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Lagenar potentials of the vocal plainfin midshipman fish, *Porichthys notatus*

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Abstract

The plainfin midshipman fish (*Porichthys notatus*) is a species of marine teleost that produces acoustic signals that are important for mediating social behavior. The auditory sensitivity of the saccule is well established in this species, but the sensitivity and function of the midshipman's putative auditory lagena are unknown. Here, we characterize the auditory-evoked potentials from hair cells in the lagena of reproductive type I males to determine the frequency response and auditory sensitivity of the lagena to behaviorally relevant acoustic stimuli. Lagenar potentials were recorded from the caudal and medial region of the lagena, while acoustic stimuli were presented by an underwater speaker. Our results indicate that the midshipman lagena has a similar low-frequency sensitivity to that of the midshipman saccule based on sound pressure and acceleration (re: 1 μPa and 1 ms^{-2} , respectively), but the thresholds of the lagena were higher across all frequencies tested. The relatively high auditory thresholds of the lagena may be important for encoding high levels of behaviorally relevant acoustic stimuli when close to a sound source.

Keywords

Auditory-evoked potentials; Lagena; Hair cell tuning; Hearing

Introduction

Many teleost fishes have evolved adaptations for the production and reception of social acoustic communication signals (Ladich 2004; Bass and Ladich 2008; Kelley and Bass 2010; Fine and Parmentier 2015). Batrachoidid fishes (i.e., midshipman and toadfishes) are good examples of soniferous fishes that have evolved a number of physiological, endocrinological, and morphological adaptations for intraspecific acoustic communication

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and social behaviors, especially during courtship and reproduction (Bass and McKibben 2003; Forlano et al. 2016; Mohr et al. 2017). The plainfin midshipman (*Porichthys notatus*) has become a model organism for investigating the neural basis of acoustic communication, as this species provides a highly tractable model for examining auditory-driven social behavior and its underlying neurophysiology (Bass et al. 1999; Sisneros 2009a; Forlano et al. 2015).

The plainfin midshipman is a nocturnal marine teleost species that employs a nest-guarding reproductive strategy in which type I or “guarder” males reside under rocky nests in the intertidal zone and produce multi-harmonic advertisement calls during the breeding season to attract reproductive females that are gravid (full of eggs) (Maruska and Sisneros 2015; Bose et al. 2018). In contrast, there exists a second male morph, known as a type II or “sneaker” male, that employs a cuckoldry reproductive strategy in which type II males mimic females to gain entrance to a nest to “sneak spawn” and steal fertilizations from nesting type I males and their mates (Brantley and Bass 1994; Sisneros 2009b; Forlano et al. 2015). While all morphs produce the distinct short duration, broadband “grunts” during social agonistic interactions (Bass et al. 1999; Sisneros 2009a), only the nesting type I males produce long-duration vocal signals, such as the broadband agonistic “growls” used in nest defense and the multi-harmonic advertisement calls that attract females during breeding (Brantley and Bass 1994; Bass and McKibben 2003; Forlano et al. 2016).

Like most teleost fishes, midshipman have an inner ear which consists of three semicircular canals that encode angular momentum (vestibular function) and three otolithic end organs (saccul, lagena, and utricle) that may serve an auditory and/or vestibular (positional) function, however, to what extent each end organ contributes to audition is unknown and likely varies between taxa (Platt and Popper 1981; Popper and Fay 1993; Schulz-Mirbach et al. 2018). The otolithic end organs essentially act as biological accelerometers that are sensitive to acoustic particle motion (de Vries 1950; Fay 1984). Of the three end organs, the saccul is often the largest and most implicated in terms of hearing for most teleost fishes (Popper and Fay 1993). While a number of studies have characterized the response properties of the teleost saccul and its auditory afferents, very few studies have examined the auditory response properties and acoustic function of the teleost lagena and utricle (Schulz-Mirbach et al. 2018). A small number of studies suggest that both the lagena and utricle are capable of coding acoustic particle motion (Lu et al. 2003, 2004; Meyer et al. 2004, 2010; Maruska and Mensinger 2015), but the possible acoustic functions of the lagena and utricle are still not yet well understood.

The main goal of this study was to characterize the auditory-evoked potentials from the inner ear lagena of the plainfin midshipman and determine the auditory sensitivity and frequency response of lagenar hair cells to behaviorally relevant auditory stimuli. In this study, we focus on the lagenar potentials of type I males collected during the reproductive season to establish baseline data on the response characteristics of lagena for this male morph and to better understand the contribution of this inner ear end organ to overall auditory sensitivity and hearing.

