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RESEARCH ARTICLE

Sexually dimorphic swim bladder extensions enhance the auditory sensitivity of female plainfin midshipman fish, *Porichthys notatus*

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ABSTRACT

The plainfin midshipman fish, *Porichthys notatus*, is a seasonally breeding, nocturnal marine teleost fish that produces acoustic signals for intraspecific social communication. Females rely on audition to detect and locate 'singing' males that produce multiharmonic advertisement calls in the shallow-water, intertidal breeding environments. Previous work showed that females possess sexually dimorphic, horn-like rostral swim bladder extensions that extend toward the primary auditory end organs, the saccule and lagena. Here, we tested the hypothesis that the rostral swim bladder extensions in females increase auditory sensitivity to sound pressure and higher frequencies, which potentially could enhance mate detection and localization in shallow-water habitats. We recorded the auditory evoked potentials that originated from hair cell receptors in the saccule of control females with intact swim bladders and compared them with those from treated females (swim bladders removed) and type I males (intact swim bladders lacking rostral extensions). Saccular potentials were recorded from hair cell populations *in vivo* while behaviorally relevant pure-tone stimuli (75–1005 Hz) were presented by an underwater speaker. The results indicate that control females were approximately 5–11 dB re. 1 µPa more sensitive to sound pressure than treated females and type I males at the frequencies tested. A higher percentage of the evoked saccular potentials were recorded from control females at frequencies >305 Hz than from treated females and type I males. This enhanced sensitivity in females to sound pressure and higher frequencies may facilitate the acquisition of auditory information needed for conspecific localization and mate choice decisions during the breeding season.

KEY WORDS: Saccule, Hearing, Gas bladder, Communication

INTRODUCTION

Rostral swim bladder extensions have been reported in a number of teleost fishes across a variety of families including Batrachoididae, Holocentridae, Gadidae, Gerreidae, Sciaenidae, Chaetodontidae, Cichlidae and Serrasalmidae (Nelson, 1955; Braun and Grande, 2008; Parmentier et al., 2011; Tricas and Boyle, 2015; Tricas and Webb, 2016; Ladich, 2016; Mohr et al., 2017; Boyle and Herrel, 2018). These swim bladder morphological adaptations are thought to increase auditory sensitivity to sound pressure and higher frequencies by decreasing the distance between the swim bladder and inner ear, which then allows sound pressure-induced vibrations of the swim bladder to be detected by the inner ear auditory end organs (Popper and Coombs, 1980; Braun and Grande, 2008). In some fishes, this indirect mechanism of sound pressure detection via the swim bladder may be important for the detection and localization of behaviorally relevant acoustic stimuli (Coffin et al., 2014).

Sound pressure detection by fishes is thought to be a more recently derived characteristic of fish auditory systems while particle motion detection is consider to be the more ancestral state with all or most fishes being capable of using their otolithic end organs as inertial accelerometers to detect the direct displacement of the fish by local particle motion. The most common pressure-mediated mode of fish hearing involves the swim bladder and an otophysic connection. Otophysan fishes (e.g. goldfish) have evolved skeletal adaptations (i.e. Weberian ossicles) that connect the anterior part of the swim bladder to the inner ear, with the swim bladder acting as a crude 'ear drum' that captures sound pressure energy and then transduces it to the inner ear via the otophysic connection. While otophysan fishes are believed to be sensitive to sound pressure throughout their hearing range (Fay et al., 2002), all other fishes are thought to possess a 'continuum' of pressure detection mechanisms that range from fish with highly specialized otophysic connections (e.g. goldfish, catfish and relatives), to fish with the swim bladder close but not connecting to the inner ear (e.g. Atlantic cod), to fish with the swim bladder far from the inner ear (e.g. salmonids), to fish with no swim bladder or gas bubble (e.g. flatfish and sharks) (Popper and Fay, 2011).

A recent micro-computerized tomography study revealed that plainfin midshipman fish (*Porichthys notatus*) possess intrasexual and intersexual dimorphic swim bladder extensions that project toward the inner ear saccule and lagena (Mohr et al., 2017). Similar examples of modified swim bladders are found in squirrelfish (Family Holocentridae) and cod (Family Gadidae), which have paired rostral swim bladder extensions that project toward the inner ear, and these fish exhibit increasing sound pressure sensitivity the closer the swim bladder is to the saccule and lagena (Coombs and Popper, 1979; Chapman and Hawkins, 1973). The horn-like swim bladder extensions in squirrelfish and cod decrease the distance between the swim bladder and the auditory end organs (i.e. saccule and lagena) to more effectively detect the local particle motion generated by the pressure wave-induced vibrations of the swim bladder when exposed to sound. Thus, this indirect mechanism of swim bladder-mediated pressure detection is thought to increase auditory sensitivity and extend the upper range of frequencies that these fish can detect (Popper and Coombs, 1980; Braun and Grande, 2008).

The detection of sound pressure by fishes is also thought to be important for the localization of sound sources. The dominant theories for fish sound localization maintain that the detection and

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processing of both sound pressure and particle motion are necessary for successful sound source localization (Schuijf and Buwalda, 1975; Rogers et al., 1988; Sisneros and Rogers, 2016). Recent behavioral experiments with the plainfin midshipman suggest that the swim bladder is necessary for near-field sound localization (Coffin et al., 2014). Reproductive female midshipman primarily rely on audition to detect and locate potential mates during the breeding season. Nesting males produce an advertisement call or 'hum' to attract females for courtship and spawning, and the playback of natural and synthetic hums can evoke strong phonotaxic responses in females that are gravid (full of eggs) (McKibben and Bass, 1998; Zeddies et al., 2010, 2012). Coffin et al. (2014) showed that females with surgically deflated swim bladders with no access to sound pressure cues had a very low probability of locating sound sources. The majority of the females (95%) that localized sound sources had at least partially inflated swim bladders, suggesting that pressure reception is critical for sound source localization, at least in shallow water environments like those where midshipman breed (Coffin et al., 2014).

The objective of this study was to determine whether the sexually dimorphic, rostral swim bladder extensions observed in female midshipman function to enhance auditory sensitivity to sound pressure and higher frequencies. We hypothesized that the rostral swim bladder extensions found in females (Mohr et al., 2017) afford greater sound pressure sensitivity and extend the upper range of frequencies detected by the midshipman auditory system, which will likely facilitate the increased detection and localization of conspecifics in shallow water habitats. We predicted that females with intact swim bladders (control group) would have greater sensitivity to sound pressure and higher frequencies than females with swim bladders removed (treated group). In addition, we compared the sound pressure sensitivity of control females with intact swim bladders with that of type I males with intact swim bladders lacking rostral extensions. We interpret our results as relating to possible adaptations of the plainfin midshipman for social acoustic communication and conspecific detection and localization.

