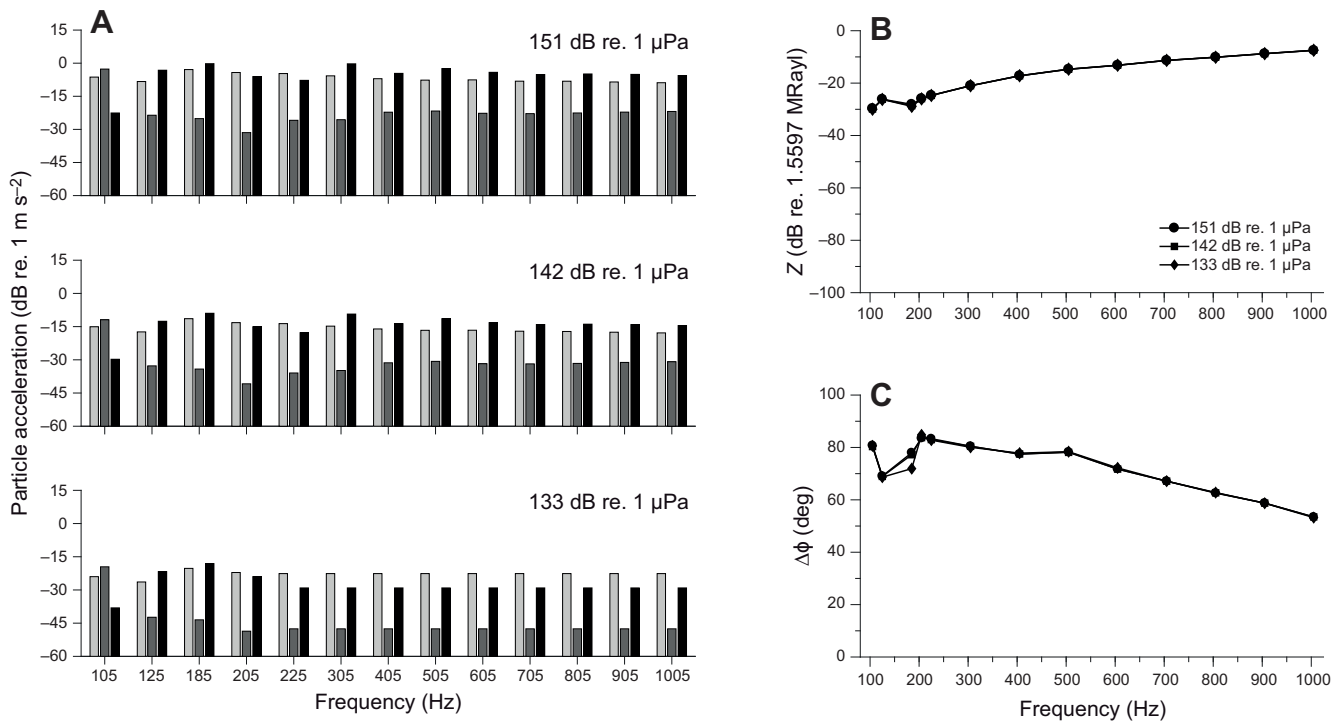
dominate bandwidth frequencies contained within type I male midshipman advertisement vocalizations and avoid any potential interference associated with acoustic tank resonance frequencies and electrical noise (60 Hz and its harmonics).

Prior to each physiology experiment, calibration of the acoustic stimuli was performed by positioning a mini-hydrophone (model 8103, Bruel and Kjaer, Naerum, Denmark), which was connected to a conditioning amplifier (gain=100 mV Pa<sup>-1</sup>, Nexis 2692-0S1, Bruel and Kjaer), 10 cm perpendicular from the face of the underwater speaker and 4 cm below the water's surface to coincide with the position of the midshipman inner ear during auditory evoked hair cell potential measurements. Acoustic stimuli were calibrated by measuring the peak-to-peak (*p–p*) voltage ( $V_{p-p}$ ) amplitude on an oscilloscope (Tektronix, Beaverton, OR, USA), and then equalized in sound pressure level (SPL; dB re. 1 μPa) using a custom-written MATLAB (MathWorks Inc.) script, which measured the power spectral density for all tested frequencies. The signal ( $V_{p-p}$ ) sent to the speaker was scaled until the measured peak-to-peak SPL (SPL<sub>*p–p*</sub>) output from the speaker was 130±0.5 dB re. 1 μPa.

Particle acceleration (dB re. 1 m s<sup>-2</sup>) measurements were conducted using a calibrated neutrally buoyant waterproofed triaxial accelerometer (Model VW3567A12; sensitivity at 100 Hz: 10.42 mV/m s<sup>-2</sup> (*x*-axis), 10.03 mV/m s<sup>-2</sup> (*y*-axis), 10.37 mV/m s<sup>-2</sup> (*z*-axis); PCB Piezotronics, Depew, NY, USA) that connected to a signal conditioner (Model: 482A16; PCB Piezotronics), which was used to amplify the signal (gain=×100/axis). Measurements were then sent to a data acquisition system (Model NI USB-6009; National Instruments, Austin, TX, USA) and visualized using LabVIEW software (National Instruments). Particle acceleration (dB re. 1 m s<sup>-2</sup>) measurements were made by placing the triaxial accelerometer 4 cm below the water's surface and 10 cm perpendicular from the cone of the speaker, which corresponded with position of the midshipman inner ear during testing, and was calibrated in response to each tested frequency across the entire intensity range. Using a custom-written LabVIEW (National Instruments) script, particle motion amplitude measurements ( $V_{p-p}$ ) for each axis (*x*-, *y*- and *z*-axis) were corrected for the gain (sensitivity) of the accelerometer. Fig. 3A illustrates the variation of stimulus particle acceleration (dB re. 1 m s<sup>-2</sup>) along the *x*-, *y*- and *z*-axes at three sound pressure levels (dB re. 1 μPa) tested within our experimental tank.