Methods

Experimental animals

Adult type I male midshipman (*P. notatus*) were hand-collected from rocky nests in the intertidal zone during low tide at Seal Rock in Brinnon, WA during the midshipman reproductive season (August 2017 and May–June 2018). Soon after collection, animals were transported to the University of Washington in Seattle, WA, where they were maintained in salt-water aquaria at 13–16 °C and fed a diet of defrosted shrimp every 2–4 days. Lagenar potential recordings were conducted 38–52 days after collection for animals recorded during 2017, while lagenar potentials were recorded within 22 days of capture for animals collected during 2018. The reproductive state of the animal was determined by measuring the gonadosomatic index (GSI) using the following equation according to Tompkins and Simmons (2002): $100 \times (\text{gonad mass} / (\text{body mass} - \text{gonad mass}))$.

Acoustic stimulus generation and calibration

Acoustic stimuli were generated using the reference output signal of a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA) that was delivered to an audio amplifier (BG-1120, TOA Electronics, Inc, South San Francisco) controlling an underwater monopole speaker (AQ339 Aquasonic Speaker, Clark Synthesis, Littleton, CA, USA). The acoustic stimuli consisted of eight repetitions of single 500-ms pure tones at a rate of one every 1.5 s. Pure tones were presented at 85, 105, 125, 165, 205, 245, 305, 345, 405, 445, and 505 Hz; the presentation order of the single tones was randomized. These frequencies were chosen, because they encompass the bandwidth of the dominant frequencies contained within the type I male midshipman's advertisement call, but also avoid frequencies associated with acoustic tank resonances and electrical noise (60 Hz and its harmonics), both of which could potentially interfere with the lagenar potential measurements.

Acoustic stimuli calibrations were performed before each physiology experiment. During the calibration procedure, a mini-hydrophone (8103, Bruel and Kjaer, Naerum, Denmark), which was connected to a conditioning amplifier (gain = 100 mV/Pa, Nexus 2692–0S1, Bruel and Kjaer, Naerum, Denmark), was placed 10 cm above the underwater speaker (and 3 cm below the water surface) in the same position, where the fish's inner ear would be located. Auditory stimuli were equalized in SPL using a MATLAB (Math-Works Inc., Natick, MA, USA) script that measured the power spectral density at each tested frequency. The voltage signal sent to the speaker was scaled until the measured SPL output from the speaker was within 0.5 dB of the desired peak-to-peak amplitude (pk–pk) of 130 dB re: 1 μPa . The sound level of 130 dB SPL_{pk–pk} was chosen for the calibration procedure, because it is significantly above the level of the background noise, is biologically relevant, and has been the standard calibration sound level used in the previous studies using this experimental setup (Sisneros 2007, 2009b; Alderks and Sisneros 2011; Bhandiwad et al. 2017). Figure 1a shows representative pressure waveforms and corresponding power spectra for all acoustic stimuli tested. Recordings were made using a mini-hydrophone (8103, Bruel and Kjaer, Naerum, Denmark) connected to a hand-held recorder (Zoom H2, Tokyo, Japan) and were analyzed using Adobe Audition (ver.1.0, Adobe Systems). This was done to verify that the

acoustic stimulus emitted from the underwater speaker matched the stimulus that was received by the fish in the position occupied by the fish's ear.

Particle acceleration measurements were collected using a neutrally buoyant, triaxial accelerometer [PCB model VW356A12, PCB Piezotronics, Depew, NY, USA; 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)]. Particle acceleration measurements were made at the corresponding sound pressure levels tested to construct the threshold tuning curves based on particle motion. To calibrate the particle acceleration levels, the accelerometer was placed at the same position as the fish's head, and then, the particle acceleration (re: 1 ms⁻²) measurements were recorded for each of the three accelerometer axes (*x*, *y*, and *z*) at every frequency and sound pressure level used in this study. The accelerometer's three dimensions corresponded to the following anatomical positions: *x* = anterior/posterior, *y* = left/right, and *z* = dorsal/ventral. A PCB signal conditioner (model 482A16, PCB Piezotronics, Depew, NY, USA) was used to amplify the signal (gain = 100 × for each axis). Particle acceleration ($a = \text{ms}^{-2}$) was calculated using the following equation: $a = \text{mV}_{\text{peak-to-peak}}/S$, where S = accelerometer sensitivity (mV/ms⁻²) for the corresponding *x*-, *y*-, or *z*-axis (see triaxial sensitivities reported above). Figure 1b illustrates the variation of stimulus acceleration (dB re: 1 ms⁻²) in the *x*, *y*, and *z* directions at the position, where the fish's head was during experimentation at three different sound pressure levels (139, 145, and 151 dB re: 1 μPa) tested.