MATERIALS AND METHODS

Animal collection and care

The 49 adult plainfin midshipman fish, *Porichthys notatus* Girard 1854, used in this study were hand-collected during low tide from exposed nests in the rocky intertidal zone in the summer (May–July) midshipman breeding season of 2016. Among those fish, 34 (20 females and 14 type I males) were collected from Tomales Bay, CA, USA, during late May 2016 and the remaining 15 animals were females collected near Seal Rock in Brinnon, WA, USA, during early July 2016. Soon after field collection, fish were transported to the University of Washington in Seattle, WA, USA, where they were kept in saltwater aquaria at 13–15°C with a 16 h:8 h light:dark photoperiod that simulated the ambient summer photoperiod. Fish were fed with defrosted shrimp every 2–4 days. Before each physiology experiment, the standard length (SL) and body mass (BM) of each individual was recorded, and then after each experiment the reproductive state and sex of the individual was confirmed by visual inspection of the gonads and by measurement of the gonadosomatic index (GSI). GSI was calculated as 100×gonad mass/(body mass−gonad mass), according to Tomkins and Simmons (2002). The SL, BM and GSI ranges of females and type I males reported were well within the ranges reported for both sexes in previous studies (Sisneros, 2007, 2009a; Rohmann and Bass, 2011).

Saccular potential recordings were performed within 15 days of collection from the field for females, whereas type I males were recorded approximately 2 months after collection while being maintained in captivity. All experimental procedures followed National Institutes of Health guidelines for the care and use of animals and were approved by the University of Washington Institutional Animal Care and Use Committee.

Stimulus generation and calibration

The methodology used in the present study followed that of previously published work (Sisneros, 2007, 2009a; Alderks and Sisneros, 2011; Bhandiwad et al., 2017). Acoustic stimuli were generated by a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA) that sent the signal to an audio amplifier and then to an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA). Prior to each experiment, calibration of the acoustic stimuli was performed by positioning a mini-hydrophone (model 8103, Bruel & Kjaer, Naerum, Denmark) 10 cm above the underwater speaker, at the position normally occupied by the fish's inner ear during the recordings. Stimuli were calibrated by peak-to-peak voltage measurements on an oscilloscope, and then equalized in sound pressure level (SPL) using an iterative MATLAB (MathWorks Inc., Natick, MA, USA) script that measured power spectral density for all frequencies (75–1005 Hz) from recordings taken through the mini-hydrophone. At each iteration, the voltage signal sent to the underwater speaker was scaled until the measured SPL output at each frequency tested was within ±2 dB of the desired amplitude. Acoustic stimuli were 500 ms pure tones presented at 30 Hz increments from 75 to 105 Hz, at 40 Hz increments from 105 to 385 Hz, and at 100 Hz increments from 505 to 1005 Hz. Note these frequencies were chosen in order to avoid frequencies associated with the harmonics of 60 Hz noise and resonant frequencies of the experimental tank. We presented eight repetitions of each tone at a rate of one every 1.5 s. Each recording session began with blank test trials (no acoustic stimulus) followed by the presentation of a single-tone (frequency) stimulus that was randomly selected. In order to measure threshold tuning responses, pure-tone stimuli were presented at SPLs from 100 to 151 dB re. 1 µPa in incremental steps of 3 dB.

Batrachoidid fish, such as the plainfin midshipman, lack specialized structures for hearing (e.g. Weberian ossicles) and they are thought to primarily detect acoustic particle motion (Popper and Fay, 2011). Consistent with previous studies (Casper and Mann, 2006; Wysocki et al., 2009; Bhandiwad et al., 2017), we thus report hearing thresholds in terms of both sound pressure and particle acceleration levels. Acceleration measurements were collected in three dimensions relative to fish orientation inside the tank – x (anterior/posterior), y (left/right) and z (dorsal/ventral) – using a custom-modified three-dimensional underwater accelerometer [PCB model VW356A12, sensitivity of 10.4 mV/(m s^{-2}) x-axis, 9.6 mV/(m s^{-2}) y-axis, 10.14 mV/(m s^{-2}) z-axis; PCB Piezotronics, Depew, NY, USA] that was encased in syntactic foam and epoxy to make it neutrally buoyant. For each sound level, particle acceleration was relatively constant and greatest in the z-axis relative to that in the axes (x and y) orthogonal to the speaker motion. Moreover, particle acceleration in all three dimensions scaled linearly across the SPLs tested (see Bhandiwad et al., 2017, for more details). Because of this linear relationship between sound pressure and particle motion at all frequencies tested, best-fit linear transformations were used to determine the equivalent particle motion measurements in all three axes for each frequency. Then, particle motion threshold, reported as the combined magnitude vector, was calculated as $20\log[\sqrt{(x^2+y^2+z^2)}]$ (see Wysocki et al., 2009; Vasconcelos et al., 2011).

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Swim bladder removal experiments

Animals were anesthetized by immersion in a 0.025% (250 mg l^{-1}) ethyl-*p*-aminobenzoate saltwater bath solution for approximately 5–7 min followed by an intramuscular injection of cisatracurium besylate (~3 mg kg^{-1} BM) for immobilization. Before proceeding to the swim bladder removal surgery, fish were injected with bupivacaine (~1 mg kg^{-1} BM) at the incision site for local analgesia. A small incision (~1.5 cm) was made on the sidewall of the body at the midpoint of the body length (i.e. next to the place where the swim bladder is located in the body cavity). After incision, sterile forceps and a cauterizer (Acu-Tip®, Practicon, Greenville, NC, USA) were used to remove the connective tissue of the swim bladder that was attached to the inside body cavity wall. Then, the swim bladder was deflated using a sterile scalpel blade, and gently removed from the body cavity with the forceps. Once the swim bladder (Fig. 1) had been removed, the body wall incision was closed with running sutures. After surgical treatment, all animals were monitored for signs of stress such as color loss or changes in blood flow in the sensory auditory epithelium. No such signs of stress were noted for any of the animals used in physiology experiments. A total of 17 females underwent this surgical procedure and were considered as treated individuals, whereas 18 control females were subject to the identical process but without swim bladder deflation and removal (in order to ensure that all experimental fish were exposed to the same surgery-related stress). In addition, the 14 type I males were also subjected to the same surgical process as control females.

Saccular potential recordings

Immediately after the swim bladder removal surgery, the inner ear saccule was exposed by removing skin, muscle and bone just dorsal to the otic capsules. The surgical procedures were similar to those used in previous studies (Sisneros, 2007, 2009a; Alderks and Sisneros, 2011; Bhandiwad et al., 2017). Once opened, the cranial cavity was filled with cold teleost Ringer's solution in order to prevent drying. A hydrophobic dam (~2–3 cm high) made of denture cream was built around the craniotomy to enable the fish to be submerged just below the water surface without exposing the brain and inner ears to salt water. During the recording, fresh, chilled salt water (14±1°C) was pumped into the mouth and over the gills of the experimental fish. Animals were periodically checked visually to verify blood flow in the dorsal vasculature of the brain and inner ear organs to ensure they were still alive. The experimental fish were placed in the center of a Nalgene tank (40 cm diameter) and suspended by a custom-built acrylic stereotaxic head-holder that was positioned 10 cm above the underwater speaker. In this way, the saccules were approximately 3 cm below the water surface. The speaker was embedded in a layer of gravel placed on the bottom of the recording tank so that only the top 2 cm of the speaker projected upwards into the water column. The water temperature was maintained between 14 and 15°C for the duration of the recording session. The distance between the water surface and the surface of the speaker was 13 cm. The tank was positioned on an inflated pneumatic table housed inside an acoustic isolation chamber (Industrial Acoustics, New York, NY, USA). All of the recording and stimulus generation equipment were located outside this chamber.