### Acoustic impedance measurements

The small dimensions (40 cm diameter, 20 cm water depth) and material (Nalgene plastic) of the experimental testing tank directly influenced the acoustic environment in which auditory evoked potential recordings were performed. Therefore, as suggested by Popper and Fay (2011) and more recently by Popper et al. (2019), the acoustic impedance (*Z*) of the experimental tank environment should be measured and compared with the acoustic impedance of seawater in a free-field environment, thus allowing for more meaningful comparisons of different experimental tank acoustic environments in other physiology and behavior studies. The *Z* is the complex ratio of sound pressure to particle velocity and is expressed in Rayls (where 1 Rayl=1 Pa s m<sup>-1</sup>) and was determined in the experimental test tank across all tested frequencies at three sound pressure levels (151, 142 and 133 dB re. 1 μPa). The experimental tank's *Z* was measured and then compared with the *Z* of 'theoretical seawater' ( $Z_{\text{theoretical seawater}}=1.559$  MRayls) in a free-field environment with a salinity of 35 ppt at 15°C (Bradley and Wilson, 1966; Erbe, 2011). Additionally, the phase ( $\phi$ ) of the complex *Z* was also determined across all test frequencies at three



**Fig. 3. Acoustic characteristics of the experimental speaker and tank.** (A) Particle acceleration levels from the underwater speaker measured along each axis (x-axis: light gray; y-axis: dark gray; z-axis: black). (B) Acoustic impedance ( $Z$ ), which is the complex ratio of sound pressure to particle velocity and is expressed in Rayls (1 Rayl=1 Pa s<sup>-1</sup>). (C) Phase difference ( $\Delta\phi$ ) between the pressure and particle velocity wave. All measurements were made using a triaxial accelerometer placed in the center of the tank at the position of the fish head during testing. Additionally, measurements were made at three sound pressure levels (SPL; 151, 142 and 133 dB re. 1 μPa) for all tested frequencies (105, 125, 185, 205, 225, 305, 405, 505, 605, 705, 805, 905 and 1005 Hz).

sound pressure levels (151, 142 and 133 dB re. 1 μPa) by comparing the phase difference between the particle velocity and sound pressure waves. All measurements and analyses for  $Z$  and  $\phi$  of the complex acoustic impedance were similar to those in previously published studies (Colleye et al., 2019; Vetter et al., 2019).

The  $Z$  of our experimental tank was determined by simultaneously measuring the sound pressure (dB re. 1 μPa) and particle acceleration (dB re. 1 m s<sup>-2</sup>) for each tested frequency. Simultaneous measurements were conducted at the position that would normally be occupied by the midshipman inner ear during the physiology experiment using a mini-hydrophone (model 8103, Bruel and Kjaer) connected to a conditioning amplifier (gain=100 mV Pa<sup>-1</sup>, Nexis 2692-0S1, Bruel and Kjaer) to record sound pressure (dB re. 1 μPa), whereas particle acceleration (dB re. 1 m s<sup>-2</sup>) was measured using a calibrated neutrally buoyant waterproofed triaxial accelerometer [Model VW3567A12; sensitivity at 100 Hz: 10.42 mV/m s<sup>-2</sup> ( $x$ -axis), 10.03 mV/m s<sup>-2</sup> ( $y$ -axis), 10.37 mV/m s<sup>-2</sup> ( $z$ -axis); PCB Piezotronics] connected to a signal conditioner (Model: 482A16; PCB Piezotronics) that amplified the particle acceleration signal (gain= $\times 100$ /axis). Particle acceleration (dB re. 1 m s<sup>-2</sup>) and sound pressure (dB re. 1 μPa) measurements were recorded using a data acquisition system (NI myDAQ 16 bit analog to digital conversion at 200 kS s<sup>-1</sup>, National Instruments) that was controlled by a custom-written program in LabVIEW software (NI LabVIEW 2016, National Instrument). Analysis of the complex acoustical impedance followed Colleye et al. (2019) and Vetter et al. (2019).

The complex phase of  $Z$  is equal to the phase difference ( $\Delta\Phi_{p,v}$ ) between the particle velocity ( $v$ ) and the pressure ( $p$ ). The phase ( $\Phi$ ) of the complex  $Z$  in our experimental test tank was determined by measuring the phase difference ( $\Delta\Phi$ ) between the particle

acceleration ( $a$ ) and pressure ( $p$ ), where  $\Delta\Phi_{p,a} = \Phi_p - \Phi_a$ . All measurements were recorded with a data acquisition system (NI myDAQ 16 bit analog to digital conversion at 200 kS s<sup>-1</sup>, National Instruments) that was controlled by a custom-written program in LabVIEW software (NI LabVIEW 2016, National Instruments). For sinusoid waves, such as the pure tones examined in our study, the phase of particle acceleration ( $a$ ) will always lead the phase of particle velocity ( $v$ ) by 90 deg. Therefore, the phase difference ( $\Delta\Phi_{p,v}$ ) between the particle velocity and acoustic pressure waves was determined by:

$$\Delta\Phi_{p,v} = \Delta\Phi_{p,a} + 90. \quad (1)$$

All measurements were within the near-field approximation; however, we do not expect a simple relationship between velocity and pressure because of the complex nature of our experimental tank conditions. Fig. 3 displays both the  $Z$  (Fig. 3B) and  $\Delta\Phi_{p,v}$  (Fig. 3C) at all frequencies examined for three sound pressure levels (151, 142 and 133 dB re. 1 μPa) along the  $x$ -axis (rostral-caudal). Acoustic impedance and  $\Delta\Phi_{p,v}$  along the  $y$ - (lateral) and  $z$ -axes (dorsal-ventral), respectively, are also provided (Fig. S1).

