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RESEARCH ARTICLE

Swim bladder enhances lagenar sensitivity to sound pressure and higher frequencies in female plainfin midshipman (*Porichthys notatus*)

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ABSTRACT

The plainfin midshipman fish (*Porichthys notatus*) is an established model for investigating acoustic communication because the reproductive success of this species is dependent on the production and reception of social acoustic signals. Previous work showed that female midshipman have swim bladders with rostral horn-like extensions that project close to the saccule and lagena, while nesting (type I) males lack such rostral swim bladder extensions. The relative close proximity of the swim bladder to the lagena should increase auditory sensitivity to sound pressure and higher frequencies. Here, we test the hypothesis that the swim bladder of female midshipman enhances lagenar sensitivity to sound pressure and higher frequencies. Evoked potentials were recorded from auditory hair cell receptors in the lagena in reproductive females with intact (control condition) and removed (treated condition) swim bladders while pure tone stimuli (85–1005 Hz) were presented by an underwater speaker. Females with intact swim bladders had auditory thresholds 3–6 dB lower than females without swim bladders over a range of frequencies from 85 to 405 Hz. At frequencies from 545 to 1005 Hz, only females with intact swim bladders had measurable auditory thresholds (150–153 dB re. 1 µPa). The higher percentage of evoked lagenar potentials recorded in control females at frequencies >505 Hz indicates that the swim bladder extends the bandwidth of detectable frequencies. These findings reveal that the swim bladders in female midshipman can enhance lagenar sensitivity to sound pressure and higher frequencies, which may be important for the detection of behaviorally relevant social signals.

KEY WORDS: Communication, Hearing, Acoustic signal, Inner ear, Auditory threshold

INTRODUCTION

The inner ear of bony fishes consists of three semicircular canals and three otolithic end organs: saccule, utricle and lagena. The semicircular canals detect angular acceleration or rotational movement of the head and function as part of the vestibular system, while the otolithic end organs act as biological accelerometers that detect linear acceleration and respond directly to displacement by acoustic particle motion (de Vries, 1950; Fay and Popper, 1980; Fay, 1984; Sisneros and Rogers, 2016). The three otolithic end organs are thought to have both auditory and vestibular function (Schulz-Mirbach et al., 2019). Previous research suggests that the saccule (e.g. Cohen and Winn, 1967; Enger et al., 1973; Lu et al., 1998, 2010; Lu and Xu, 2002; Sisneros, 2007, 2009a; Vasconcelos et al., 2011) and lagena (Sand, 1974; Lu et al., 2003; Meyer et al., 2010; Vetter et al., 2019) are primarily responsible for sound detection, while the utricle probably has both vestibular (Riley and Moorman, 2000) and auditory function (Lu et al., 2004; Maruska and Mensinger, 2015). However, otolithic end organ function has only been studied in relatively few species and the contribution of each end organ to audition is likely to vary greatly across taxa given the diverse morphology of the inner ear among the approximately 26,000 species of teleost fishes (Nelson et al., 2016).

All fishes are thought to have the ability to detect acoustic particle motion via their otolithic end organs, but some teleost species have evolved hearing specializations that allow for the ability to also detect sound pressure. Sound pressure detection in fishes requires an acoustic coupling between the inner ear and a gas-filled chamber. Many species capable of sound pressure detection have evolved a secondary function of the swim bladder to act as an acoustic organ in addition to its primary function of regulating buoyancy. In these species, the swim bladder must either be directly connected to or come within close proximity to the otolithic end organs of the inner ear. The most common example of species with a direct acoustic coupling between the swim bladder and inner ear is found in otophysan fishes (e.g. Siluriformes or catfish and Cypriniformes or goldfish and carp). Otophysans have modified vertebrae, called Weberian ossicles, which form a direct physical connection between the swim bladder and the endolymph of one or more of the otolith organs. Pressure detection can also occur when the swim bladder and inner ear are situated close enough to allow for the transfer of acoustic energy from swim bladder vibrations to the inner ear, which results in local particle motion that stimulates the otolithic end organs (Rogers et al., 1988b). Species in which the swim bladder is located far from the inner ear (e.g. salmonids) or species that lack a swim bladder altogether (e.g. Gobiiformes or elasmobranchs) are only capable of sound detection via direct stimulation of the otolithic end organs by particle motion. These species tend to have a narrower bandwidth of detectable frequencies than those capable of pressure detection (for reviews, see Popper and Fay, 2011; Ladich, 2016; Schulz-Mirbach et al., 2019).

Recent work suggests that the plainfin midshipman fish (*Porichthys notatus*) is adapted for pressure-mediated hearing. The plainfin midshipman is a marine teleost species that uses vocalizations for communication and has become a model for studying fish hearing (Bass et al., 1999; Sisneros, 2009b; Forlano et al., 2015). Particularly, female midshipman rely on their auditory sense to detect and locate the advertisement calls of nesting males during spawning. Mohr et al. (2017) demonstrated that females have

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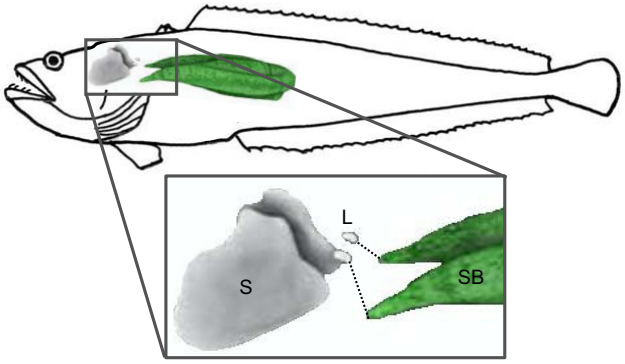

Fig. 1. Drawing of a female plainfin midshipman with a representative micro-computed tomography scan showing a lateral view of the swim bladder, saccule and lagena. SB, swim bladder; S, saccule; L, lagena. Inset: dotted lines indicate close proximity of rostral swim bladder extensions to the lagena. Drawing adapted from Robertson (2015) and micro-computed tomography scan modified from Mohr et al. (2017).

sexually dimorphic, rostral swim bladder extensions that project close to the saccule (mean distance 2.59 mm) and lagena (mean distance 2.89 mm), while nesting males lack such extensions of the swim bladder. Recently, Colleye et al. (2019) showed that females with intact swim bladders had auditory saccular potential thresholds that were 5–11 dB (re. 1 µPa) lower than females with removed swim bladders. These results indicate that the presence of the horn-like, swim bladder extensions in females enhances the sound pressure sensitivity of the saccule. The lagena in the midshipman is caudal to both the utricle and saccule and is close in proximity to the rostral swim bladder extensions (Fig. 1) (Mohr et al., 2017). Thus, the location of the lagena relative to the rostral swim bladder extensions suggests that the lagena may also contribute to sound pressure sensitivity and enhance bandwidth detection in female midshipman.