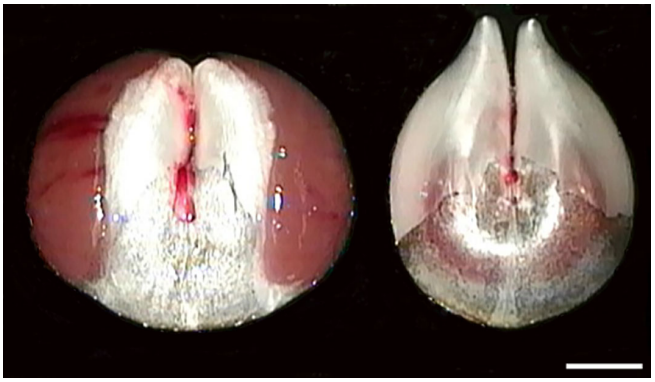

Fig. 1. Intersexual swim bladder dimorphism in the plainfin midshipman fish (*Porichthys notatus*). Dissected swim bladders from a type I male midshipman with a standard length (SL) of 17.0 cm (left) and a female midshipman with a SL of 13.4 cm (right). Note that the females have prominent rostral swim bladder extensions while the type I males have enlarged red sonic muscles (attached to the swim bladder), used to produce multiharmonic advertisement calls during the breeding season. Scale bar: 5 mm.

Saccular potential recordings were performed using glass electrodes (3.0–7.0 MΩ) filled with a 3 mol l^{-1} KCl solution. The electrodes were visually guided into the endolymph of the saccule and positioned in the space between the sagitta (i.e. the otolith) and the macula (i.e. the sensory epithelium) in the middle/caudal region of the saccule. More precisely, the tip of the electrode was placed roughly 2–5 mm from the closest hair cell bed of the saccular macula. Both left and right saccules were used in the present study. Recording fidelity was assessed by comparing the magnitude of the saccular potentials recorded during the blank (no stimulus presented) and auditory stimulus test trials. The auditory stimuli presented were of equal amplitude (with ±1–2 dB) at a given sound level across all frequencies tested. Electrode signals were band-pass filtered (80–3000 Hz, SR650, Stanford Research Systems), preamplified 10 times (model 5A, Getting Instruments, San Diego, CA, USA), inputted into the lock-in amplifier (10 times, SR830, Stanford Research Systems) and then stored on a computer running a custom-written data acquisition and stimulus timing MATLAB script. The lock-in amplifier yielded a DC voltage output signal that was proportional to the component of the signal whose frequency was exactly locked to the reference frequency. The reference frequency was set to the second harmonic of the stimulation frequency signal (i.e. 2 times the fundamental frequency). The lock-in amplifier filtered out noise signals at frequencies other than the reference. We used this reference frequency because the greatest evoked potentials from teleost inner ear saccule hair cells occur at twice the sound stimulus frequency because of the non-linear response and opposite orientation of hair cell populations within the saccule (Cohen and Winn, 1967; Sisneros, 2007).

Acoustic impedance measurements

As the acoustic environment in which evoked potentials were recorded was influenced by the small dimensions and material of the experimental test tank, we determined the tank's acoustic impedance as suggested by Popper and Fay (2011). The acoustic impedance (Z), expressed in Rayls [where 1 Rayl=1 (Pa s) m^{-1}], is the complex ratio of sound pressure to particle velocity. We determined the acoustic impedance by simultaneously measuring sound pressure and particle motion, using a hydrophone (8103, Bruel & Kjaer) and a neutrally buoyant, tri-axial accelerometer [PCB model VW356A12, PCB Piezotronics, Depew, NY, USA; sensitivity at 100 Hz: 11.02 mV/(m s^{-2}) (x-axis), 10.03 mV/(m s^{-2}) (y-axis), 10.37 mV/(m s^{-2}) (z-axis)] at each frequency evaluated for three SPLs: 133, 142 and 151 dB re. 1 µPa. Both the hydrophone and accelerometer were centered above the speaker and placed in the middle of the water column, directly between the top of the

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speaker and the surface of the water. A conditioning amplifier (Nexus 2692-0S1, Bruel & Kjaer) and a signal conditioner (gain=100×; model 482A16, PCB Piezotronics) were used to amplify the acoustic signals for the hydrophone and accelerometer, respectively. The amplified peak-to-peak (p–p) voltage measurements for both sound pressure and particle motion were recorded using a data acquisition system (NI myDAQ 16 bit analog to digital conversion at 200 kS s^{-1}, National Instruments, Austin, TX, USA) that was controlled by a custom-written program in LabVIEW software (NI LabVIEW 2016, National Instruments). The acoustic impedance was determined based on acceleration only in the *z*-axis as the majority of the acceleration was observed in this axis for the frequencies and SPLs evaluated. The absolute value of acoustic impedance was determined by dividing the amplitude of the pressure wave by the amplitude of the particle velocity wave. We then compared it with that of a free-field underwater environment (unbound conditions) with a salinity of 35 ppt at 15°C (Bradley and Wilson, 1966; Erbe, 2011). 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 MRayl (Bradley and Wilson, 1966; Erbe, 2011). Finally, the phase (Φ) of the complex acoustic impedance was determined by comparing 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.

To calculate the acoustic impedance, the SPL was first determined using the following equation: SPL=mV_{p-p}/sc, where sc is the scale factor (mV Pa^{-1}) from the conditioning amplifier and mV_{p-p} is measured peak-to-peak voltage from the recorded signal via the hydrophone. Particle acceleration (a=m s^{-2}) was calculated using the equation: a=mV_{p-p}/S, where S is accelerometer sensitivity [mV/(m s^{-2})] for the *z*-axis. From the amplitude of the particle acceleration waveform, the amplitude of the particle velocity waveform (v=m s^{-1}) was calculated using the following equation: $v=a/2\pi f$ (Nedelec et al., 2016), where f is frequency (Hz). For each frequency and sound level, the absolute magnitude of acoustic impedance [ratio of pressure to particle velocity, (Pa s) m^{-1}] was then expressed logarithmically relative to the acoustic impedance in a free-field of seawater (Z=1.559 MRayl; 15°C; salinity 35 ppt) 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 MRayl [1×10^6 (Pa s) m^{-1}] (Bradley and Wilson, 1966; Erbe, 2011) (Fig. 2).

To assess the phase of the complex acoustical impedance in our test tank, we also directly measured the phase difference ($\Delta\Phi p,a$) between particle acceleration (a) and pressure (p) using the accelerometer and hydrophone. Measurements were recorded using the same data acquisition system (NI myDAQ) and LabVIEW software. For sinusoid waves, such as the pure tones examined, the phase of particle acceleration (a) will always lead the phase of particle velocity (v) by 90 deg. Therefore, the phase difference between the particle velocity and acoustic pressure waves was determined using the following equation: $\Delta\Phi p,v=\Delta\Phi p,a+90$ deg where $\Delta\Phi p,a=\Phi p-\Phi a$ (Fig. 2).

