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Seasonal plasticity of auditory saccular sensitivity in “sneaker” type II male plainfin midshipman fish, *Porichthys notatus*

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Abstract Adult female and nesting (type I) male midshipman fish (*Porichthys notatus*) exhibit an adaptive form of auditory plasticity for the enhanced detection of social acoustic signals. Whether this adaptive plasticity also occurs in “sneaker” type II males is unknown. Here, we characterize auditory-evoked potentials recorded from hair cells in the saccule of reproductive and non-reproductive “sneaker” type II male midshipman to determine whether this sexual phenotype exhibits seasonal, reproductive state-dependent changes in auditory sensitivity and frequency response to behaviorally relevant auditory stimuli. Saccular potentials were recorded from the middle and caudal region of the saccule while sound was presented via an underwater speaker. Our results indicate saccular hair cells from reproductive type II males had thresholds based on measures of sound pressure and acceleration (re. 1 μ Pa and 1 ms^{-2} , respectively) that were \sim 8–21 dB lower than non-reproductive type II males across a broad range of frequencies, which include the dominant higher frequencies in type I male vocalizations. This increase in type II

auditory sensitivity may potentially facilitate eavesdropping by sneaker males and their assessment of vocal type I males for the selection of cuckoldry sites during the breeding season.

Keywords Hearing · Particle acceleration · Teleost · Saccule · Hair cells

Introduction

Many teleost fishes use acoustic signals for social communication, and in many species, the use of these social acoustic signals is essential for successful courtship and reproduction (Bass and McKibben 2003; Ladich 2004; Fine and Parmentier 2015). Recent studies have shown that many teleosts possess both behavioral and neural adaptations for the production and perception of acoustic signals that are necessary for reproduction and survival (Bass and McKibben 2003; Bass and Ladich 2008; Kelley and Bass 2010). One vocal teleost that is a good species for investigating neural mechanisms of vocal production and signal perception is the plainfin midshipman fish, *Porichthys notatus* (Bass et al. 1999; Sisneros 2009a; Forlano et al. 2015). This nocturnally active marine teleost produces a relatively simple repertoire of acoustic signals for intraspecific social communication that includes “grunts”, “growls” and “hums” (Bass et al. 1999; Sisneros 2009a). While all three adult sexual phenotypes (female and two male sexual phenotypes: types I and II) are known to produce the short-duration, broadband agonistic “grunts”, only type I “singing” males build nests and produce the long-duration, broadband agonistic “growls” and multiharmonic advertisement calls or “hums” during the summer breeding season (Bass and McKibben 2003; Brantley and Bass 1994).

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Previous studies have shown that both the female and type I male auditory systems are seasonally adapted to detect the broadband agonistic calls and the dominant harmonic components of the advertisement calls produced by type I males during the breeding season (Sisneros and Bass 2003; Rohmann and Bass 2011). These adaptations are potentially important for conspecific detection and localization, mate choice decisions and intraspecific male competition (Sisneros 2009a, b).

In contrast to “singing” type I males, “sneaker” type II males employ an alternative reproductive strategy that does not require building a nest or producing an advertisement call to attract mates. Instead, type II males, which have neuroendocrine traits similar to that of females, engage in satellite or sneak-spawning to steal fertilizations from type I males that are actively courting females in the nest (Brantley and Bass 1994). While females and type I males are known to be better suited to detect midshipman vocalizations during the breeding season (Sisneros and Bass 2003; Sisneros 2009b; Rohmann and Bass 2011), the auditory sensitivity of sneaker type II males has yet to be characterized. There is no a priori reason to expect that seasonal plasticity of midshipman auditory sensitivity would be limited to females and type I males.

The sensitivity of auditory systems in fishes can be measured in terms of acoustic particle motion and pressure. All fishes are likely sensitive to the particle motion component of sound via their otolithic end organs which essentially function as inertial accelerometers (de Vries 1950; Fay 1984). This common mode of hearing in fishes enables them to detect particle motion, the directional vector component of sound, as opposed to sound pressure, which is a scalar quantity containing no directional information. Some more recently derived fishes such as Otophysan fishes (e.g., goldfish, carp, zebrafish, etc.) have evolved pressure sensitivity as a result of having a specific connection between the inner ear and an air bubble (e.g., the swim bladder) (Fay and Popper 1980; Blaxter 1981; Schellart and Popper 1992). Recently Popper and Fay (2011) proposed that all future fish hearing studies should independently characterize particle motion and sound pressure sensitivities of fish regardless of any hearing specializations that fish may possess (e.g., accessory hearing structures such as Weberian ossicles that connect the swim bladder to the inner ear). Previous auditory physiology studies of the plainfin midshipman fish have primarily reported auditory responses with regard only to sound pressure (McKibben and Bass 1999, 2001; Sisneros et al. 2004; Sisneros and Bass 2003, 2005; Sisneros 2007, 2009b; Alderks and Sisneros 2011; Rohmann and Bass 2011; but for the exception see Weeg et al. 2002). Thus, better characterization of particle motion sensitivity in this species and across the different sexual phenotypes is warranted.