A recent study by Vetter et al. (2019) showed that the auditory evoked potentials recorded from the lagena in male midshipman had a similar frequency response range to that of the midshipman saccule (Sisneros, 2007). Here, we examine if the rostral swim bladder extensions in females enhance the auditory sensitivity of the lagena to sound pressure and higher frequencies. We characterize the evoked lagenar potentials from reproductive females with intact swim bladders (control condition) and compare them with reproductive females with removed swim bladders (treated condition). We hypothesize that the swim bladder in females will facilitate sound pressure detection by the lagena, and predict that females with intact swim bladders will have greater lagenar sensitivity to sound pressure and higher frequencies compared with females whose swim bladders are surgically removed. Our findings are interpreted as they relate to the detection of biologically relevant acoustic signals during social and reproductive related behaviors.

MATERIALS AND METHODS

Experimental animals

Adult female plainfin midshipman, *Porichthys notatus* Girard 1854 (N=30), were hand collected from rocky nests in the intertidal zone during low tide at Seal Rock in Brinnon, WA, USA during the midshipman reproductive season (May–June 2018) and transported in aerated coolers to the University of Washington in Seattle, WA. Fish were maintained in salt water aquaria at 13–16°C with a 16 h:8 h light:dark photoperiod, and fed a diet of defrosted shrimp every 2–4 days. The auditory physiology experiments were performed within 14 days of capture. Prior to experimentation, the standard length (L) and body mass (M_b) were measured and these metrics were used to determine the gonadosomatic index (GSI). The GSI, as described by Tompkins and Simmons (2002), is a measure of reproductive state and is calculated as: 100×[gonad mass/(body mass−gonad mass)].

Generation and calibration of acoustic stimulus

The acoustic stimuli were generated using a lock-in amplifier (SR830; Stanford Research Systems, Sunnyvale, CA, USA) that sent the signal to an audio amplifier and then to an underwater monopole speaker (AQ339; Aquasonic Speaker, Clark Synthesis, Littleton, CA, USA). 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 set frequencies between 85 and 1005 Hz, and the presentation order of the single tones was randomized. Specifically, the frequencies examined were: 85, 105, 125, 165, 205, 245, 305, 345, 405, 445, 505, 545, 605, 645, 705, 745, 805, 845, 905, 945 and 1005 Hz.

Prior to each physiology experiment, acoustic stimuli calibrations were performed using a mini-hydrophone (8103; Bruel and Kjaer, Naerum, Denmark) connected to a conditioning amplifier (gain=100 mV Pa^{-1}, Nexus, 2692-0S1, Bruel and Kjaer). The hydrophone was placed 10 cm above the underwater speaker, in the same position where the experimental fish's inner ear would be located (3 cm below the water surface). Acoustic stimuli were equalized in sound pressure level (SPL) using a MATLAB (MathWorks Inc., Natick, MA, USA) script that measured the power spectral density for all frequencies examined. The voltage signal sent from the lock-in amplifier was scaled until the measured SPL output from the speaker was within 0.5 dB of 130 dB re. 1 µPa (peak-to-peak amplitude). This sound level (130 dB re. 1 µPa) was chosen because it is significantly above the background noise, is biologically relevant, and has been the standard calibration sound level utilized in previous studies using the same experimental set-up (Sisneros, 2007, 2009a; Alderks and Sisneros, 2011; Coffin et al., 2012; Bhandiwad et al., 2017; Colleye et al., 2019; Vetter et al., 2019). In order to protect the equipment, the highest sound pressure evaluated was 154 dB re. 1 µPa.

Particle acceleration measurements were made at the corresponding sound pressure levels to construct the threshold tuning curves based on particle motion. A triaxial accelerometer [PCB model VW356A12; PCB Piezotronics, Depew, NY, USA; sensitivity (S) at 100 Hz: 10.42 mV/(m s^{-2}) (x-axis), 9.65 mV/(m s^{-2}) (y-axis), 10.14 mV/(m s^{-2}) (z-axis)] was used to collect particle acceleration measurements. The accelerometer was encased in syntactic foam and epoxy to make it neutrally buoyant, and was placed in the same position as the fish's head. Measurements (re. 1 m s^{-2}) were made at every frequency and sound pressure level evaluated in this study for each of the three accelerometer axes (x, y, z), which corresponded to the following anatomical positions: x=anterior/posterior, y=left/right and z=dorsal/ventral. A PCB signal conditioner (model 482A16; PCB Piezotronics) was used to amplify the signal (gain=100× for each axis). Particle acceleration (a=m s^{-2}) was calculated using the equation $a=mV_{peak\text{-}to\text{-}peak}/S$, where S=accelerometer sensitivity (mV ms^{-2}) for the corresponding x-, y- or z-axis (Bhandiwad et al., 2017; Colleye et al., 2019; Vetter et al., 2019). The acoustic impedance of this acoustic tank environment has been reported previously for this speaker and experimental set-up (for more detail, see Vetter et al., 2019). The acoustical impedance is defined as the complex ratio of sound pressure to particle velocity (Bradley and Wilson, 1966; Erbe, 2011)

and, as suggested by Popper and Fay (2011), should be evaluated for all experimental tanks as the sound field in the artificial tank environment is probably affected by the tank size and materials.

Swim bladder removal procedure

Animals were first anesthetized by immersion in a solution of 0.025% ethyl-*p*-aminobenzoate in saltwater for 10–15 min. Next, fish were given intramuscular injections of cisatracurium besylate (~3 mg kg^{-1} of body mass) for immobilization and bupivacaine HCl (~1 mg kg^{-1} of body mass), an analgesic. Based on the treatment condition, either a swim bladder removal procedure (N=18; treated) or a sham surgery was performed (N=12; control). In both the swim bladder removal and sham surgery treatments, a small incision (1.5–2 cm) was made in the center of the fish's abdomen on the ventral side. To remove the swim bladder, sterile scissors were used to gently cut the connective tissue holding the swim bladder in place in the body cavity and then sterile forceps were used to remove the swim bladder from the body cavity. In both the sham surgery treatment and swim bladder removal procedure, the incision was closed with surgical sutures using a sterile needle.