### Analyses

The auditory threshold tuning curves for utricular potentials based on particle acceleration (dB re. 1 m s<sup>-2</sup>) and sound pressure (dB re. 1 μPa) were determined via input-output measurements of the evoked utricular hair cell potentials over the range of tested frequencies and amplitudes. The recorded acoustic noise floor measurements were used to establish the subthreshold levels for the utricular potentials ( $-71 \pm 1$  dB re. 1 m s<sup>-2</sup>;  $76 \pm 1$  dB re. 1 μPa). The auditory threshold for utricular potentials was defined as the lowest

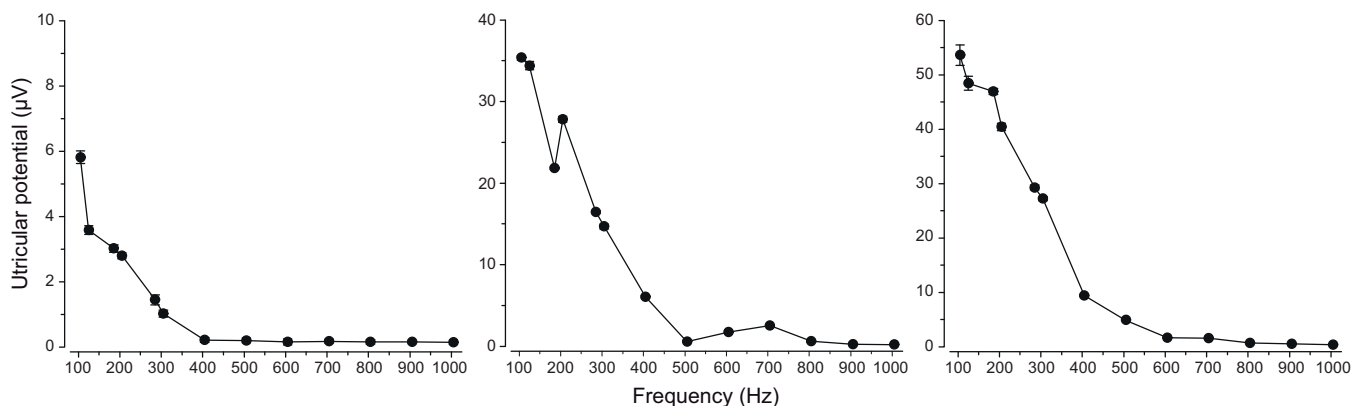
stimulus level that yielded a mean utricular evoked potential that was greater than two standard deviations above the background electrical noise measurement. The frequency that evoked the lowest utricular threshold was defined as the characteristic frequency (CF), while best frequency (BF) was defined as the frequency that elicited the highest utricular potential voltage in the iso-intensity analyses. To determine whether individuals' best frequencies differed across recording regions (rostral, medial and caudal), a non-parametric Friedman test was conducted, because of normality violations (Shapiro–Wilk normality test;  $W=0.58$ ,  $P<0.001$ ). Particle acceleration level (dB re.  $1 \text{ m s}^{-2}$ ) thresholds were calculated as the combined magnitude vector of particle acceleration in dB scale (Bhandiwad et al., 2017; Colley et al., 2019; Rogers et al., 2020; Vasconcelos et al., 2010; Vetter, 2019; Wysocki et al., 2009) as follows:

$$\text{Particle acceleration level} = 20 \log_{10} \left( \sqrt{x^2 + y^2 + z^2} \right). \quad (2)$$

## RESULTS

Auditory evoked utricular potentials were recorded from 15 adult, non-reproductive type I male midshipman fish with SL that ranged from 15.8 to 24.3 cm ( $20.4 \pm 2.6$  cm mean  $\pm$  s.d.), BM that ranged from 46.0 to 183.2 g ( $99.7 \pm 43.4$  g) and GSI that ranged from 0.3 to 2.6 ( $1.5 \pm 0.6$ ). All adult type I males tested in this study were within the size range reported in previous physiology studies for type I male midshipman (Rohmann and Bass, 2011; Sisneros, 2007; Vetter et al., 2019).

Auditory thresholds for both particle acceleration level (dB re.  $1 \text{ m s}^{-2}$ ) and SPL (dB re.  $1 \mu\text{Pa}$ ) were determined for populations of hair cell receptors in rostral ( $n=10$  records), medial ( $n=11$  records) and caudal ( $n=10$  records) regions of the utricle. Evoked utricular hair cell potentials were recorded in response to SPLs that ranged from 106 to 154 dB re.  $1 \mu\text{Pa}$ . Fig. 4 illustrates representative iso-intensity response profiles of utricular hair cell potentials in response to pure tones (105–1005 Hz) at the highest sound level tested (154 dB re.  $1 \mu\text{Pa}$ ). The iso-intensity response curves of the utricle consisted of BFs that ranged from 105 to 205 Hz, with the majority (52%) occurring at 105 Hz (Fig. 5). Because individuals' BF did not differ across recording regions (Friedman test,  $\chi^2=4.43$ , d.f.=2,  $P=0.11$ ), iso-intensity response curves from all three regions were grouped for further analysis. Additionally, a subset of recordings ( $n=12$  animals, 18 records) displayed a prominent secondary peak ranging from 185 to 505 Hz, with the majority (44%) occurring at 205 Hz (Fig. 5).



**Fig. 4.** Three representative examples of iso-intensity level curves recorded from utricular hair cells in response to single tone playbacks at a SPL of 154 dB re.  $1 \mu\text{Pa}$ . Thresholds were defined as the lowest SPL (dB re.  $1 \mu\text{Pa}$ ) needed to evoke receptor potentials at least 2 s.d. above the background electrical noise level. Data are represented as means  $\pm$  95% confidence interval; note that the confidence intervals are very small, and the bars may be obscured by the symbols.