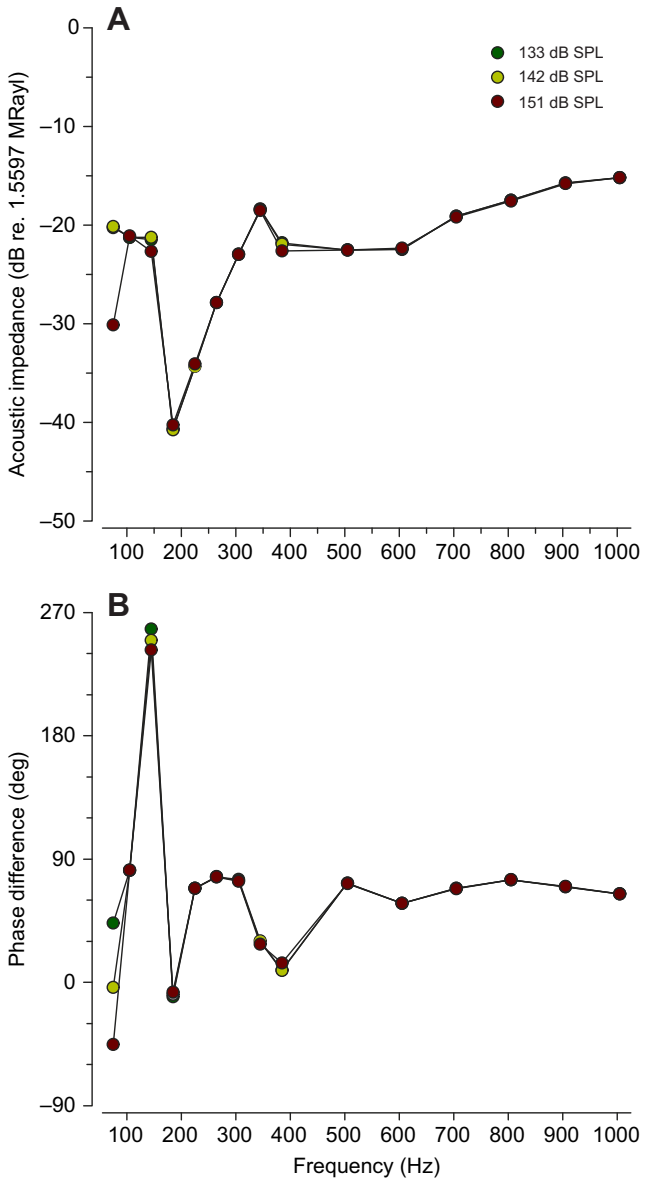

Fig. 2. Acoustic impedance and phase characteristics of the acoustic environment (i.e. the experimental tank with underwater speaker) in which the auditory physiology experiments were performed. (A) Acoustic impedance [ratio of sound pressure (dB re.1 µPa) to particle velocity (dB re. 1 m s^{-1})] in the *z*-axis relative to 1.5597 MRayl (reference impedance of a free-field in seawater with a salinity of 35 ppt and a temperature of 15°C) is plotted for all the tested frequencies at three sound pressure levels (SPLs): 133, 142 and 151 dB re. 1 µPa. Measurements were made using a mini-hydrophone and a triaxial accelerometer placed in the middle of the water column in the center of the tank. Multiple magnitude measurements (n=10) for both pressure and particle velocity were made at each frequency for the three SPLs. (B) Phase difference between the acoustic pressure and particle velocity waves. Phase measurements were made using a mini-hydrophone and a triaxial accelerometer placed in the middle of the water column in the center of the tank. Multiple phase difference measurements (n=10) were made at all the tested frequencies and at three SPLs: 133, 142 and 151 dB re. 1 µPa. Data in A and B are plotted as the mean±1 s.d.; note that the plotted s.d. bars are very small and are obscured by the symbols.

Threshold data and statistical analyses

Background noise measurements were performed prior to each saccular potential recording and used for determining the auditory threshold. These noise measurements were recorded for the eight repetitions of the stimulus at each of the tested frequencies (75–1005 Hz). They were similar to those of the saccular potential recordings but this time the speaker was turned off so that no auditory stimulus was present. The auditory threshold at each stimulus frequency was determined as the lowest stimulus level that

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evoked a response that was >2 s.d. above the background noise measurement. Any given response greater than this threshold criterion was thus considered an evoked saccular potential. Threshold tuning curves were constructed by recording the lowest stimulus level that evoked a saccular potential over the range from 100 to 151 dB re. 1 µPa in incremental steps of 3 dB. The frequency that evoked the lowest saccular potential threshold was defined as the best frequency (BF).

Differences in body size (SL), mass (BM) and reproductive state (GSI) between reproductive females collected in CA (Tomales Bay) and WA (Seal Rock, Brinnon) were determined using a two-tailed *t*-test. Because of the amount and uneven distribution of missing values that were concentrated at higher test frequencies (missing data resulted when we were unable to record an evoked potential at a particular test frequency within the experimental amplitudes used from 100 to 151 dB re. 1 µPa), the average threshold tuning curve data based on sound pressure and particle acceleration were analyzed using growth curve modeling (Alderks and Sisneros, 2011). The effects of swim bladder removal (control versus treated) and sex (male versus female) on auditory threshold were determined using an ANOVA on the regression coefficients of the growth curve modeled data followed by Bonferroni's *post hoc* test for multiple comparisons. The 95% confidence limits (CLs) of the mean thresholds (Zar, 1999) were calculated and were also used to determine whether the mean evoked saccular thresholds differed between control females, treated females and type I males at each frequency (i.e. overlapping 95% CLs were considered not significantly different). Differences in BF of the evoked saccular potentials between control females, treated females and type I males were determined using a one-way ANOVA followed by Tukey's test for multiple comparisons. All statistical analyses were performed with SPSS v.24 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, Inc. USA). Significance level was determined at $P<0.05$.

RESULTS

Evoked saccular potentials were recorded from 49 adult midshipman fish: 35 females with a size range of 11.7–16.3 cm SL (mean±s.d. SL 14.1±1.3 cm, BM 33.8±9.7 g and GSI 15.3±9.1) and 14 type I males with a size range of 13.5–20.3 cm SL (mean±s.d. SL 16.2±2.2 cm, BM 59.8±30.3 g and GSI 2.1±0.8). Morphological analyses were conducted on the 35 female midshipman used in the swim bladder removal experiments. For the 18 control females (individuals with sham swim bladder removal), the size range was 11.7–16.3 cm SL (mean±s.d. SL 14.2±1.3 cm, BM 35.6±10.2 g and GSI 15.5±8.8); for the 17 treated females (individuals with swim bladder removed), the size range was 11.9–16.1 cm SL (mean±s.d. SL 13.9±1.2 cm, BM 31.8±8.6 g and GSI 15.2±9.4). There was no difference in SL (*t*-test, $t=0.876$, d.f.=33, $P=0.39$), BM (*t*-test, $t=1.191$, d.f.=33, $P=0.24$) and GSI (*t*-test, $t=0.082$, d.f.=33, $P=0.93$) between control and treated females.