Lagenar potential measurements

The procedure for recording potentials from the lagena was similar to that used by Vetter et al. (2019) and to the method used to record from the midshipman saccule in previous studies (Sisneros, 2007, 2009a; Alderks and Sisneros, 2011; Coffin et al., 2012; Bhandiwad et al., 2017; Colleye et al., 2019). Immediately after the swim bladder removal or sham surgery, the lagena was exposed via a dorsal craniotomy and the cranial cavity was filled with cold teleost Ringer solution to prevent drying of the inner ear end organs. To protect the surgery preparation from saltwater contamination, a 3–4 cm hydrophobic dam of denture cream was then built around the craniotomy. The fish was 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 underwater speaker (AQ339, a monopole). The speaker was embedded in a ~4 cm layer of gravel on the bottom of the tank and positioned in the center. Throughout the recording, fish were ventilated with chilled (14.5–15.5°C) saltwater, which was continuously pumped through the mouth and over the gills. The experimental tank was maintained on an inflated pneumatic, vibration-isolation table, housed inside an acoustical isolation chamber (Industrial Acoustics, New York, NY, USA). All 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 mol l^{-1} 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 to 3 kHz) and amplified (10×) again using a digital filter (model SR 650; Stanford Research Systems) before finally being sent to a lock-in amplifier (SR830, Stanford Research Systems). 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; Coffin et al., 2012; Bhandiwad et al., 2017; Colleye et al., 2019; Vetter et al., 2019). All experimental procedures followed NIH guidelines for the care and use of animals and were approved by the University of Washington Institutional Care and Use Committee.

Threshold determination and statistical analyses

Background noise measurements were performed prior to recording lagenar potentials and were used in determining the auditory threshold. The auditory threshold at each stimulus frequency was designated as the lowest stimulus level that yielded an averaged evoked lagena potential that was greater than two standard deviations above the average background measurement. 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. Particle acceleration thresholds were reported as the combined magnitude vector that was calculated as $20\log\sqrt{(x^2+y^2+z^2)}$ (Wysocki et al., 2009; Vasconcelos et al., 2011; Bhandiwad et al., 2017; Colleye et al., 2019; Vetter et al., 2019). The characteristic frequency (CF) is defined as the frequency that evoked the lowest lagenar threshold.

Parametric (t-test) or non-parametric (Mann–Whitney U) tests were used based on the Shapiro–Wilk test for normality (P<0.05) and performed in SigmaPlot (version 12.5; SYSTAT, Chicago, IL, USA). The auditory threshold tuning curves based on pressure and acceleration were modeled in RStudio (version 3.6.0) using a logarithmic regression model with the following packages: car and lme4 (The R Foundation for Statistical Computing). We only compared the frequencies for which both groups yielded more than one data point (85–445 Hz). The significance level was determined at P<0.05.

RESULTS

Evoked potentials were recorded from the lagenar epithelium in 30 adult female plainfin midshipman with a size range of 15.7–19.2 cm (L=17.5±1.0 cm, M_b=68.3±13.6 g, GSI=18.4±9.1; means±s.d.). There was no significant difference in L (t-test, t=0.33, d.f.=28, P>0.05), M_b (t-test, t=0.54, d.f.=28, P>0.05) or GSI (Shapiro–Wilk, P<0.05; Mann–Whitney U=101.0, t=179.0, N_1=12, N_2=18, P>0.05) between the sham surgery (control) and treated fish (swim bladder removal). For the control condition, 12 individual fish were tested, yielding a total of 17 auditory threshold tuning curves (for some fish, evoked potentials were recorded from both left and right lagenae) (Table 1). We tested 18 individual fish with the swim bladder removed (treated fish) and had a total of 30 tuning curves.

Auditory thresholds for both sound pressure and particle acceleration were determined for populations of hair cells in the lagena for both control and treated female midshipman. For both control and treated fish, lagenar potentials above threshold were recorded in response to sound pressures ranging from 127 to 154 dB re. 1 µPa. For control fish, evoked potentials above threshold were recorded in response to all frequencies evaluated (85–1005 Hz); however, in the females that had swim bladders removed, potentials were only recorded up to 505 Hz. Fig. 2 shows representative iso-level response profiles for a range (low, middle, high) of evoked lagenar potentials at the highest sound pressure level tested (154 dB re. 1 µPa) for both the control (85–1005 Hz) and treated

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Table 1. Summary of mean evoked potentials recorded from the hair cells of the lagena in control (swim bladder intact) and treated (swim bladder removal condition) midshipman at 154 dB re. 1 µPa

	Mean evoked potential (µV)		
Frequency (Hz)	Control	Treated	*P*-value
85	33±8.9 (*N*=12, 17)	13.9±4.8 (*N*=18, 30)	<0.001* (*U*=84, *t*=580)
105	15±7.0 (*N*=12, 17)	5.2±2.0 (*N*=18, 30)	<0.001* (*U*=90, *t*=573)
125	10±6.3 (*N*=12, 17)	3.3±0.9 (*N*=18, 30)	<0.001* (*U*=103, *t*=560)
165	11±5.1 (*N*=12, 17)	4.5±1.5 (*N*=18, 30)	<0.05* (*U*=145, *t*=471)
205	8±4.8 (*N*=12, 17)	2.4±1.4 (*N*=18, 27)	<0.05* (*U*=113, *t*=482)
245	7±5.2 (*N*=11, 16)	2.0±0.8 (*N*=16, 23)	<0.05* (*U*=88, *t*=400)
305	6±3.8 (*N*=10, 15)	1.7±0.6 (*N*=12, 114)	<0.05* (*U*=43, *t*=121)
345	4±2.3 (*N*=9, 13)	1.4±0.3 (*N*=9, 10)	<0.005* (*U*=14, *t*=59)
405	5±3.3 (*N*=8, 12)	1.3±0.4 (*N*=8, 8)	<0.05* (*U*=117, *t*=45)
445	3±1.5 (*N*=8, 11)	1.0±0.3 (*N*=4, 4)	<0.05* (*U*=0, *t*=6)
505	2±1.4 (*N*=8, 11)	1.1‡ (*N*=1, 1)	–
545	2±1.1 (*N*=8, 11)	–	–
605	5±4.4 (*N*=8, 11)	–	–
645	4±2.5 (*N*=8, 11)	–	–
705	4±3.3 (*N*=8, 11)	–	–
745	2±1.3 (*N*=8, 10)	–	–
805	5±4.2 (*N*=6, 7)	–	–
845	2±0.9 (*N*=6, 7)	–	–
905	2±1.3 (*N*=6, 6)	–	–
945	3±1.8 (*N*=5, 5)	–	–
1005	1±0.9 (*N*=4, 4)	–	–