Lagenar potential measurements

The method for recording the lagenar potentials was similar to that used to characterize the evoked potentials from the midshipman saccule (Sisneros 2007, 2009b; Alderks and Sisneros 2011; Bhandiwad et al. 2017). Animals were first anesthetized by immersion in a 0.025% ethyl *p*-aminobenzoate (benzocaine) salt-water bath followed by intramuscular injections of cisatracurium besylate (~ 3 mg/kg of body mass) and bupivacaine HCL (~ 1 mg/kg of body mass) for immobilization and analgesia, respectively. Next, the lagena was exposed via a dorsal craniotomy (Fig. 2) and the cranial cavity and inner ear was filled with cold teleost Ringer solution to prevent drying of the inner ear end organs. A 3–4 cm hydrophobic dam of denture cream was built around the exposed craniotomy. This prevented the surgery prep from becoming contaminated with salt water when the fish was lowered below the tank water line. The fish was then positioned in the center of a circular (40 cm diameter) experimental tank with a custom built acrylic head holder that allowed the fish to be suspended below the waterline and positioned, such that inner ear lagena was approximately 3 cm below the water surface and 10 cm above the under-water speaker. The monopole AQ339 underwater speaker was positioned on the bottom of the tank and embedded in a ~ 4.5 cm layer of small rock substrate. Once secured in the acrylic head holder, fish were then perfused with chilled, fresh salt water (12–14 °C) by a small silicone tube that was insert into the mouth of fish, which provided a continuous flow of recirculated salt water across the gills throughout the experiment. The experimental tank was maintained on an inflated pneumatic, vibration–isolation table inside an acoustical isolation chamber (Industrial Acoustics, New York, NY, USA), while the other stimulus generation and recording equipment were housed outside the isolation chamber.

Evoked potentials from the lagenar hair cells were recorded using glass microelectrodes filled with 3 M KCl (1.0–10.0 MΩ) positioned in the endolymph near the sensory epithelia of the lagena. The microelectrodes were always positioned near hair cells in the caudal to medial region of the lagena. The recorded analog evoked potentials were first pre-amplified (10 ×, model 5A, Getting Instruments, San Diego, CA, USA), and then bandpass filtered (70 Hz–3 kHz) and amplified (10 ×) again using a digital filter (model SR 650, Stanford Research Systems, Sunnyvale, CA, USA) before finally being sent to a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA). The lock-in amplifier yields an output signal that reflects the relative amplitude of the lagena's hair cell response to each pure tone stimulus. Because opposing hair cell orientations yield a maximum evoked potential at twice the sound stimulus frequency (Zotterman 1943; Cohen and Winn 1967; Furukawa and Ishii 1967; Hama 1969; Sisneros 2007), the lagenar potential was defined as the amplitude of the hair cell response at the second harmonic of the stimulus frequency (Cohen and Winn 1967; Sisneros 2007; Bhandiwad et al. 2017). This double-frequency response was first reported in the lateral line of the ruffe (*Acerina ceruna*; Jielof et al. 1952) and in the saccule of the gold-fish (*Carassius auratus*; Furukawa and Ishii 1967), perch (*Perca fluviatilis*; Sand 1973), and common carp (*Cyprinus carpio*; Fay 1974). Because of the opposed orientated hair cells in the end organ, the hair cells produce two evoked potentials during every stimulus cycle, which has been effectively demonstrated in the midshipman saccule [see Fig. 2 in both Sisneros (2007) and in Bhandiwad et al. (2017)]. The greatest evoked potential occurring at the second harmonic of the acoustic stimulus (i.e., twice the stimulus frequency) has also been reported in a number of other teleost hearing studies using the auditory-evoked potential (AEP) technique (e.g., Mann et al. 2001; Higgs et al. 2001; Egner and Mann 2005; Casper and Mann 2006; Maruska et al. 2007; Vetter et al. 2018). With the reference frequency of the lock-in amplifier set to the second harmonic of the stimulus frequency, noise or other signals evoked at frequencies other than the second harmonic reference frequency were rejected by the lock-in amplifier and did not affect the lagenar potential measurement. Therefore, the lagenar potentials recorded in this study represent the evoked potentials that originated from at least two regional populations of hair cells with opposing orientations in the lagena. Both the data acquisition and the stimulus timing were controlled by a custom MATLAB script. Each recording session began with control trials of background noise measurements (no auditory stimulus present) followed by stimulus trials at the various tested frequencies and amplitudes.

Acoustic impedance measurement

Because the sound field in our experimental tank is likely determined by the tank dimensions and composition (e.g., Nalgene plastic) and is unlike the natural environmental condition, we report here the acoustic impedance of the test tank as suggested by Popper and Fay (2011) and compare it to the acoustical impedance of theoretical “seawater” ($Z = 1.559$ MRayls) in a free-field environment (unbounded conditions) with a salinity of 35 ppt at 15 °C (Bradley and Wilson 1966; Erbe 2011). The acoustic impedance of our experimental tank was determined by simultaneously measuring both sound pressure and particle acceleration at each tested frequency and sound level using a mini-hydrophone (8103, Bruel and Kjaer, Naerum, Denmark) and a neutrally buoyant, triaxial accelerometer [PCB model