Auditory thresholds based on sound pressure and particle acceleration were constructed for whole populations of hair cells in the saccule for control and treated females, and type I males (which do not have rostral swim bladder extensions). The threshold tuning curves for the saccular potentials of females and males based on sound pressure generally consisted of response profiles with lowest thresholds at 75 Hz that gradually increased to highest thresholds at frequencies ≥805 Hz (Fig. 3). BFs ranged from 75 to 105 Hz for all fish, with the majority of BFs occurring at 75 Hz (control females 91%, treated females 73%, type I males 96%), but there were significant differences in BF among the three groups

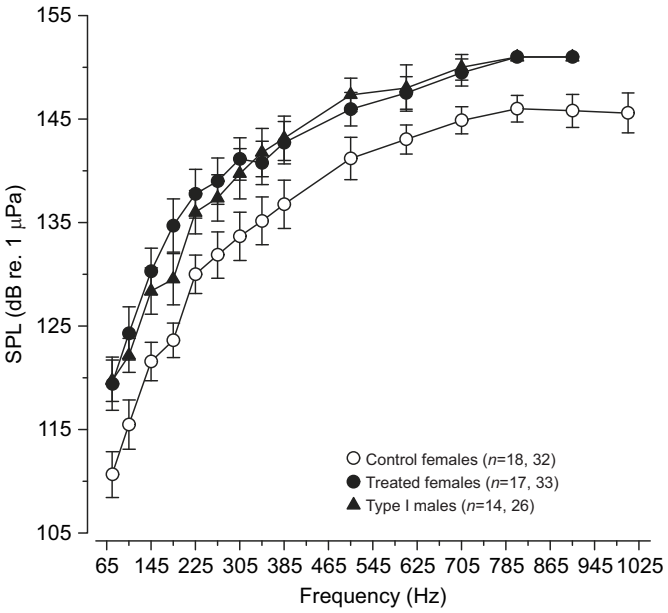

Fig. 3. Auditory threshold tuning curve based on sound pressure for the evoked potentials recorded from hair cells in the midshipman saccule. All SPL data are plotted as the mean±95% confidence interval and the number of animals and records is indicated in parentheses for each group: control females (with intact swim bladders with rostral extensions), treated females (with removed swim bladders) and type I males (with swim bladders that do not have rostral extensions). Auditory threshold for each stimulus frequency was defined as the lowest stimulus intensity in dB re. 1 Pa that evoked a saccular potential that was >2 s.d. above the background noise measurement.

(one-way ANOVA, $F=3.946$, d.f.=2, 88, $P=0.02$). Mean BFs were slightly but significantly higher in treated females than in type I males (83.2 versus 76.1 Hz, Tukey's test, $P<0.05$); however, mean BFs did not differ between control and treated females (77.8 versus 83.2 Hz, Tukey's test, $P>0.05$) or between control females and type I males (77.8 versus 76.1 Hz, Tukey's test, $P>0.05$).

In order to compare the mean threshold tuning curves based on sound pressure, we applied a logarithmic regression model because it provided the best fit for the majority of the data (mean±s.d. $R^2=0.72\pm0.07$, minimum $R^2=0.64$, maximum $R^2=0.78$). There were no effects of swim bladder removal (control versus treated) and sex (male versus female) on saccular tuning profiles based on slope (ANOVA, $F=2.518$, d.f.=2, 999, $P=0.08$). In contrast, the saccular tuning profiles based on intercept (level of auditory threshold) showed significant differences (ANOVA, $F=9.356$, d.f.=2, 999, $P<0.001$) between control and treated females (Bonferroni's test, $P<0.001$) as well as between control females and type I males (Bonferroni's test, $P<0.05$). In addition, there were no differences in auditory sensitivity (threshold levels) of saccular tuning between treated females and type I males based on intercept (Bonferroni's test, $P>0.05$). These findings from saccular tuning profile comparisons were supported by the fact that the auditory thresholds of the saccular hair cells from control females were approximately 5–11 dB re.1 µPa and 5–9 dB re.1 µPa lower than those of treated females and type I males at frequencies from 75 to 905 Hz, respectively (Fig. 4), whereas the differences in auditory saccular sensitivity between treated females and type I males were approximately 0.5–3.0 dB re. 1 µPa at frequencies from 75 to 705 Hz (Fig. 3).

The threshold tuning curves for the saccular potentials of control females, treated females and type I males based on particle acceleration consisted of tuning profiles with lowest thresholds at

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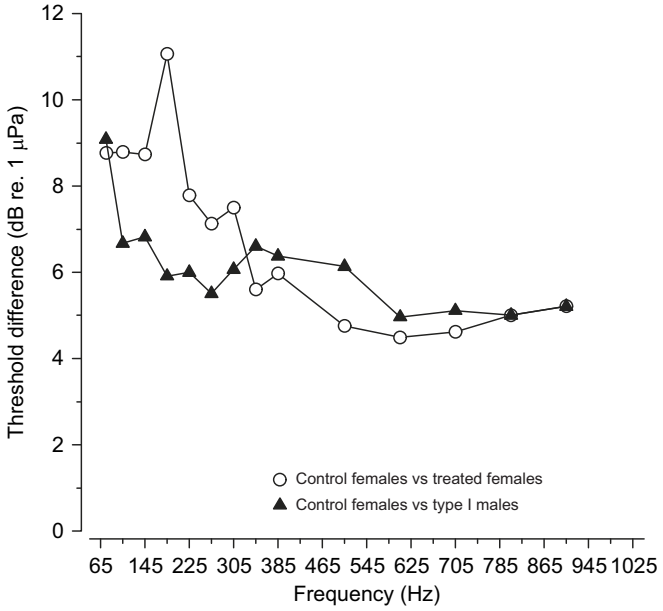

Fig. 4. Comparison of sound pressure sensitivity between female midshipman with (control) and without (treated) swim bladders, and control females and type I males. Shown here is a plot of the difference in auditory saccular thresholds at each frequency tested between control females and the two other tested groups (treated females and type I males).

105 Hz that gradually increased to highest thresholds at frequencies ≥505 Hz (Fig. 5). BFs ranged from 75 to 145 Hz for all fish, with the majority at 105 Hz (control females 72%, treated females 55%, type I males 89%). The BF means showed significant differences (one-way ANOVA, F=7.169, d.f.=2, 88, P=0.001) with treated females having significantly lower auditory thresholds than both control females (91.4 versus 105.3 Hz, Tukey's test, P<0.01) and type I males (91.4 versus 101.5 Hz, Tukey's test, P<0.05). However, mean BF did not differ between control females and type I males (105.3 versus 101.5 Hz, Tukey's test, P>0.05).

In order to compare the average threshold tuning curves based on acceleration, we also applied a logarithmic regression model because it provided the best fit for the majority of the data (mean± s.d. R^2=0.73±0.02, minimum R^2=0.71, maximum R^2=0.75). There were no differences in saccular tuning profiles between the three groups (control females, treated females and type I males) based on slopes (ANOVA, F=2.171, d.f.=2, 999, P=0.11) and intercept (ANOVA, F=0.7639, d.f.=2, 999, P=0.47). Thus, the three groups of midshipman show similar saccular tuning profiles based on particle acceleration (Fig. 5).