For each frequency evaluated, the mean evoked potential (±95% confidence interval, CI) between control and treated fish in response to the highest sound pressure level evaluated (154 dB re. 1 µPa) is shown. The threshold was defined as the lowest sound pressure level (dB re. 1 µPa) required to evoke a potential 2 s.d. above background noise. The total number of individual fish for which we were able to record evoked potentials is shown for both the control and treated fish (first *N* value). Furthermore, as we were able to record from both left and right lagenae in some individual fish, the total number of records for which evoked potentials were elicited (second *N* value) is also shown. To compare the mean evoked potentials between the two conditions, non-parametric Mann–Whitney *U*-tests were performed for the frequencies that yielded more than one data point for both groups (85–445 Hz) and the *P*-values are shown. *Significant differences between the control and treated condition ($P<0.05$). Data are displayed as means±95% CI (‡except for the single fish in the treatment group at 505 Hz).

(85–505 Hz) females. For control fish, mean evoked potentials varied from 32.6±8.9 µV (95% confidence interval, CI) at 85 Hz to 1.3±0.9 µV at 1005 Hz (Table 1). Evoked potentials were significantly ($P<0.05$) lower in treated fish (swim bladder removal condition) with mean potentials ranging from 13.9±4.8 µV at 85 Hz to 1.0±0.3 µV at 445 Hz (Table 1).

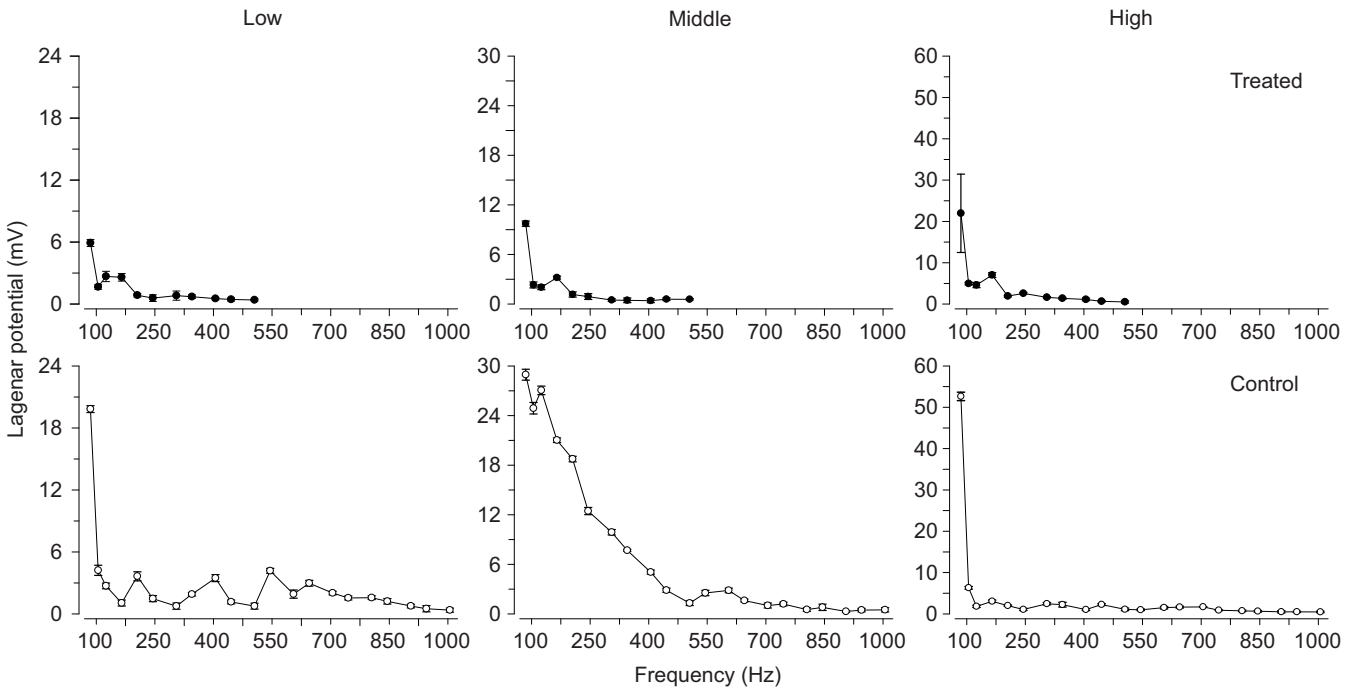

Fig. 2. Representative examples of iso-intensity curves recorded from the lagena in response to pure tone frequencies at the highest sound pressure level tested (154 dB re. 1 µPa). A range of evoked potentials (low, middle and high) are shown for both control (intact swim bladder condition; bottom) and treated (removed swim bladder condition; top) females. The data are displayed as means±95% CI.