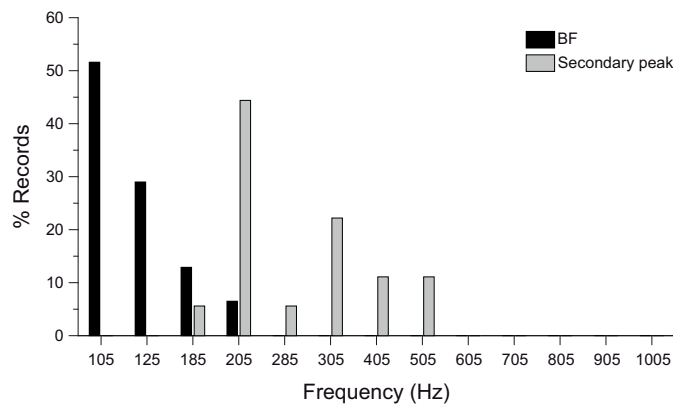
Auditory threshold curves based on particle acceleration level (dB re.  $1 \text{ m s}^{-2}$ ) and SPL (dB re.  $1 \mu\text{Pa}$ ) were constructed from utricular potentials recorded from rostral, medial and caudal regions of the utricle. Fig. 6 illustrates representative individual auditory threshold tuning curves based on particle acceleration level (dB re.  $1 \text{ m s}^{-2}$ ) and SPL (dB re.  $1 \mu\text{Pa}$ ). Across all recordings, the CFs ranged from 105 to 205 Hz for both particle acceleration level (median CF 105 Hz) and SPL (median CF 105 Hz) tuning curves (Fig. 7). Lowest utricular auditory thresholds occurred at 105 Hz (lowest frequency tested; mean particle acceleration threshold  $-32$  dB re.  $1 \text{ m s}^{-2}$ , mean sound pressure threshold 119 dB re.  $1 \mu\text{Pa}$ ) and gradually rose to highest threshold levels at 605–1005 Hz (mean particle acceleration threshold range  $-5$  to  $-4$  dB re.  $1 \text{ m s}^{-2}$  and mean sound pressure threshold range 146 to 150 dB re.  $1 \mu\text{Pa}$ ) (Fig. 7).

In addition, utricular potentials were consistently ( $\geq 95\%$ ) recorded at sound levels [relative to particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) and sound pressure (dB re.  $1 \mu\text{Pa}$ )] above threshold at frequencies from 105 to 705 Hz in the 31 recordings collected from the 15 non-reproductive type I males (Fig. 8). The percentage of recordings with evoked utricular potentials at sound levels above threshold from 805 to 1005 Hz decreased from 84% to 65%. In sum, relatively high percentages (84–95%) of evoked utricular potentials were recorded across a range of frequencies from 105 to 805 Hz.

## DISCUSSION

The goal of this study was to characterize the auditory evoked potentials of hair cells in the utricle of non-reproductive type I male midshipman to test the hypothesis that the utricle is sensitive to behaviorally relevant acoustic stimuli. We show based on the utricular tuning profiles for particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) and sound pressure (dB re.  $1 \mu\text{Pa}$ ) that the utricle is highly sensitive to a broad range of behaviorally relevant, particle motion stimuli in the horizontal plane and that the midshipman utricle is capable of detecting the dominant higher frequencies contained within conspecific social signals.

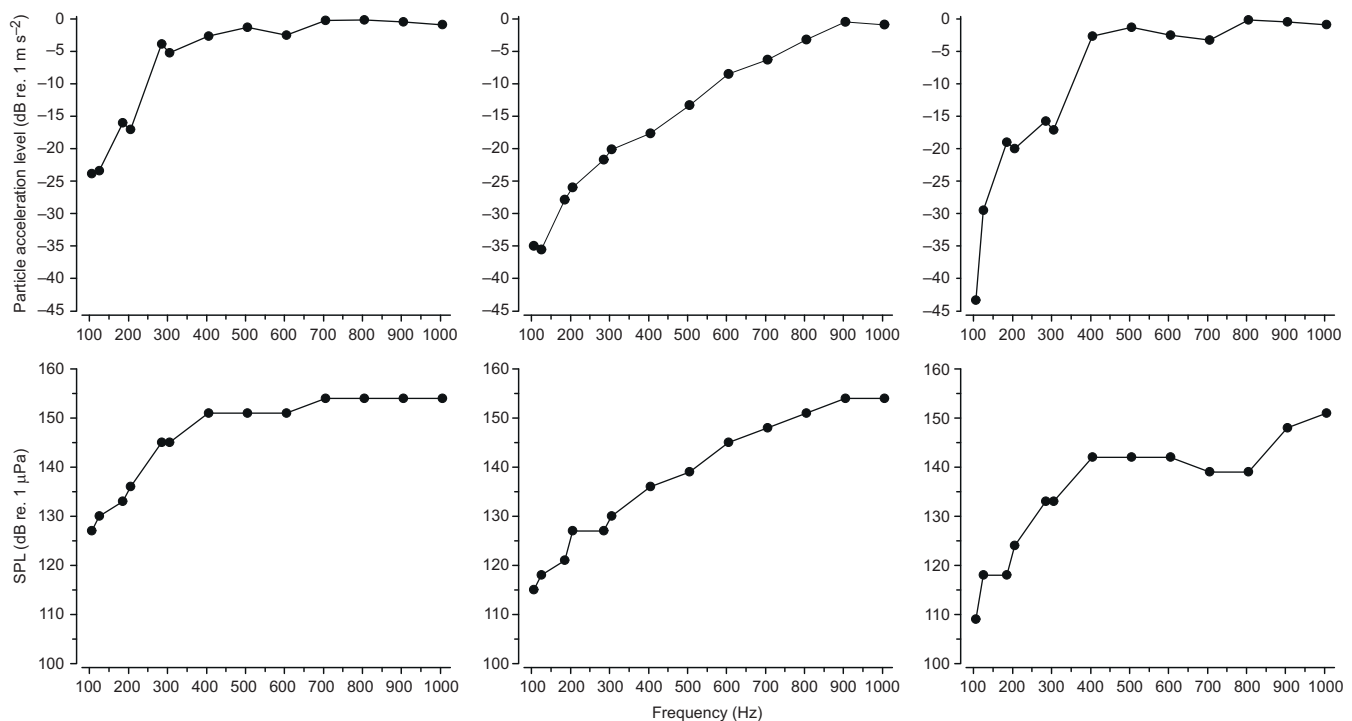
The utricle of the midshipman, like the other inner ear end organs, contains a dense calcium carbonate otolith that rests on a sensory bed of hair cells, which acts as an inertial accelerometer that is sensitive to particle motion and responds to linear acceleration. We show that the utricle is relatively sensitive to particle motion across a broad range of frequencies with lowest particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) thresholds occurring at 105 Hz (mean threshold  $-32$  dB re.