In addition to the above-noted differences in saccular tuning (relative to sound pressure and acceleration), we also observed differences in the highest detectable frequency among the three midshipman groups (Fig. 6). For the 18 control females, we observed evoked saccular potentials in all of the recordings (n=32) from 75 to 385 Hz (100%) while the percentage of recordings that had evoked potentials from 505 to 705 Hz dropped to 81–94%, and then to 66–75% from 805 to 905 Hz. At the highest frequency tested (1005 Hz), 31% (n=10) of the recordings had detectable evoked saccular potentials. For the 17 treated females, we observed evoked saccular potentials in all of the recordings (n=33) up to 185 Hz (100%), while the percentage of recordings that had evoked potentials from 225 to 385 Hz dropped to 76–97%, then to 18–67% from 505 to 705 Hz, and then to 3–6% from 805 to 905 Hz. No evoked saccular potentials were recorded at 1005 Hz. For the type I males, we observed evoked saccular potentials in all of the recordings (n=26) up to 265 Hz (100%), while the percentage of recordings that had evoked potentials from 305 to 385 Hz dropped

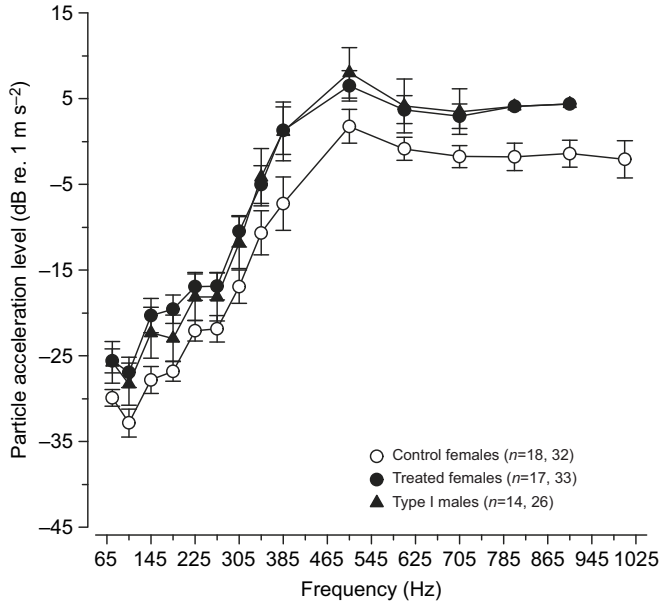

Fig. 5. Auditory threshold tuning curve based on particle acceleration for the evoked potentials recorded from hair cells in the midshipman saccule. All data are plotted as the mean±95% confidence interval and the number of animals and records is indicated in parentheses for each group: control females, treated females and type I males. Auditory threshold for each stimulus frequency was defined as the lowest stimulus level in dB re. 1 m s^{-2} that evoked a saccular potential that was >2 s.d. above the background noise measurement.

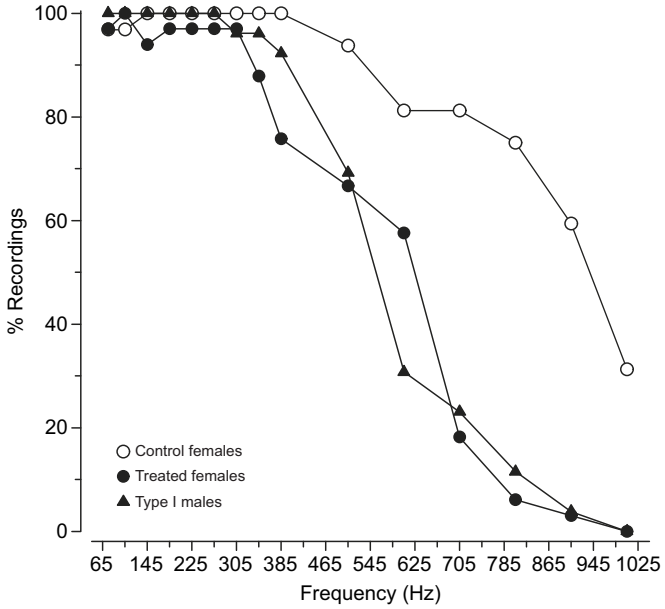

Fig. 6. Distribution of the percentage of saccular potential recordings that displayed significant thresholds above background noise for each tested frequency for each group. Data are shown for control females (with intact swim bladders with rostral extensions), treated females (without swim bladders) and type I males (with swim bladders that do not have rostral extensions).

to 92–96%, then to 23–69% from 505 to 705 Hz, and then to 4–12% from 805–905 Hz. No evoked saccular potentials were recorded at the highest tested frequency of 1005 Hz. Thus, the control females with swim bladders intact were observed to have a relatively high percentage (66–75%) of evoked saccular potentials at 805 and 905 Hz and were also observed to have 31% evoked potential recordings at the highest recorded frequency of 1005 Hz. In contrast, we observed no evoked saccular potentials in treated females and type I males at the highest frequency tested (1005 Hz) and ≤6% evoked potential recordings at 905 Hz (Fig. 6).

DISCUSSION

The aim of this study was to determine whether the sexually dimorphic rostral swim bladder extensions observed in female midshipman function to enhance auditory sensitivity to sound pressure and higher frequencies. We showed that control females (with intact swim bladders) were significantly more sensitive to sound pressure than were treated females (with removed swim bladders) and type I males (with swim bladders that do not have rostral extensions). In addition, control females were also more sensitive to the highest frequencies tested compared with treated females and type I males. Thus, based on our data we suggest that the rostral swim bladder extensions found in females do afford greater sensitivity to sound pressure and higher frequencies. In the following discussion, we interpret our results as they relate to the reception of sound pressure signals in female plainfin midshipman and how the rostral swim bladder extensions may serve to enhance the detection and localization of vocalizing males during the midshipman breeding season.

The swim bladders in most teleost fishes are thought to primarily function in buoyancy regulation (Pelster, 2011) and to act as an oxygen reservoir (Blaxter et al., 1979), but in some teleosts the swim bladder can also serve a secondary function for sound production and/or sound reception (Popper et al., 2003; Fine and Parmentier, 2015). As an acoustic organ, the swim bladder can facilitate the reception of acoustic stimuli via the indirect stimulation of the inner ear by sound pressure (Fay and Popper, 1980; Popper et al., 2003). In this scenario, the swim bladder acts as a pressure-to-displacement transducer when the gas-filled swim bladder begins to oscillate as a result of the sound-induced changes in acoustic pressure which cause the compression and rarefication movement of particles in the medium. The resulting oscillations of the swim bladder wall will act as a secondary sound source that reradiates sound energy in the form of local particle motion, which then can effectively stimulate the particle motion-sensitive inner ear end organs depending on the proximity of the swim bladder to the auditory end organ(s) (e.g. the saccule, lagena and in some cases the utricle). Pressure-sensitive fish often either have a direct linkage of the swim bladder to the inner ear via skeletal elements or ossicles known as Weberian ossicles (e.g. Otophysan fishes) or have their swim bladders (often with anterior swim bladder extensions) in close proximity to the auditory end organ(s) (e.g. Holocentridae, Gadidae, Gerreidae, Sciaenidae, Chaetodontidae, Cichlidae and Serrasalmidae; Nelson, 1955; Braun and Grande, 2008; Parmentier et al., 2011; Tricas and Boyle, 2015; Tricas and Webb, 2016; Ladich, 2016; Boyle and Herrel, 2018). Having the swim bladder in close proximity to the inner ear not only increases sensitivity to pressure indirectly but also extends the upper range of frequency sensitivity. Because acoustic particle motion attenuates more rapidly than sound pressure, pressure-sensitive fish are able to detect higher acoustic frequencies at a greater distance from the sound source (Bass and Clark, 2003; Popper et al., 2003; Hawkins and Popper, 2018). Thus, this difference in the propagation properties of underwater sound may have in part driven the evolution of accessory hearing morphologies in pressure-sensitive teleosts, leading to the enhanced sensitivity and bandwidth (Ladich, 2000, 2013; Ladich and Schulz-Mirbach, 2016; Boyle and Herrel, 2018).