VW356A12, PCB Piezotronics, Depew, NY, USA; 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)]. The hydrophone and accelerometer were placed directly over the center of the speaker in the middle of the water column (i.e., the sensors were equidistant between the speaker and water surface). Because the majority of the acoustic energy from the speaker was contained in the vertical (*z*-axis) direction, acceleration was only measured in the *z*-axis for measurements of tank impedance. Acoustic signals were amplified using a PCB signal conditioner (model 482A16, PCB Piezotronics, Depew, NY, USA) for accelerometer measurements (gain = 100 ×), while a conditioning amplifier (Nexus 2692-0S1, Bruel and Kjaer, Naerum, Denmark) was used for hydrophone measurements (gain = 100 mV/Pa). The amplified peak-to-peak (pk-pk) voltage measurements for both sound pressure and particle motion were measured and recorded using a data acquisition system (NI myDAQ 16 bit analog-to-digital conversion at 200kS/s, National Instruments, Austin, TX, USA) that was controlled by a custom program in the LabVIEW software (NI LabVIEW 2016, National Instruments, Austin, TX, USA).

The acoustical impedance (*Z*) is defined as the complex ratio of sound pressure to particle velocity and is expressed in Rayls [1 Rayl = 1 (Pa s)/m]. The absolute value of acoustic impedance is determined by dividing the amplitude of the pressure wave by the amplitude of the particle velocity wave. For planar sound waves traveling through a free-field of non-viscous seawater at a salinity of 35 ppt and a temperature of 15 °C, the absolute value of the acoustic impedance is independent of frequency at *Z* = 1.559 MRayls (Bradley and Wilson 1966; Erbe 2011). The phase (*Φ*) of the complex acoustical impedance is determined by the phase difference between the particle velocity wave and the pressure wave. For free-field, planar, sound waves, the particle velocity wave is in phase with the pressure wave, the phase difference is zero, and the acoustic impedance is entirely real.

In our analysis, sound pressure level (Pa) was first calculated using the following equation: SPL = mV_{pk-pk}/sc, where sc = the scale factor (mV/Pa) from the conditioning amplifier and mV_{pk-pk} is measured peak-to-peak voltage from the recorded signal via the hydrophone. Next, the particle acceleration (*a* = ms⁻²) was calculated using the equation: *a* = mV_{pk-pk}/*S*, where *S* = accelerometer sensitivity (mV/ms⁻²) for the *z*-axis. The amplitude of the particle velocity waveform (*v* = ms⁻¹) was calculated from the amplitude of the particle acceleration waveform using the following equation: *v* = *a*/2π*f* (Nedelec et al. 2016), where *f* = frequency in Hz, and *a* = the amplitude of the measured acceleration waveform via the accelerometer. The absolute magnitude of acoustic impedance [ratio of pressure to particle velocity, (Pa s)/m] for a given frequency and sound level was then expressed logarithmically relative to the acoustic impedance in a free-field of seawater (*Z* = 1.559 MRayls) with a salinity of 35 ppt at 15 °C (Bradley and Wilson 1966; Erbe 2011) using the following equation: dB (re: 1.5597 MRayl) = 20 log [(sound pressure/particle velocity)/1.5597 MRayl], where the tank impedance (sound pressure/particle velocity) is expressed in MRayls (1 × 10⁶ Pa s/m) (Bradley and Wilson 1966; Erbe 2011).

The complex phase of the acoustic impedance is equal to the phase difference between the particle velocity (*v*) and the pressure (*p*). To assess the phase of the complex acoustical impedance in our test tank, we also directly measured the phase difference (*Φ*) between the

particle acceleration (a) and pressure, where $\Phi_{p,a} = \Phi_p - \Phi_a$. For a sinusoidal wave, the phase of particle acceleration will always lead the phase of particle velocity (v) by 90° , $\Phi_a - \Phi_v = 90^\circ$. Therefore, the phase difference between the particle velocity and pressure can be determined using the following equation: $\Phi_{p,v} = \Phi_{p,a} + 90^\circ$. In the far field from a spherical sound source, we would expect particle velocity to be in phase with pressure, yielding: $\Phi_{v,p} = 0^\circ$. In the near field for a spherical sound source, we would expect particle acceleration to be in phase with pressure, yielding: $\Phi_{p,a} = 0^\circ$ and $\Phi_{p,v} = 90^\circ$ (Harris and van Bergeijk 1962). Our measurements are well within the near-field approximation, but we do not expect a simple relationship between velocity and pressure, because of the confined tank environment. Figure 3 shows the both the acoustic impedance (Z ; Fig. 3a) and $\Phi_{p,v}$ (Fig. 3b) at all frequencies examined for three relevant sound pressure levels: 136, 145, and 151 dB re: 1 μ Pa.