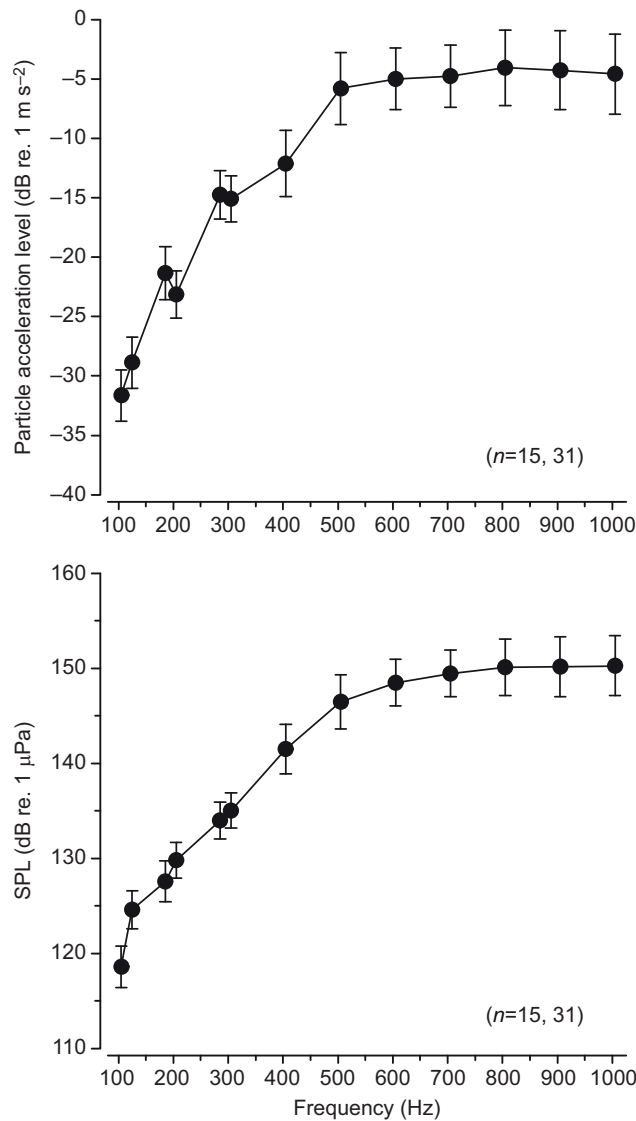
**Fig. 5. Best frequency and secondary peak histograms of the midshipman utricular potentials evoked in response to 154 dB re. 1  $\mu$ Pa pure tones.** Best frequency (BF) was defined as the frequency that elicited the highest utricular potential voltage in the iso-intensity analyses, while the secondary peak was characterized as the second highest utricular potential for recordings that had more than one prominent peak present in their iso-intensity level curve.

1  $\text{m s}^{-2}$ ) and highest thresholds between 605 and 1005 Hz (mean threshold range  $-5$  to  $-4$  dB re. 1  $\text{m s}^{-2}$ ) (Fig. 7). Surprisingly, utricular particle motion (dB re. 1  $\text{m s}^{-2}$ ) sensitivity of type I males is remarkably similar to that of the saccule in type I males at frequencies  $\leq 305$  Hz; however, at frequencies  $>305$  Hz, the utricle may be even more particle motion (dB re. 1  $\text{m s}^{-2}$ ) sensitive than the saccule, at least in type I males (Colley et al., 2019) (Fig. 9). Another important difference in particle motion (dB re. 1  $\text{m s}^{-2}$ ) sensitivity between the utricle and saccule is the directional axis of sensitivity. The utricle is oriented in the horizontal plane (see Fig. 1; but also see CT scans for *P. notatus* in the Virtual Natural History

Museum: <http://131.220.133.140/VNHM/>), with the utricular hair cells also oriented in the horizontal plane ( $x$ - and  $y$ -axes) (and see fig. 6 in Coffin et al., 2012); thus, the utricle is likely to be highly directionally sensitive to particle motion stimuli in the horizontal plane. Here, we show based on utricular potential thresholds that the utricle was highly sensitive to the particle motion stimuli produced by the underwater speaker positioned in the horizontal plane, which emitted the majority of the particle motion magnitude in the  $x$ - and  $y$ -axis (Fig. 3). In contrast, the midshipman saccule is primarily oriented in the vertical plane ( $z$ -axis), with hair cell orientation patterns in both the vertical and horizontal planes ( $x$ - and  $y$ -axis), with a corresponding directional sensitivity in both the vertical and horizontal planes (Weeg et al., 2002; Coffin et al., 2012). Thus, the horizontal directional sensitivity and high gain of the utricle likely complement the directional sensitivity and gain of the saccule to enhance the ability of the midshipman inner ear to detect and localize biologically relevant acoustic stimuli including conspecific vocalizations. Previous work has shown that reproductive state-dependent changes occur in the saccular sensitivity of males (type I and II) and females such that reproductive animals become better suited than non-reproductive animals to detect conspecific vocalizations (Bhandiwad et al., 2017; Rohmann and Bass, 2011; Sisneros, 2009b; Sisneros and Bass, 2003). Our data suggest that the particle motion (dB re. 1  $\text{m s}^{-2}$ ) sensitivity of the utricle in non-reproductive type I males is already well suited to detect conspecific type I male vocal signals including the broadband agonistic growls and multiharmonic advertisement calls. Future studies that employ a shaker table system such as that used by Fay (1984) will be needed to verify the directional sensitivity of the utricle. In addition, future investigations that examine reproductive state-dependent changes in utricular sensitivity will be instrumental in determining whether midshipman also exhibits seasonal enhancement of utricular sensitivity for the detection of social relevant acoustic stimuli.