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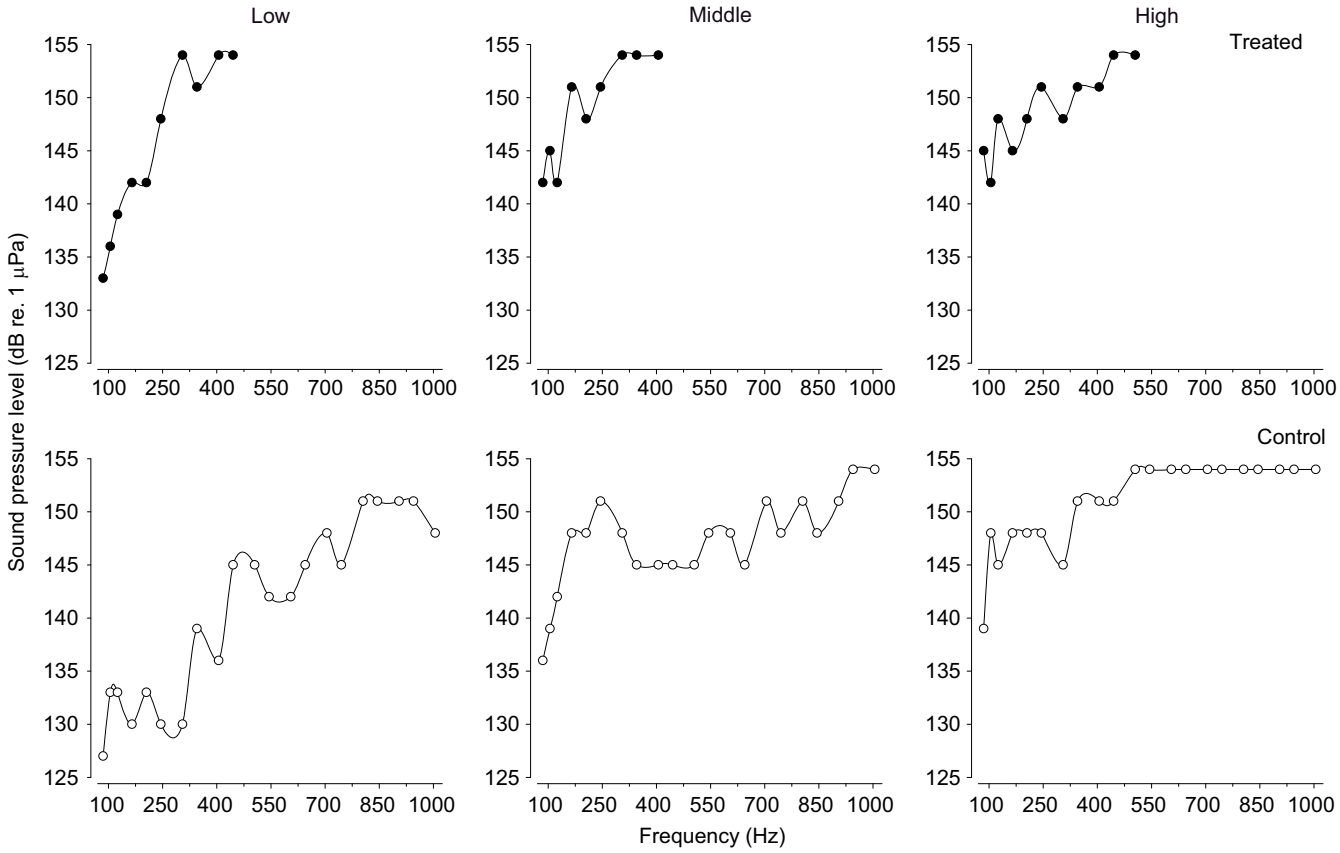

Fig. 3. Representative examples of individual auditory threshold tuning curves based on evoked potentials recorded from the lagena of control and treated female midshipman. A range of characteristic frequencies (low, middle, high) are shown for both control (intact swim bladder condition; bottom) and treated (removed swim bladder condition; top) females. Thresholds were defined as the lowest sound pressure level (dB re. 1 µPa) to evoke a lagenar potential at least 2 s.d. above background noise.

Fig. 3 shows representative tuning curves based on sound pressure sensitivity for both the control and treated females. For the control fish, the threshold tuning curves based on sound pressure consisted of response profiles with lowest thresholds ≤165 Hz (mean thresholds±95% CI: 133.4±2.4 to 145.0±3.7 dB re. 1 µPa) that gradually increased to highest thresholds at frequencies ≥705 Hz (mean thresholds: 150.7±1.4 to 152.5±4.8 dB re. 1 µPa) (Table 1). The treated females also had the lowest thresholds at frequencies ≤165 Hz (mean thresholds: 139±1.9 to 148±2.2 dB re. 1 µPa) and the highest thresholds were observed at frequencies ≥305 Hz (mean thresholds: 152±1.3 to 154±0.0 dB re. 1 µPa) (Table 1). Characteristic frequencies (CFs) ranged from 85 to 165 Hz in both control and treated females, with the majority of CFs occurring at 85 Hz (control=89%, treated=80%) and there was no significant difference in CFs between the two groups (Shapiro–Wilk, $P<0.05$; Mann–Whitney $U=279.0$, $t=487.0$, $N_1=19$, $N_2=35$, $P<0.05$). The range of CFs (low, medium and high) are shown for both control and treated females in Fig. 3.

To compare the mean threshold tuning curves, we applied a logarithmic regression model for both the control (adjusted $r^2=0.30$) and treated (adjusted $r^2=0.42$) conditions up to 445 Hz (i.e. only for the frequencies that yielded more than one data point for both groups). Mean lagenar tuning curves based on slope ($P<0.05$) and intercept ($P<0.001$) were significantly different between control and treated females (ANOVA, $F=57.8$, d.f.=3,354, $P<0.001$). Fig. 4 shows the mean auditory threshold tuning curves based on sound pressure for lagenar hair cells in the control and treated fish. Control females with intact swim bladders had auditory thresholds that were ∼3–6 dB re. 1 µPa lower than treated females without swim bladders at frequencies from 85 to 505 Hz. At frequencies greater than 505 Hz, only females with intact swim bladders had measurable

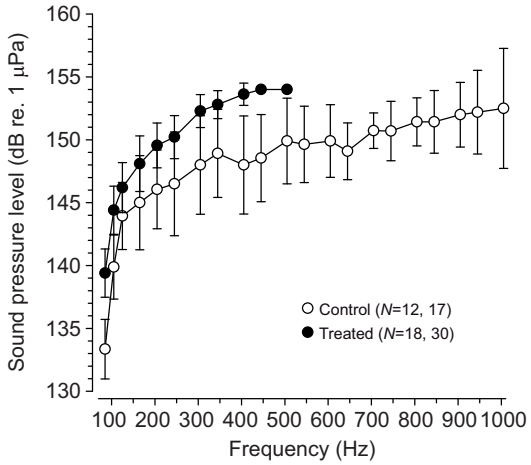

Fig. 4. Auditory threshold tuning curves based on sound pressure levels for lagenar potentials recorded in control and treated female midshipman. The auditory threshold was defined as the lowest sound pressure level (dB re. 1 µPa) needed to evoke a lagenar potential that was at least 2 s.d. above background noise. Data are represented as means±95% CI. For both control (intact swim bladder condition) and treated (removed swim bladder condition) fish, the number of individual animals (first *N* value) and records (second *N* value) is indicated in parentheses.