Data analyses

Auditory threshold tuning curves based on sound pressure and particle acceleration were constructed by characterizing the input–output measurements of the evoked lagenar potentials over the range of stimulus amplitudes and frequencies tested. The recorded background noise measurements were used to establish the subthreshold lagenar potential response levels and determine the auditory thresholds at each frequency. The auditory threshold at each stimulus frequency was designated as the lowest stimulus level that yielded an averaged evoked lagenar potential that was greater than two standard deviations above the average background measurement. The frequency that evoked the lowest lagenar potentials, and thus had the lowest thresholds, was defined as the characteristic frequency (CF). Particle acceleration thresholds were reported as the combined magnitude vector that was calculated using the following equation (Wysocki et al. 2009; Vasconcelos et al. 2011; Bhandiwad et al. 2017): $20 \log [(x^2 + y^2 + z^2)]$.

Results

Evoked lagenar potentials were recorded from 21 adult type I midshipman fish that ranged in size from 13.0 to 20.2 cm SL [mean SL = 16.0 ± 2.2 SD cm, mean body mass = 55.2 ± 29.9 SD g, and mean gonadosomatic index (GSI) = 1.27 ± 0.71 SD]. All the fish used in this study were within the size range reported in the previous physiology studies for adult type I midshipman (Sisneros 2007; Rohmann and Bass 2011). Adult type I males can be distinguished from adult type IIs based on the presence of well-developed sonic muscles and lower mean GSI in type I males. Type II males are typically characterized as having smaller standard lengths (SL range 8.0–16.0 cm, mean SL = 9.9 ± 1.4 SD) and larger gonads (mean GSI = 12.3 ± 5.4 SD) than type I males (Bhandiwad et al. 2017).

Auditory thresholds for both sound pressure level and acoustic particle motion were determined for populations of hair cells in type I males recorded from the caudal–medial region of the lagenar. Lagenar potentials were evoked in response to a wide range of sound pressure levels from 121 to 154 dB re: 1 μ Pa. Figure 4 shows representative iso-level response profiles of evoked lagenar potentials to single tones (85–505 Hz) at the highest sound level tested (154 dB re: 1 μ Pa). Figure 5a shows representative tuning curves of

lagenar hair cells based on the sound pressure levels tested. In general, the lagenar threshold tuning curves for sound pressure consisted of tuning profiles with the lowest thresholds at 85 Hz (mean threshold = 136 dB re: 1 μ Pa) and 105 Hz (mean threshold = 143 dB re: 1 μ Pa) that gradually increased to highest thresholds at frequencies 205 Hz (mean thresholds 150–152 dB re: 1 μ Pa) (Fig. 6a). The averaged threshold tuning curve for lagenar hair cells in type I males based on sound pressure is summarized in Fig. 6a. Characteristic frequency (CF; defined as the frequency that evoked the lowest lagenar potential threshold) ranged from 85 to 105 Hz and the median CF based on sound pressure was 85 Hz.

The lagenar threshold tuning profiles based particle acceleration differed in shape compared to the sound pressure profiles. Figure 5b shows representative tuning curves of lagenar hair cells based on the particle acceleration levels tested. Both 85 Hz (mean threshold = -9.7 dB re: 1 ms^{-2} or 110.3 dB re: 1 μms^{-2}) and 125 Hz (mean threshold = -4.3 dB re: 1 ms^{-2} or 115.7 dB re: 1 μms^{-2}) had the lowest acceleration thresholds, while the highest thresholds were sometimes observed at 105 Hz (mean threshold = -3.9 dB re: 1 ms^{-2} or 123.9 dB re: 1 μms^{-2}) or 165 Hz (mean threshold = 7.3 dB re: 1 ms^{-2} or 127.3 dB re: 1 μms^{-2}), in addition to frequencies 205 Hz (mean thresholds 0.05–3.4 dB re: 1 ms^{-2} or 120.1–123.5 dB re: 1 μms^{-2}). The averaged threshold tuning curve based on acceleration is summarized in Fig. 6b. Characteristic frequencies based on acceleration were observed at 85 Hz and 125 Hz, with a median CF of 125 Hz.

The lagenar potentials evoked above threshold (relative to sound pressure and acceleration) were recorded across the range of frequencies tested from 85 to 505 Hz, but the lagenar potentials were most consistently evoked from 85 to 205 Hz (100%) in the 30 recordings made from the 21 type I males (Fig. 7). The percentage of recordings that had evoked lagenar potentials at 245–305 Hz dropped to 87–90% and then to 40–43% at 405–445 Hz. At the highest frequency tested (505 Hz), only 10% ($n = 3$) of the recordings had detectable evoked lagenar potentials (Fig. 7). Thus, the number of recorded evoked lagenar potentials precipitously decreased at frequencies > 345 Hz.