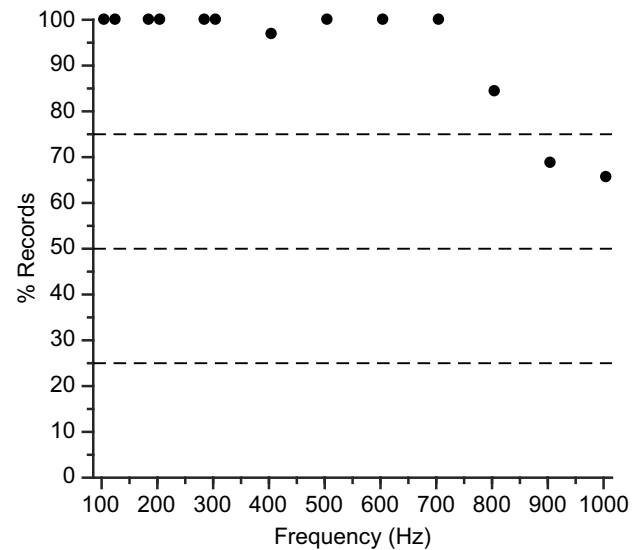


**Fig. 6. Representative auditory threshold tuning curves for particle acceleration level (top) and SPL (bottom).** Tuning curves were constructed using the non-reproductive type I male midshipman utricular evoked response. Thresholds were defined as the lowest SPL (dB re. 1  $\mu$ Pa) needed to evoke a utricular potential that was at least 2 s.d. above the background electrical noise level.



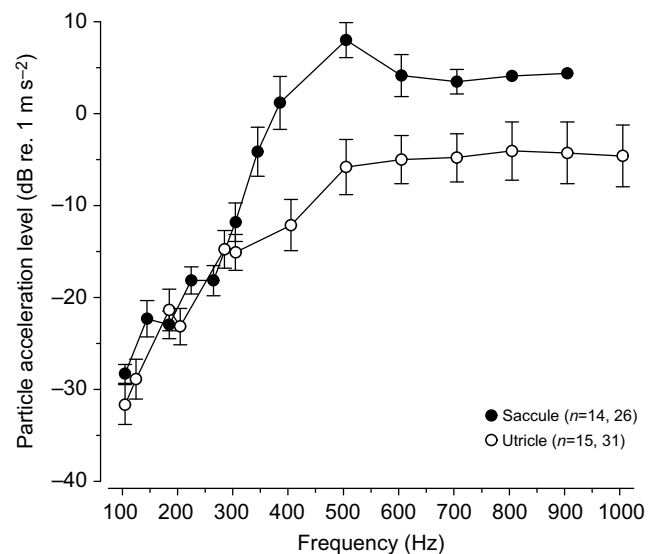
**Fig. 7. Particle acceleration level (top) and SPL (bottom) auditory threshold tuning curves recorded from midshipman utricular hair cells.** The auditory thresholds were defined as the lowest auditory stimulus level needed to evoke utricular potentials that were at least 2 s.d. above the background electrical noise level. All data are plotted as means $\pm$ 95% confidence interval. The number of animals and records is indicated in parentheses.

The low-pass filter tuning characteristics of the midshipman utricle reported in this study were similar to those reported for other teleost fishes. Lu et al. (2004) showed using a shaker table system that the non-soniferous sleeper goby (*Dormitator latifrons*) had similar low-pass tuning characteristics for the utricular afferents, which exhibited CFs ranging from  $\leq 50$  to 400 Hz with a median CF of 80 Hz. Lu et al. (2004) also reported that the best sensitivity of the utricular afferents occurred along the horizontal axis and ranged from  $-70$  to  $-40$  dB re.  $1g$  with a mean particle acceleration threshold of  $-52$  dB re.  $1g$ , which was about 30 dB less sensitive than that reported for sleeper goby saccular afferents (Lu et al., 2010). In the sleeper goby, the mean particle acceleration threshold (mean  $-52$  dB re.  $1g$ ) of utricular afferents was similar to the mean particle acceleration threshold for utricular potentials in midshipman (most sensitive frequency: 105 Hz, mean threshold  $-32$  dB re.  $1m s^{-2}$ , or approximately  $-52$  dB re.  $1g$ ). In addition,



**Fig. 8. Distribution of the percentage of utricular potential recordings that displayed evoked potential thresholds above background electrical noise levels for each frequency tested.** Note that utricular potentials were consistently ( $\geq 95\%$ ) recorded at sound levels [relative to particle acceleration (dB re.  $1m s^{-2}$ ) and SPL (dB re.  $1\mu Pa$ )] above threshold at frequencies from 105 to 705 Hz in the 31 recordings collected from the 15 non-reproductive type I males; however, they were not always detected, even at the highest SPL (dB re.  $1\mu Pa$ ) tested, for test frequencies  $>705$  Hz.

the range of CFs reported for midshipman utricular potentials (105–205 Hz) was similar to and overlapped with that reported for the sleeper goby (Lu et al., 2004). Similarly, Maruska and



**Fig. 9. Particle acceleration sensitivity comparison of type I male plainfin midshipman saccular and utricular hair cell auditory threshold tuning curves.** Saccular auditory thresholds were recorded from reproductive type I males that were collected during the reproductive summer but were held in captivity for greater than 2 months before the auditory thresholds of the saccule were measured (Colley et al., 2019). Therefore, the saccular thresholds for reproductive type I males may be less sensitive than saccular thresholds recorded from recently collected summer reproductive type I males. The auditory thresholds were defined as the lowest auditory stimulus level needed to evoke utricular potentials that were at least 2 s.d. above the background electrical noise level. All data are plotted as means $\pm$ 95% confidence interval. The number of animals and records is indicated in parentheses. Auditory saccular threshold tuning data for reproductive type I males was adapted from Colley et al. (2019).

Mensing (2015) showed that utricular afferents in free-swimming oyster toadfish (*Opsanus tau*) responded best to low frequencies from 80 to 200 Hz and were sensitive to the playbacks of conspecific boatwhistles and grunts, which had fundamental frequencies that ranged from 80 to 180 Hz. Although there is very limited data regarding the response characteristics of the utricle in fishes, our midshipman data and that of the toadfish and sleeper goby suggest the auditory utricle is highly sensitive to low-frequency linear acceleration in the horizontal plane and that the utricle is capable of detecting conspecific vocalizations. Whether these utricular response characteristics are conserved in other various fish species needs to be examined in future work.