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auditory thresholds (150–153 dB re. 1 µPa) from 545 to 1005 Hz, while no measurable thresholds were recorded for sound pressure levels up to 154 dB for treated females over the same frequency range (note that 154 dB re. 1 µPa was the highest sound level used in this study in order to avoid damage to the underwater speaker).

Evoked lagenar potentials were elicited at mean particle acceleration levels ranging from −12.3±2.8 to 11.7±0.15 dB re. 1 m s^{-2} (mean±95% CI) for both control and treated fish. The general tuning profiles based on acceleration for both control and treated fish had lowest thresholds at ≤165 Hz and gradually increased to the highest thresholds at frequencies ≥705 Hz for control females and ≥305 Hz for treated females. CFs based on particle acceleration ranged from 85 to 165 Hz with the majority of CFs occurring at 85 Hz (control=89%, treated=80%) and there was no significant difference in CFs between the two groups (Shapiro–Wilk, P<0.05; Mann–Whitney U=580.5, N_1=34, N_2=35, P=0.852). Fig. 5 shows the average threshold tuning curves based on acceleration for control and treated females. Control fish had thresholds that were ∼5–7 dB re. 1 m s^{-2} lower across the range of frequencies from 85 to 505 Hz compared with the treated fish that had their swim bladders removed. Again, we used a logarithmic regression model to compare the mean threshold tuning curves based on particle motion for both control (adjusted r^2=0.27) and treated (adjusted r^2=0.35) fish up to 445 Hz. Mean lagenar tuning curves relative to acceleration were also significantly different between control and treated females based on slope (P<0.05) and intercept (P<0.001) (ANOVA, F=44.7, d.f.=3,354, P<0.001).

Evoked lagenar potentials (relative to sound pressure and acceleration) were most consistently recorded from 85 to 165 Hz (100%) of the 47 recordings made from all 30 animals (Fig. 6, Table 1). For the control fish, lagenar potentials were recorded from 100% of fish from 85 to 205 Hz. The percentage of evoked potential recordings for control fish then dropped from 94 to 71% between 245 and 405 Hz, respectively. From 445 to 745 Hz, the percentage of recordings stayed consistently between 59 and 65%, but then dropped again from 41 to 30% between 745 and 945 Hz, respectively. At the highest frequency examined (1005 Hz), evoked potentials were detected in 24% of animals (N=4) with intact swim bladders. In contrast, lagenar potentials were only recorded in response to frequencies between 85 and 505 Hz in fish with removed swim bladders (treated). In treated fish, evoked potentials were most consistently recorded from 85 to 165 Hz (100%), and the percentage of recordings that had evoked potentials at 205 to 245 Hz dropped from 90 to 77% and then from 33 to 13% between 345 and 445 Hz, respectively. At 505 Hz, only one animal (3%) had detectable evoked lagenar potentials with the swim bladder removed.

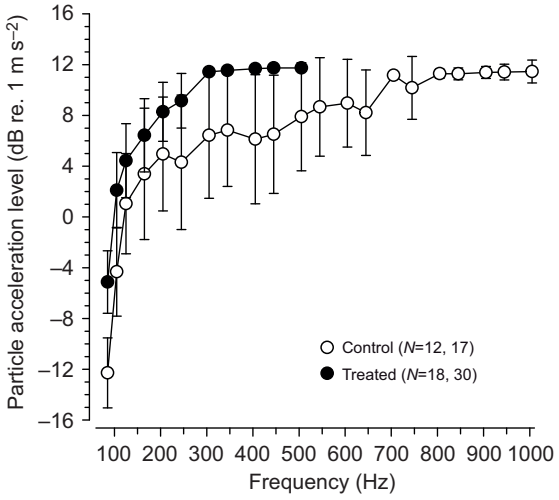

Fig. 5. Auditory threshold tuning curves based on particle acceleration levels for lagenar potentials recorded in control and treated female midshipman. Particle acceleration measurements were made at each sound pressure level evaluated and thresholds for particle acceleration were constructed at the corresponding sound pressure levels. Data are represented as means±95% CI. The number of individual animals (first *N* value) and records (second *N* value) for both control (intact swim bladder condition) and treated (removed swim bladder condition) fish is indicated in parentheses.

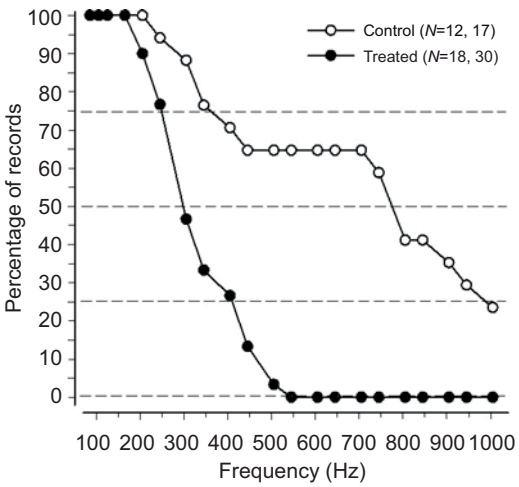

Fig. 6. Percentage of lagenar potentials evoked above threshold at each tested frequency for control and treated fish. Lagenar potentials were observed in all recordings at frequencies from 85 to 205 Hz in control (intact swim bladder condition; *N*=17) fish and at frequencies from 85 to 165 Hz in treated (removed swim bladder condition; *N*=30) fish. However, at frequencies >205 Hz in control fish or >165 Hz in treated fish, lagenar potentials were not always detected, even at the highest sound pressure evaluated. The number of individual animals (first *N* value) and records (second *N* value) for both treatment groups is indicated in parentheses.

DISCUSSION

The objective of this study was to determine if the swim bladder in female plainfin midshipman enhances the auditory sensitivity of the lagena to sound pressure and higher frequencies. We show that females with intact swim bladders had relatively high lagenar sensitivity across the range of frequencies tested (80–1005 Hz) with a maximum frequency detection up to 1005 Hz (highest frequency tested). In contrast, females with removed swim bladders had relatively low lagenar sensitivity and a maximum frequency detection only up to 505 Hz. Our results reveal that the presence of the sexually dimorphic swim bladders in females affords an increase in gain for the lagena such that it is more sensitive to sound pressure, especially at high frequencies. In this discussion, we consider the role of the swim bladder as an acoustic organ and how it is used to detect sound pressure cues and higher frequencies, both of which are likely to be important for reproductive females in detecting biologically relevant signals during social and reproductive behaviors.