The results from our study support the hypothesis that the rostral swim bladder extensions in female midshipman enhance auditory sensitivity to sound pressure and higher frequencies. We show that control females with intact swim bladders were approximately 5–11 dB re. 1 µPa more sensitive (based on sound pressure) than treated females without swim bladders and type I males without swim bladder extensions across all frequencies tested. Control females also had a greater bandwidth of sensitivity with higher percentages of evoked saccular potentials recorded at the highest tested frequencies (805, 905, 1005 Hz) compared with treated females and type I males. Based on these data, we suggest that the enhanced sensitivity to sound pressure and higher frequencies in control females was directly related to the presence of swim bladders with rostral extensions, and these changes (1.8–3.5 times greater) in auditory sensitivity will likely increase the probability of detection of the higher harmonic frequencies in the male advertisement call and extend the range at which females can localize calling mates. Previously, we showed that the distance between the rostral swim bladder extensions and the sagitta (saccular otolith) in females was shorter than that in type I males, such that the distance between the horn-like swim bladder extensions in females was less than 3 mm compared with 4.7 mm for the same measurement in type I males (Mohr et al., 2017). In other studies of pressure-sensitive fishes such as sciaenids (Ramcharitar et al., 2006), cichlids (Schulz-Mirbach et al., 2012) and ophidiids (Kéver et al., 2014), enhanced sound pressure sensitivity at higher frequencies (>700 Hz) is associated with the swim bladder being less than 3 mm away from the otic capsule, which contains the auditory end organs (e.g. saccule and lagena). In the holocentrids (squirrelfishes or soldierfishes), the degree of sensitivity to sound pressure and higher frequencies depends on how close the swim bladder is to the inner ear. Greatest sensitivity to sound pressure and higher frequencies was found in the genus *Myripristis*, which has a swim bladder with elongated rostral extensions that contact the auditory bulla adjacent to the saccule (Coombs and Popper, 1979). Interestingly, type II or 'sneaker' male midshipman also possess elongated rostral swim bladder extensions that project to within ∼2 mm of the otic capsule, which suggests that this male morph is also likely sensitive to sound pressure and higher frequencies (Mohr et al., 2017). Future studies that investigate the sound pressure sensitivity in the midshipman male morphs (i.e. types I and II) would provide valuable insight into the potential adaptations of the midshipman swim bladder as an acoustic organ for sound production and reception in males with divergent social and reproductive behaviors related to their bioacoustic ecology.

Shallow-water environments, like those where midshipman breed during the summer, limit the propagation and detection of behaviorally relevant, low-frequency acoustic stimuli. Such environmental constraints may have been a selective factor in the evolution of the inner ear in many teleost species including midshipman that have accessory hearing morphologies for increased sensitivity to sound pressure and higher frequencies. As discussed by Ladich (1999, 2000), the evolution of hearing specializations for the detection of sound pressure and higher frequencies mostly occurs in fishes that inhabit shallow, quiet water environments. One important factor that affects the propagation of sound transmission and its frequency content is water depth. Low-frequency sounds are quickly

attenuated in shallow-water environments as a result of the cut-off frequency of sound transmission, which is affected by the repeated interaction of the long wavelengths with the water surface and bottom substrate (Rogers and Cox, 1988). Thus, limited sound propagation occurs below the characteristic cut-off frequency at a given depth. In shallow-water environments, there exists an inverse relationship between water depth and the cut-off frequency of sound transmission such that as water depth decreases, the cut-off frequency increases (Rogers and Cox, 1988; Bass and Clark, 2003). Thus, this environmental constraint of the attenuation of low-frequency sounds in shallow water may have been a selective force for inner ear adaptations to detect the higher frequencies that exist and propagate in shallow-water environments.

Another factor that may have influenced the evolution of accessory hearing morphologies in fishes is the detection of ambient environmental sounds in underwater soundscapes (Lugli, 2019). Ambient sounds from underwater soundscapes are likely to contain important information about the environment that allows animals to perceive the auditory scene and behave appropriately to different sound sources (Fay, 2009). Auditory scene analysis of biotic and abiotic sound sources in natural underwater soundscapes may allow fish to detect potential prey, avoid predators and orient within the environment (Fay and Popper, 2000; Simpson et al., 2005). Natural ambient sounds or environmental 'noise' in underwater soundscapes can act as a source of acoustic illumination or 'acoustic daylight' (Buckingham and Berkhout, 1992; Buckingham, 1999). The concept of acoustic daylight draws upon the analogy of terrestrial environments being bathed by natural sunlight that can provide useful information when imaging the environment. Thus, the concept of acoustic daylight suggests that ambient noise from underwater soundscapes can be exploited for imaging the acoustic environment by fish and other marine organisms to obtain information about the environment's contents. Unfortunately, very little information is known about the natural soundscapes that fish inhabit but, recently, efforts to address this have been made for fish species including the plainfin midshipman and zebrafish (McIver et al., 2014; Halliday et al., 2018; Lara and Vasconcelos, 2019).