Discussion

The purpose of this study was to obtain quantitative baseline data regarding the auditory sensitivity and frequency response characteristics of the lagena in the plainfin midshipman, using the evoked potential recording technique. Our results provide new quantitative data about the sound pressure and particle motion sensitivity, dynamic range, and frequency response characteristics of lagenar hair cells in reproductive type I male midshipman. We show that the midshipman lagena has a similar frequency response range as the midshipman saccule, but the thresholds based on sound pressure and acceleration are considerably higher for the lagena than that reported for the midshipman saccule (Sisneros 2007; Bhandiwad et al. 2017). In this discussion, we compare the frequency response and auditory sensitivity of the midshipman lagena with that reported for other fishes and then assess the auditory lagenar sensitivity of type I males in relation with that of their vocal-acoustic social behavior.

The observed auditory lagenar sensitivity of type I male midshipman based on acceleration was very similar to that reported for other teleost fishes. Lu et al. (2003) showed using a shaker table system that the auditory afferents of the lagena in the sleeper goby (*Dormitator latifrons*) were primarily sensitive to linear accelerations that simulated underwater acoustic particle motion. The lagenar epithelia in the sleeper goby was found to be oriented vertically with the lagenar afferents having best response axes of directional sensitivity centered on the longitudinal axis in the horizontal plane, but distributed in a variety of axes in the mid-sagittal plane. In addition, the lagenar afferents were found to be less sensitive (~ 35 dB) than that of saccular afferents (Lu et al. 1998; Lu and Xu 2002) and the lagena of the sleeper goby had two ranges of peak sensitivity: one range of CFs ~ 50 Hz and other range of CFs from 80 to 125 Hz (Lu et al. 2003). Lu et al. (2003) concluded that the sleeper goby lagena likely plays a role in sound source localization in regard to directional sensitivity in the elevation, especially at relatively high stimulus levels, where the saccule and its afferents responses are often saturated. Similarly, Meyer et al. (2010) also showed that the lagena of the non-teleost ray-finned (bony) lake sturgeon (*Acipenser fulvescens*) was primarily sensitive to low-frequency acoustic stimuli with a majority (59%) of the lagenar afferents having best frequencies at 100 Hz and only 10% of the lagenar afferents having best frequencies > 141 Hz. Using the evoked potential recording technique (similar to that used in present study), Sand (1973) showed that the lagena of the perch (*Perca fluviatilis*) was primarily sensitive to acoustic stimuli in the vertical plane at frequencies of 100 and 200 Hz. In our study, we showed that the peak frequency sensitivity of the mid-shipman lagena based on acceleration, with CFs at 85 and 125 Hz in type I males, was similar to that reported for other “hearing non-specialist” fishes (Lu et al. 2003; Meyer et al. 2010). In addition, the lagena also displayed low-pass filter characteristics similar to that of the saccule in type I males. The tuning profiles of the lagenar potentials were similar in shape and had a similar range of frequency sensitivity to that reported for midshipman saccular potentials (Sisneros 2007). However, the acceleration thresholds of the lagena were much higher than that reported for the saccule in type II male midshipman. Bhandiwad et al. (2017) showed that the saccular potential thresholds of type II males ranged from -44 dB re: 1 ms^{-2} at 85 Hz to -24 dB re: 1 ms^{-2} at 345 Hz, which were lower compared to the lagenar potential thresholds of type I males that ranged from approximately -10 dB re: 1 ms^{-2} at 85 Hz to 2.5 dB re: 1 ms^{-2} at 345 Hz. Thus, the relative difference in acceleration thresholds between the midshipman saccule and lagena is approximately 26–34 dB over the range of frequencies from 85 to 345 Hz. These large differences in acceleration thresholds are in agreement with Lu et al. (2003) who suggested that the less sensitive lagena may be important for encoding direction at relatively high sound levels close to the sound source when the saccule and its afferents are likely to be overstimulated and saturated. If, as suggested by Sand (1973) and Lu et al. (2003), the lagena is primarily sensitive to vertical (z -axis) acceleration, then some of the irregularities observed in our acceleration threshold tuning curves might be due to the directional sensitivity of the midshipman lagenar hair cells. We consistently observed relatively high acceleration thresholds for the lagenar potentials at 105 Hz and 165 Hz (Fig. 6). These frequencies (105 and 165 Hz) also produced relatively high y -axis (horizontal) acceleration levels compared to the z -axis (vertical) and x -axis acceleration levels from the sound source. At 105 Hz, the greatest particle motion occurred in the y -axis (approximately