Our results support the hypothesis that the swim bladder in reproductive female midshipman enhances the auditory sensitivity of the lagena to sound pressure. The swim bladder in female midshipman is known to serve as an acoustic organ that allows sound pressure-induced vibrations of the swim bladder to be detected by the inner ear saccule (Colleye et al., 2019). The swim

bladder in midshipman and other pressure-sensitive fish can act as a pressure-to-displacement transducer to stimulate the particle-motion sensitive otolithic end organs, depending on the proximity of the swim bladder to the three auditory end organs (saccule, lagena and utricle). In female midshipman, the horn-like extensions of the swim bladder project close to the saccule (mean distance 2.59 mm) and the lagena (mean distance 2.89 mm) (Mohr et al., 2017). Our result of swim bladder-enhanced lagenar sensitivity to sound pressure in females is in agreement with previous observations of pressure sensitivity being associated with having a swim bladder in close proximity to an auditory end organ. In other pressure-sensitive fishes, the swim bladder can be found relatively close to auditory end organs, often less than 3 mm away from the saccule and/or lagena (Ramcharitar et al., 2006; Schulz-Mirbach et al., 2012; Kéver et al., 2014). In our study, we report that the swim bladder increased lagenar sensitivity to sound pressure by a factor of 1.4 to 2 times (or 3 to 6 dB re. 1 µPa) over a range of frequencies from 85 to 405 Hz, compared with fish with no swim bladders. Although the variation in the threshold data (shown by the 95% CI; Fig. 4), resulted in adjusted r^2 values for the logarithmic regression models that were somewhat low (0.27–0.48), the most compelling finding from this study was the extended bandwidth of frequency detection at socially relevant sound levels in the control group. Control females with swim bladders had measurable sound pressure thresholds (mean thresholds 133–153 dB re. 1 µPa) up to 1005 Hz, while females without swim bladders only had measurable pressure thresholds (mean thresholds 139–154 dB re. 1 µPa) up to 505 Hz. Our findings are consistent with our previous study (Colleye et al., 2019), in which we report that swim bladder enhanced pressure sensitivity of the saccule, the most sensitive midshipman auditory end organ. In the Colleye et al. (2019) study, we showed that the swim bladder in reproductive females increased the sensitivity of the saccule to sound pressure by a factor of 1.8 to 3.5 times (or 5–11 dB re. 1 µPa) over a range of frequencies from 75 to 1005 Hz, compared with females with no swim bladders. The findings from our study and that of Colleye et al. (2019) support the hypothesis that the sexually dimorphic swim bladder of female midshipman enhances the sensitivity of the lagena and saccule to sound pressure.

Results from our study also indicate that the swim bladder plays an important role in expanding the gain and upper bandwidth limit of frequency sensitivity in the lagena. We show that fish with removed swim bladders had a maximum frequency detection of 505 Hz, while control fish with intact swim bladders had a maximum frequency detection up to 1005 Hz. Furthermore, we observed that control females with intact swim bladders had greater evoked potentials (Table 1) and a much greater percentage of evoked lagenar potentials over a range of frequencies from 205 to 505 Hz, compared with treated females with removed swim bladders (Fig. 6). It is possible that females without swim bladders may exhibit measurable lagenar thresholds at frequencies >505 Hz using sound pressure levels greater than 154 dB re. 1 µPa (the highest levels used in this study), but we chose to limit auditory stimuli to sound pressure levels of ≤154 dB re. 1 µPa in order to avoid damage to our underwater speaker. The advertisement calls of type I males are known to be as loud as 154–161 dB re. 1 µPa at the entrance of a midshipman nest (Bass and Clark, 2003; Vetter et al., 2019, fig. 8), but it remains unclear at what levels sound pressure becomes too intense and not behaviorally relevant. The observed increase in maximum frequency detection of the lagena in control females with intact swim bladders compared with treated females with removed swim bladders was similar to that reported for the midshipman saccule (Colleye et al., 2019). Colleye et al. (2019) showed that control females with intact swim bladders had a greater percentage of the evoked saccular potentials above 305 Hz, compared with treated females with removed swim bladders. Increased sensitivity to higher frequencies has also been reported in other fish species that possess rostral swim bladder extensions that project to the inner ear. For instance, in squirrelfishes (genus *Myripristis*), Coombs and Popper (1979) reported that species with the smallest distance between the swim bladder and the inner ear had the greatest sensitivity to sound pressure and higher frequencies. In addition, Atlantic cod (*Gadus morhua*) and other members of the *Gadidae* family also have rostral swim bladder extensions that are associated with enhanced frequency sensitivity, especially above 100 Hz (Chapman and Hawkins, 1973; Sand and Enger, 1973; Offutt, 1974). Thus, the rostral swim bladder extensions found in species such as the plainfin midshipman, Atlantic cod and squirrelfishes are probably conserved morphological adaptations that enhance the auditory sensitivity of the fish inner ear to sound pressure cues and higher frequencies.

Studies evaluating the impact of swim bladder deflation on hearing sensitivity have similarly reported increased thresholds in species with specialized connections between the swim bladder and inner ear including: brown bullhead (*Ameiurus nebulosus*; Kleerekoper and Roggenkamp, 1959), Atlantic codfish (Offutt, 1974), channel catfish (*Ictalurus punctatus*; Fay and Popper, 1975), roach (*Rutilus rutilus*; Lamming and Morrow, 1981), goldfish (*Carassius auratus*; Yan et al., 2000), New Zealand bigeye (*Pempheris adspersa*; Radford et al., 2013) and walking catfish (*Clarias batrachus*; Shao et al., 2014). Yan et al. (2000) examined the effect of swim bladder deflation on hearing in oyster toadfish (*Opsanus tau*), a species in the same family as plainfin midshipman (Batrachoididae), and reported no change in hearing sensitivity. However, unlike midshipman, oyster toadfish do not have swim bladder extensions or any other known coupling between the swim bladder and the inner ear. In contrast, Tricas and Boyle (2015) deflated only the anterior swim bladder horns in two species of butterflyfishes (*Chaetodon multicinctus* and *Chaetodon auriga*) and reported 5–20 dB increases in hearing thresholds. Here, we report a decrease in sound pressure sensitivity, especially at the higher frequencies, in female midshipman with surgically removed swim bladders. We also report a decrease in bandwidth of treated females with removed swim bladders but this is probably because we chose not to test treated females at sound pressure levels greater than 154 dB re. 1 µPa in order to avoid damaging our underwater speaker. A similar bandwidth of frequency sensitivity in treated females probably exists, but at higher sound pressure levels. Future research could compare both saccular and lagenar sensitivity in female midshipman with ablated anterior swim bladder horns to that of male midshipman, which do not have rostral swim bladder extensions.