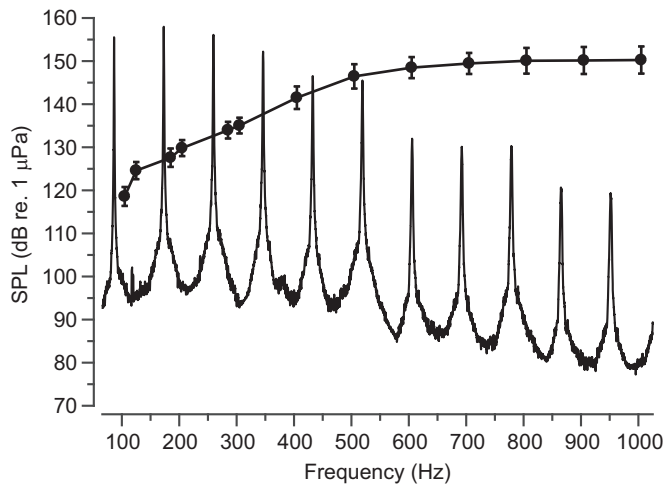
The results from our study also indicate that the midshipman utricle has a similar frequency response range to that of the midshipman lagena; however, the auditory thresholds based on particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) and sound pressure (dB re.  $1 \mu\text{Pa}$ ) are considerably lower for the utricle (i.e. more sensitive) than those reported for the midshipman lagena (Vetter et al., 2019). Lowest particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) thresholds for the lagena in non-reproductive type I males occurred at 85 Hz (mean threshold  $-9.7 \text{ dB re. } 1 \text{ m s}^{-2}$ ) and 125 Hz (mean threshold  $-4.3 \text{ dB re. } 1 \text{ m s}^{-2}$ ), while the highest thresholds for the lagena occurred at 165 Hz (mean threshold  $7.3 \text{ dB re. } 1 \text{ m s}^{-2}$ ) with particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) thresholds decreasing to mean threshold levels of 0.05 to  $3.4 \text{ dB re. } 1 \text{ m s}^{-2}$  from 205 to 505 Hz (Vetter et al., 2019). The recent work by Vetter et al. (2019) suggests that the relatively high thresholds of the lagena may be important for the detection of high intensity levels of behaviorally relevant acoustic stimuli close to a sound source when the saccule and its afferents are likely overstimulated and saturated. In contrast, the particle acceleration thresholds (dB re.  $1 \text{ m s}^{-2}$ ) of the utricle in non-reproductive type I males were very similar to those of the saccule in reproductive type I males at frequencies  $\leq 305 \text{ Hz}$ , but at frequencies  $> 305 \text{ Hz}$  the utricle may be even more sensitive (Colley et al., 2019) (Fig. 9). One possible explanation for this difference in particle motion sensitivity at frequencies  $> 305 \text{ Hz}$  between the utricle in non-reproductive type I males and the saccule of reproductive type I males may be the different times at which saccular recordings from reproductive type I males were made. In the study by Colley et al. (2019), reproductive type I males were collected during the summer but were held in captivity for greater than 2 months before the auditory thresholds of the saccule were measured. Sisneros and Bass (2003) showed that reproductive midshipman maintained in captivity longer than 25 days exhibit decreased saccular sensitivity to frequencies greater than 300 Hz. Thus, the saccular thresholds for type I males reported by Colley et al. (2019) may actually be higher (i.e. less sensitive) than saccular thresholds from recently collected summer reproductive type I males. Alternatively, the differences in particle acceleration (dB re.  $1 \text{ m s}^{-2}$ ) thresholds between the utricle and saccule at frequencies  $> 305 \text{ Hz}$  in type I males may be related to differences in the intrinsic response properties of the hair cells in the two different auditory end organs. We show based on iso-intensity response curves that the utricles of non-reproductive type I males exhibited BFs that ranged from 105 to 205 Hz with the majority of BFs (52%) occurring at 105 Hz. In addition, a number of the utricle recordings from type I males exhibited a prominent secondary peak in the evoked potentials at frequencies that ranged from 185 to 505 Hz, with the majority of the secondary peaks occurring at 205 Hz (Fig. 5). In contrast, the saccules of non-reproductive midshipman (females and type I males) exhibited BFs that ranged from 75 to 145 Hz, with majority of BFs ( $> 63\%$ ) occurring at 75 Hz (Colley et al., 2019;

Sisneros, 2007). A prominent secondary peak in the iso-intensity response curves was also observed in the saccular recordings of non-reproductive midshipman that ranged from 95 to 205 Hz, with the majority of secondary peaks occurring at 135 to 145 Hz (mean 140 Hz) (Sisneros, 2007). Future studies that investigate the intracellular recordings of hair cells from the midshipman inner ear will provide valuable insight into whether the electrical tuning properties of hair cells are different between the utricle and saccule.