20 dB higher than the *z*-axis and *x*-axis particle motion) (see Fig. 1b). *Y*-axis acceleration levels were greatest at frequencies < 245 Hz, while *z*-axis acceleration levels were greatest at frequencies > 205 Hz (where *z*-axis acceleration levels were consistently 20 dB higher than *y*-axis and *x*-axis acceleration levels). In addition, the orientation of the sensory epithelium in the mid-shipman lagena is primarily in the vertical (*z*-axis) plane (Vetter and Sisneros, personal observation; but also see CT scans for *P. notatus* in the *Virtual Natural History Museum*: <http://131.220.133.140/VNHM/>). Thus, the midshipman lagena is likely to also be sensitive to accelerations in the vertical (*z*-) axis of elevation. Taken together, the relatively high acceleration thresholds of the lagenar potentials at frequencies < 205 Hz (especially at 105 Hz and 165 Hz) may be due to a disproportionate amount of particle acceleration produced by the speaker in the horizontal (*y*-) axis which may not have effectively stimulated lagena hair cells that are sensitive to vertical accelerations. Therefore, because of these irregularities in particle motion within the tank at stimulus frequencies < 205 Hz, it is difficult to interpret the acceleration tuning curve and the sensitivity of the lagena at frequencies < 205 Hz. However, our results provide general support for the previous findings that midshipman are most sensitive to frequencies (85–505 Hz) that are contained with the dominant harmonics of the male advertisement call (Sisneros 2007; Bhandiwad et al. 2017). Future studies that employ a shaker table system will be needed to more precisely characterize the acceleration thresholds and determine the directional sensitivity of the midshipman lagena.

Although the auditory end organs of most teleost fishes primarily serve as biological accelerometers to detect particle motion (de Vries 1950; Hawkins 1993; Sisneros and Rogers 2016; Schulz-Mirbach et al. 2018), some fish have evolved adaptations that allow them to detect pressure. For instance, Otophysan fishes (e.g., goldfish, zebrafish, catfishes, and relatives) possess specialized structures or skeletal adaptations (e.g., the Weberian ossicles in goldfish) that allow for the transfer of acoustic energy from the swim bladder to the inner ear. While plainfin midshipman do not possess such hearing specializations, like Weberian ossicles, they do have swim bladder adaptations that may allow them to potentially detect sound pressure indirectly. A recent study by Mohr et al. (2017) demonstrated that midshipman possess intra- and intersexual dimorphisms of the swim bladder that may facilitate pressure detection. Like other batrachoidid fishes, midshipman have a *U*-shaped swim bladder; however, females and type II males have rostral extensions or “horns” that project close to the saccule and lagena, while the swim bladder of type I males does not have the distinct horns. Therefore, we predict that the lagena and saccule, and their interaction with the swim bladder in female and type II males, would likely provide enhanced sound pressure detection. Future studies that investigate the evoked potentials of the lagena and saccule in midshipman females and type II males, especially in fish with intact and ablated swim bladders, would be useful in determining whether the swim bladder enhances sound pressure sensitivity and extends the range of frequency sensitivity of the midshipman inner ear.

In addition, otolith size may be a potential adaptation to enhance sound detection and contribute to an end organ’s high-frequency sensitivity. Recently, Boyle and Herrel (2018) postulated that the lower mass utricular and lagenar otoliths found in serrasalmids

(otophysans including piranhas and pacus), which inhabit environments with fast moving water, may allow end organs to retain high-frequency sensitivity in low-frequency noisy environments to effectively enhance hearing sensitivity. Future studies that examine the size and mass of midshipman lagenar and utricle otoliths may provide additional insight into how oto-lith mass contributes to auditory sensitivity in teleost fishes.

Audition plays a major role in the social behavior of midshipman as the midshipman's reproductive ecology is dependent on the production and reception of social acoustic signals. Midshipman have evolved a number of adaptations that enhance auditory sensitivity during the reproductive season to increase the detection and localization of conspecifics. Previous studies have shown that all three morphs undergo seasonal, reproductive-state-dependent changes in auditory saccular sensitivity that facilitate the enhanced detection of the higher frequency components in midshipman social signals (Sisneros and Bass 2003; Sisneros 2009a, b; Rohmann and Bass 2011; Bhandiwad et al. 2017). The enhanced detection of the dominant higher frequency components in midshipman social signals is thought to be adaptive, because higher frequencies propagate further in shallow water environments, like those where midshipman breed during the summer (Fine and Lenhardt 1983; Rogers and Cox 1988; Bass and Clark 2003). Although the present results suggest that the thresholds for the lagena are higher than those reported for the midshipman saccule, they still fall below the peak sound pressure levels contained within the dominant harmonic frequencies of male advertisement call (Fig. 8). Thus, it is possible that the lagena has some function in acoustic signal detection and communication, especially when the animal is close to the sound source. Whether the lagena also undergoes seasonal reproductive-state-dependent changes in auditory sensitivity is unknown. Future studies that investigate seasonal changes in auditory lagenar sensitivity for enhanced detection of social signals will provide valuable insight into whether plasticity of the midshipman auditory system also extends to the lagena.