Our results support the hypothesis that the lagena functions as an accessory auditory end organ to the saccule that can act to extend the dynamic sensitivity range of the inner ear to auditory stimuli (Lu et al., 2003; Khorevin, 2008; Vetter, 2019; Vetter et al., 2019). In the midshipman, the larger saccule is more sensitive and has lower evoked potential thresholds than the smaller lagena (Sisneros, 2007; Vetter et al., 2019). The size of the end organ, which is often correlated with the surface area of auditory epithelia and the number of hair cells contained within the end organ, is known to be related to the end organ's overall auditory sensitivity (Corwin, 1983; Coffin et al., 2012; Lu and DeSmidt, 2013). Thus, the differences in auditory sensitivity between the midshipman saccule and lagena are likely to

be related, in part, to their differences in size. Previous studies that have characterized the sensitivity of the saccule and the lagena in the midshipman under the same experimental conditions have reported lower evoked potential thresholds from hair cells in the saccule compared with the lagena (Sisneros, 2007; Colleye et al., 2019; Vetter et al., 2019). For example, in male plainfin midshipman, lagenar thresholds were approximately 12–23 dB re. 1 µPa higher (i.e. less sensitive) than saccular thresholds across a range of frequencies from 85 to 345 Hz (Sisneros, 2007; Vetter et al., 2019). Similarly, in reproductive females, the lagenar thresholds reported in the present study were approximately 5–24 dB re. 1 µPa higher than those previously reported for the saccule across a range of frequencies from 105 to 1005 Hz (Colleye et al., 2019). Relatively few studies have evaluated the auditory sensitivities of all three end organs (saccule, lagena and utricle) within a single species using the same experimental set-up. Notably, Lu et al. (2003, 2004, 2010) characterized the auditory afferent sensitivities of all three end organs in the sleeper goby (*Dormitator latifrons*) under similar conditions using a shaker table system. In these studies, the thresholds were characterized based on acceleration, as sleeper gobies do not have swim bladders and are probably not pressure sensitive. For the sleeper goby, both the saccule and lagena afferents had overlapping ranges of characteristic frequencies (CF) (saccule: CF <50–400 Hz; lagena: CF <50 Hz and 80–125 Hz; utricle: CF ≤50–400 Hz), but the saccule was more broadly tuned compared with the lagena (Lu et al., 2003, 2004, 2010). Furthermore, Lu and Zu (2002) also found that bilateral removal of the saccule in the sleeper goby resulted in significant hearing loss (13–35 dB re. 1 µm) at various frequencies, but nevertheless the fish were still capable of sound detection, probably relying on the lagena and possibly the utricle to detect auditory stimuli. Although there are differences in the hearing abilities between midshipman and sleeper goby, both of these examples reveal that the lagena has higher thresholds (i.e. less sensitive) than the saccule but has a bandwidth of frequency sensitivity similar to that of the saccule, which is likely to contribute to hearing by extending the dynamic range of inner ear sensitivity to biologically relevant acoustic stimuli, especially when close to a sound source (Lu et al., 2003; Khorevin, 2008; Vetter, 2019; Vetter et al., 2019). To better understand the role of individual end organs in fish hearing, future research should focus efforts to characterize each end organ's auditory sensitivity independently under similar experimental conditions on a species-by-species basis, especially given the high diversity of fish inner ears and swim bladder morphologies across taxa (Schulz-Mirbach et al., 2019).

The swim bladder enhanced lagenar sensitivity to sound pressure, and higher frequencies observed in female midshipman may be adaptive for sound source localization and the assessment of conspecific mates in complex, shallow-water acoustic environments, like those where midshipman breed during the summer. During the summer reproductive season, plainfin midshipman migrate into the shallow intertidal waters along the west coast of North America and can be found in calm shallow bays and estuaries. From their rocky shelters, type I or 'nesting' males produce multi-harmonic male advertisement calls that contain significant acoustic energy in the higher frequency harmonics that range up to 1000 Hz (Bass et al., 1999; Vetter et al., 2019). While low frequencies propagate in deep water environments, the low-frequency components of the male advertisement call, including the fundamental frequency (approximately 80–100 Hz), are often below the cut-off frequency of sound transmission and will attenuate rapidly in shallow water environments (Rogers and Cox, 1988a; Bass et al., 1999). Thus, in the midshipman shallow-water breeding grounds, only the higher frequency harmonics of the advertisement call will propagate, and therefore the swim bladder enhanced sensitivity of the lagena is likely to aid in the ability of females to detect and localize calling males. Furthermore, the spawning area can be loud and noisy during breeding, as male nests are often clustered close together and the sound pressure level of a single type I male midshipman call can be as loud as 154–161 dB re. 1 µPa near the nest opening (Bass and Clark, 2003; Vetter et al., 2019). During courtship when females approach the nest, sound pressure levels of individual male advertisement calls can be approximately 10–40 dB re. 1 µPa greater than the reported mean evoked potential thresholds of the saccule (Sisneros, 2009b; Colleye et al., 2019), which can potentially over-stimulate and exceed the dynamic range of the saccule and its auditory afferents. In such cases, as the lagena has higher evoked potential thresholds and a similar bandwidth to that of the saccule, it may act to extend the dynamic sensitivity range of the auditory inner ear to aid females in mate localization when close to the sound source. Finally, the swim bladder-enhanced auditory sensitivity of the lagena to higher frequencies may be adaptive for the detection of higher frequency harmonics in the male advertisement call, which may contain acoustic information related to reproductive condition-dependent indicators of mate quality (e.g. male size, body condition, health, etc.). Future studies that examine the signal characteristics of midshipman vocalizations will be instrumental in determining if the male advertisement call is an 'honest' signal and can be potentially used by females in mate choice decisions.

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Author contributions
Conceptualization: B.J.V., J.A.S.; Methodology: B.J.V., J.A.S.; Formal analysis: B.J.V., J.A.S.; Investigation: B.J.V., J.A.S.; Resources: J.A.S.; Writing - original draft: B.J.V.; Writing - review & editing: J.A.S.; Supervision: J.A.S.; Project administration: J.A.S.; Funding acquisition: B.J.V., J.A.S.

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