Our results indicate that the increased sensitivity in females to sound pressure and higher frequencies resulting from the rostral swim bladder extensions may potentially be another mechanism used to enhance the detection and localization of potential mates during the breeding season. A number of seasonal adaptations that increase female midshipman auditory sensitivity to male vocal signals have been reported (Sisneros and Bass, 2003; Sisneros et al., 2004a; Coffin et al., 2012). During the late spring and summer breeding season, plainfin midshipman migrate from deep offshore sites (at depths greater than 80 m) into shallow, rocky intertidal zones on the west coast of the USA from central California to northern Washington and on the west coast of British Columbia. Type I or 'guarder' males establish nests under rocks in the intertidal zone from which they produce multiharmonic advertisement calls or 'hums' at night to attract females for spawning (Bass et al., 1999; Sisneros, 2009b; Bose et al., 2018). Reproductive females rely on their sense of hearing to identify and locate conspecific 'singing' males. Females exhibit a number of reproductive state-dependent changes in their auditory systems that enhance their ability to detect potential mates. Sisneros and Bass (2003) showed that reproductive females exhibit seasonal changes in saccular afferent sensitivity during the breeding season such that females become better suited to detect the dominant higher harmonic components in type I male advertisement calls. Furthermore, these seasonal changes in saccular frequency sensitivity occur after females experience seasonal gonadal recrudescence that corresponds with a brief spike in circulating estradiol and testosterone levels, approximately 30 days prior to the breeding season (Sisneros et al., 2004b; Sisneros, 2009b). Sisneros et al. (2004a,b) confirmed a steroid-dependent mechanism for the seasonal changes in saccular auditory sensitivity by implanting ovariectomized, non-reproductive females with either estradiol or testosterone implants that mimicked the seasonal spike in estradiol or testosterone, which resulted in increased saccular sensitivity to the frequencies that corresponded to the dominant multi-harmonic components in the male's advertisement call. In addition, Coffin et al. (2012) showed that seasonal changes in female auditory sensitivity were concurrent with seasonal increases in saccular hair cell density. The increase in hair cell density was saccular specific and not shown to occur in the other end organs (i.e. lagena or utricle). Furthermore, the increase in saccular hair cell density was paralleled by a dramatic increase in the magnitude of the sound-evoked potentials recorded from saccular hair cells, which also corresponded to a decrease in saccular auditory thresholds (i.e. increase in sensitivity) (Coffin et al., 2012). In sum, these seasonal adaptations of the female auditory sense also likely afford greater sensitivity to sound pressure indirectly by enhancing the detection of local particle motion produced by sound pressure-induced vibrations of the swim bladder when exposed to social acoustic signals and during the sound source localization of advertising males.

In addition to increased sensitivity to sound pressure, the rostral swim bladder extensions of females also afford greater auditory sensitivity to higher frequencies, which should be adaptive for reproductive females when they migrate to inshore, shallow-water acoustic environments to breed. Interestingly, the intertidal breeding grounds where male nest sites of plainfin midshipman are most often found are in calm and protected bays (e.g. Tomales Bay, CA, USA, and the Hood Canal, WA, USA) where wave action and other sources of 'environmental noise' are often reduced. The rostral swim bladder extensions in females that enhance auditory 'gain' or sensitivity to sound pressure and higher frequencies may be especially useful for detecting the higher frequency components of male vocalizations in shallow-water environments in the presence of elevated environmental sounds (e.g. during periods of increased wave action due to wind and other weather factors). In our study, we demonstrate that the rostral swim bladder extensions can enhance saccular frequency sensitivity up to 1000 Hz, which covers the range of harmonic frequency components in the male advertisement call (Bass et al., 1999). As shown in Fig. 6, females with intact swim bladders have a higher probability of detecting frequencies from 345 to 1005 Hz compared with females without swim bladders (e.g. at 345 Hz, 100% of the records had recorded evoked saccular potentials for females with swim bladders compared with 88% of the records for females without swim bladders; and at 1005 Hz, 31% of the records had recorded evoked saccular potentials for females with swim bladders compared with 0% of the records for females without swim bladders). This enhanced high-frequency sensitivity, in part due to the rostral swim bladder extensions, may be important for the acquisition of broad-band auditory information needed for mate detection, recognition and localization in the shallow-water, midshipman breeding environments. In addition, the enhanced detection of the dominant higher frequency harmonics in male advertisement calls may also be important for mate choice decisions and the assessment of 'honest' signal information in the male's advertisement call related to reproductive condition-dependent indicators of mate quality. Future studies that investigate the signal characteristics of the male advertisement call

and its relationship to phenotypic traits including male body size (mass) will provide important insight into whether the male midshipman advertisement call is an 'honest signal' and a condition-dependent indicator of mate quality for this species.

The results from this study also indicate that type I males are significantly less sensitive to sound pressure and higher frequencies than females. Our results show that summer-caught type I males, which do not have swim bladder extensions, had similar auditory pressure sensitivity to that of females with their swim bladders removed (i.e. no significant difference between type I males with swim bladders without extensions and treated females with swim bladders removed). In contrast, type I males and treated females had significantly lower auditory pressure sensitivity (i.e. higher thresholds) compared with control females with intact swim bladders with extensions. The auditory thresholds of the saccular hair cells from type I males and treated females were approximately 5–9 dB re.1 µPa and 5–11 dB re.1 µPa higher than those of control females at frequencies from 75 to 905 Hz, respectively (Fig. 4). In addition, no evoked saccular potentials were recorded from type I males at the highest frequency tested (1005 Hz) while less than ∼6% and ∼12% of the evoked potential recordings were observed at 905 and 805 Hz, respectively. While our results for type I males (without swim bladder extensions) having the same auditory pressure sensitivity as females without swim bladders is surprising, one possible explanation for this sensitivity difference may be related to the length of time that males were maintained in captivity, which was 2 months during the summer prior to testing. Previous work by Sisneros and Bass (2003) and Rohmann and Bass (2011) showed that both reproductive females and type I males have enhanced peripheral auditory sensitivity at frequencies >140 Hz during the summer breeding season, in part due to the effects of elevated gonadal steroids (e.g. testosterone and estradiol) that peak prior to the breeding season (Sisneros et al., 2004b; Rohmann and Bass, 2011). However, Sisneros and Bass (2003) showed that reproductive animals maintained in captivity longer than 25 days exhibited a decreased sensitivity to frequencies greater than 300 Hz. Thus, the reported thresholds (especially for frequencies >300 Hz) for the intact type I males (without rostral swim bladder extensions) may be slightly higher than those of summer type I males recently collected from the field. Alternatively, the difference in auditory pressure sensitivity between type I males and females maybe more related to the relative distance between the saccule and the anterior end of the swim bladder in males and females. Mohr et al. (2017) reported that the distance between the rostral swim bladder extensions and the saccular otoliths was greater in type I males (mean distance 5.2 mm), approximately twice that in females (mean distance 2.6 mm). This greater distance between the swim bladder and saccule in type I males may be responsible for reduced detection of the local particle motion generated by the pressure wave-induced vibrations of the swim bladder when exposed to sound (i.e. mechanism of indirect pressure detection afforded by the swim bladder). In addition, the hypertrophied sonic muscles attached to the swim bladder in type I males may also play a role in dampening the local particle motion generated by the swim bladder during sound reception. Future studies that examine the sound receptivity of the swim bladder in type I males will be needed to examine the role of the swim bladder as an acoustic organ in more detail for type I males. However, the results from our current study suggest that type I males have a lower probability of detecting the higher harmonic frequency components in male advertisement calls and other conspecific vocal signals with similar broad band frequency content compared with females. Furthermore, the dominant theories of sound source localization by fishes maintain that the detection and processing of sound pressure cues are necessary for successful sound source localization (Rogers et al., 1988; Sisneros and Rogers, 2016). Given these theories of fish sound localization (e.g. the phase model; see Schuijf, 1981), our data suggest that type I males may have a reduced capacity for sound source localization compared with females. Future studies that compare the sound source localization behavior between type I male and female midshipman may provide additional insight into the role of sound pressure and the necessary cues required for successful sound source localization by fishes.

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Competing interests
The authors declare no competing or financial interests.

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

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