In summary, this study provides the first assessment of frequency detection and auditory sensitivity of the midshipman lagena for both sound pressure and particle motion. Our results indicate that the auditory sensitivity of the midshipman lagena is similar to that reported for other hearing non-specialist fishes and that the midshipman lagena has similar frequency bandwidth to that of the midshipman saccule, but with increased thresholds. The results from this study should provide valuable insight into the auditory capabilities of the lagena as well contribute to our general understanding of the teleost inner ear.

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Fig. 1.

a Representative examples of each frequency evaluated are shown with the corresponding power spectra below. Measurements were made using a hydrophone in the same place, where the fish's head was positioned. **b** Particle acceleration levels (dB re: 1 ms⁻²) from the underwater speaker measured in all three dimensions (x -axis = black, y -axis = light grey, z -axis = dark grey) at all frequencies examined for three sound pressure levels: 139, 145, and 151 dB re: 1 μ Pa. Measurements were made using a triaxial accelerometer in the same place, where the fish's head was positioned

Fig. 2. Dorsal view of the plainfin midshipman brain and inner ear. The lagena (L) has been repositioned slightly more rostral and lateral for better exposure. *C* cerebellum, *M* midbrain, *T* telencephalon, *S* saccule, and *U* utricle

Fig. 3.

Acoustic characteristics of the experimental tank and speaker. **a** Acoustic impedance [ratio of sound pressure (dB re: 1 μ Pa) to particle velocity (dB re: 1 ms^{-1})] in the z -axis (Z) relative to 1.5597 MRayl (the reference impedance for a free-field in 35 ppt salinity seawater at 15 °C) is plotted for all the frequencies examined at three sound pressure levels: 136, 145, and 151 dB re: 1 μ Pa. Measurements were made using a triaxial accelerometer placed in the center of the tank and water column. Multiple magnitude measurements ($n = 10$) for both pressure and particle velocity were made at each frequency and sound pressure level. All data are plotted as the mean $\pm$ 1 standard deviation; note that the standard deviations are very small and the standard deviation bars are obscured by the symbols. **b** Phase difference () between the pressure and particle velocity wave. Measurements were made using a triaxial accelerometer placed in the center of the tank and water column. Multiple phase

difference measurements ($n = 10$) were made at each frequency and sound pressure level. All data are plotted as the mean ± 1 standard deviation; note that the standard deviations are very small ($< 0.3^\circ$) and the standard deviation bars are obscured by the symbols

Fig. 4.

Representative examples of iso-intensity curves recorded from the lagena in response to single tone stimuli played back at the highest sound level tested (154 dB re: 1 μ Pa). The threshold was defined as the lowest sound pressure level required to evoke a potential least 2 SD above the background noise measurement. Data are represented as mean $\pm$ 95% confidence interval

Fig. 5.

Representative examples of individual auditory threshold tuning curves based on the evoked potentials recorded from the lagena of type I male midshipman. **a** Tuning curves based on the sound pressure thresholds for four individual fish. Thresholds were defined as the lowest sound pressure level (dB re: 1 μ Pa) to evoke a lagenar potential that was at least 2 SD above the back-ground noise. **b** Tuning curves based on acceleration thresholds for four individual fish

Fig. 6.

Auditory threshold tuning curves for **a** sound pressure and **b** particle acceleration based on the evoked potentials recorded from the lagena. Data are plotted as mean $\pm$ 95% confidence interval. The auditory thresholds were defined as the lowest stimulus level required to evoke a lagenar potential that was 2 SD above the background noise measurement. The number of animals and records is indicated in parentheses

Fig. 7.

Distribution of the percentage of lagelar potential recordings that displayed significant thresholds for each frequency tested. Note that between 85 and 205 Hz, potentials were observed in all 30 recordings, however, for frequencies above 205 Hz, lagelar potentials were not always detected, even at the highest sound pressure levels evaluated. The number of animals and records is indicated in parentheses

Fig. 8.

Comparison of the male midshipman lagenar tuning curve based on sound pressure with the sound pressure level of the advertisement call by a type I male midshipman. The reproductive type I male (SL = 19.2 cm; weight = 101.16 g) was collected during the summer at Seal Rock in Brinnon, WA at low tide and housed in a large indoor concrete tank (3 m diameter) at the Friday Harbor Laboratories on San Juan Island, WA, USA. The male advertisement call was recorded at night in the indoor tank with a mini-hydrophone placed directly in front of the entrance of an artificial nest (~ 0 cm from the opening). The fundamental frequency of the advertisement call is at 87 Hz (157 dB re: 1 μ Pa) with dominant harmonics at the following frequencies with their respective sound pressure levels: 173 Hz (161 dB re: 1 μ Pa), 260 Hz (158 dB re: 1 μ Pa), 346 Hz (156 dB re: 1 μ Pa), and 432 Hz (150 dB re: 1 μ Pa).