The recent work by Colley et al. (2019) showed that the plainfin midshipman is capable of pressure-mediated hearing through the use of its swim bladder, which can aid in the reception of sound pressure components of acoustic signals. Both female and type II male midshipman possess prominent horn-like extensions on the rostral ends of their swim bladders that decrease the distance between the swim bladder and the individual auditory end organs (saccule, lagena and utricle) (Mohr et al., 2017). The mean distance between the swim bladder and the saccule was less than 3 mm (mean distance in females 2.6 mm, mean distance in type II males 2.0 mm), which was half the distance for the same measurement in type I males (mean swim bladder-to-saccule distance 5.2 mm) (Mohr et al., 2017). In addition, the mean distance between the swim bladder and the lagena was also less than 3 mm (mean distance in females 2.9 mm; mean distance in type II males 2.3 mm), which was also approximately half the distance between swim bladder and lagena in type I males (mean distance 4.7 mm) (Mohr et al., 2017). This decreased distance between the swim bladder and the inner ear end organs allows the sound pressure-induced vibrations of the swim bladder to be detected by the particle motion-sensitive otolithic end organs. Colley et al. (2019) showed that in females, the rostral swim bladder extensions enhance saccular and lagenar sensitivity to sound pressure (dB re.  $1 \mu\text{Pa}$ ) and extend the upper bandwidth limit of frequency sensitivity to 1005 Hz. In other pressure-sensitive fishes, increased sensitivity to sound pressure (dB re.  $1 \mu\text{Pa}$ ) and higher frequencies is often associated with the swim bladder being in close proximity ( $< 3 \text{ mm}$ ) to the otic capsule that contains the auditory end organs (Kéver et al., 2014; Ramcharitar and Popper, 2004; Schulz-Mirbach et al., 2012). Although the mean distance between the swim bladder and the utricle is much greater than 3 mm in type I male midshipman (mean distance 8.8 mm), the mean distance between the swim bladder and the utricle in females and type II males is considerably closer (mean swim bladder–utricle distance in females 5.2 mm; in type II males 5.0 mm) (Mohr et al., 2017). Interestingly, the mean swim bladder–utricle distance in females and type II males is approximately the same distance between the saccule and swim bladder in type I males (mean distance 5.2 mm), which lack swim bladder horn extensions (Mohr et al., 2017). Whether the utricle of females and type II males is close enough to detect pressure-induced vibrations of the swim bladder and enhance utricular sensitivity to acoustic sound pressure and higher frequencies remains to be determined.

The ability to perceive behaviorally relevant social acoustic signals is critical for the reproductive success and bioacoustic ecology of the plainfin midshipman. Nocturnally active male and female midshipman rely on their auditory sense to detect and locate vocally active conspecifics during social behaviors. Previous work showed that the auditory system of the midshipman is highly adapted to detect and encode socially relevant acoustic stimuli. Males (type I and II) and females exhibit an adaptive form of auditory plasticity whereby reproductive state-dependent changes in gonadal steroids (testosterone and estrogen) act to lower the auditory thresholds (i.e. increase the sensitivity) of saccular hair cells and





**Fig. 10. Comparison of the type I male midshipman advertisement call and utricle hair cell threshold tuning curve.** Advertisement calls were recorded from a reproductive type I male midshipman (SL 19.2 cm; BM 101.2 g) collected during the summer at Seal Rock near Brinnon, WA, USA, at low tide. Recordings of the male advertisement call were made at night in a large, indoor concrete tank (3 m diameter; 14.1°C) at the University of Washington Friday Harbor Laboratories. Source level recordings were made using a mini-hydrophone that was placed directly in front of the entrance of an artificial nest. The fundamental frequency of the advertisement call was 87 Hz (155 dB re. 1  $\mu$ Pa) with dominant harmonics occurring at the following frequencies: 173 Hz (158 dB re. 1  $\mu$ Pa), 260 Hz (156 dB re. 1  $\mu$ Pa), 346 Hz (152 dB re. 1  $\mu$ Pa), 433 Hz (147 dB re. 1  $\mu$ Pa), 519 Hz (146 dB re. 1  $\mu$ Pa), 606 Hz (132 dB re. 1  $\mu$ Pa), 692 Hz (130 dB re. 1  $\mu$ Pa), 779 Hz (130 dB re. 1  $\mu$ Pa), 865 Hz (121 dB re. 1  $\mu$ Pa) and 952 Hz (119 dB re. 1  $\mu$ Pa).

their afferents by 7–14 dB (re. 1  $\mu$ Pa) over a broad range of frequencies that include the dominant higher harmonic components of advertisement and agonist calls produced by type I males (Rohmann and Bass, 2011; Sisneros, 2009a; Sisneros and Bass, 2003; Sisneros et al., 2004b). The detection of the dominant high-frequency components of midshipman vocalizations is important for acoustic communication because primarily only the higher acoustic frequencies (above the fundamental frequency of most midshipman vocal signals) propagate in the shallow water breeding environments (Bass and Clark, 2003; Forlano et al., 2016; Sisneros, 2009b). As previously mentioned, work by Vetter et al. (2019) showed that the lagena is also well adapted to detect and encode a broad range of frequencies similar to those of the saccule, but with much higher thresholds across the same bandwidth of frequency sensitivity. The relatively high auditory thresholds of the lagena may extend the dynamic sensitivity range of the inner ear and be useful for detecting high intensity levels of behaviorally relevant acoustic stimuli when close to a sound source (Khorevin, 2008; Lu et al., 2003, 2004; Vetter, 2019; Vetter et al., 2019). In contrast to the lagena, the midshipman utricle is highly sensitive to particle acceleration (dB re. 1  $\text{m s}^{-2}$ ) and its particle motion sensitivity is similar to that of the saccule at frequencies <305 Hz, and potentially even more sensitive than the saccule at frequencies from 305 to 1005 Hz (Colleye et al., 2019). The high gain and broadband frequency sensitivity of the utricle suggest that the midshipman utricle is also well suited to detect conspecific vocal signals including broadband agonistic signals and the multiharmonic advertisement calls produced by reproductive type I males (Fig. 10). Although the lagena and utricle were previously thought to serve as accessory end organs to the ‘more sensitive’ saccule, the results from our study suggest that the utricle may serve

a more important auditory function in the midshipman and be complementary to the saccule for detecting behaviorally relevant acoustic stimuli including social acoustic signals.

#### Acknowledgements

The authors would like to thank Nick Lozier, Dr Paul Forlano and Joe Welsh from the Monterey Bay Aquarium and the Moss Landing Marine Laboratory for assistance with fish collection. We would also like to thank Dr Geoff Boynton for statistical consultation, and Sujay Balebail, Ruiyu Zeng and Lauren Gardner for assistance with fish husbandry. Finally, we would like to acknowledge Dr Lane Seeley and Dr Brooke Vetter for their assistance during the initial phases of this project.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

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

#### Funding

Funding was provided by National Science Foundation grant IOS 1456700 to J.A.S., and National Science Foundation Graduate Research Fellowship Program grant DGE 1762114 to L.S.R.

#### Supplementary information

Supplementary information available online at <https://jeb.biologists.org/lookup/doi/10.1242/jeb.226464.supplemental>

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