The primary goal of this study was to characterize auditory-evoked potentials from the saccule in reproductive and non-reproductive type II male midshipman to determine whether this sexual phenotype exhibits seasonal differences in auditory threshold and frequency response of the saccular hair cells to behaviorally relevant auditory stimuli. A secondary goal was to provide a description of the acoustic particle motion sensitivity of the midshipman auditory system that could be used for future comparisons across midshipman sexual phenotypes and with other species. We hypothesized that the auditory saccular sensitivity of type II males during the breeding season would be adapted to detect the dominant frequency content of the type I male vocalizations, which would be beneficial for type II males in their selection of type I male cuckoldry sites for sneak or satellite spawning. We also compare our findings to that of the saccular potentials reported for females (Sisneros 2009b) and type I males (Rohmann and Bass 2011) and interpret our results as they relate to possible auditory adaptations for acoustic communication during the reproductive season.

Materials and methods

Experimental animals

Summer reproductive adult type II male midshipman fish (*Porichthys notatus*) were hand-collected from exposed intertidal nests at low tide in Tomales Bay, CA, USA, during the reproductive season (May–July 2010). Winter non-reproductive adult type II males were collected by otter trawl (RV Kittiwake, Bio-Marine Enterprises) at depths from 70 to 100 m in the Puget Sound near Edmonds, WA (47.82N, 122.38W) and in Shilshole Bay near Ballard, WA (47.68N, 122.41W) during the winter months (December–February) of 2007, 2010 and 2016. Note that the limited number of non-reproductive type II males used in this study was sampled over multiple years due to their scarcity during the non-reproductive winter (personal observations, Sisneros). Based on our experience, the collection of type II males during winter trawls in the waters of Puget Sound, WA, is often a rare and serendipitous event.

Type II males were distinguishable from type I males based on their standard body length (SL), gonadosomatic index (GSI) and visual inspection of their genital papilla and their swim bladder sonic muscles. The reproductive type II males used in this study were noted to have pale and underdeveloped sonic muscles instead of the typical large red sonic muscles observed in summer reproductive type I males. In addition, the SL and GSI ranges of type II males reported here were well within the ranges reported for type II males in a previous study (Grober et al. 1994) that were

collected at the same sites in Tomales Bay, CA. GSI was calculated as $100 \times \text{gonad mass} / (\text{body mass} - \text{gonad mass})$, based on Tomkins and Simmons (2002). Soon after summer field collection (<4–5 days), type II males were transported to the University of Washington in Seattle, WA, where they were housed in aquaria at 13–15 °C and kept under reverse-light cycle (light:dark—14:10 h). The experiments performed with summer reproductive type II males were conducted during the dark phase of the light/dark cycle since these fish are known to be nocturnally active. Animals were hand-fed defrosted shrimp and/or live feeder guppies two to three times a week. Auditory physiology recordings and measurements were made within 26 of collection for most animals except for two non-reproductive type II males that were recorded approximately a few months after collection while being maintained under a short-day winter photoperiod (8 h light, 16 h dark). A simple linear regression between gonadosomatic index (GSI) and days in captivity was performed to test for time-dependent changes in breeding state, with the regression coefficient (r^2) reported.

Experimental procedures

The procedures for exposing and recording from the inner ear sacculus followed those used in previous studies (Sisneros 2007, 2009b; Alderks and Sisneros 2011). Briefly, animals were initially anesthetized in a 0.025% ethyl-*p*-aminobenzoate/saltwater bath until the fish's opercula ceased movement for at least 5 min. The animals were then immobilized with an intramuscular injection of pancuronium bromide (approx. $0.5 \mu\text{g g}^{-1}$ body mass). Skin, muscle and bone were removed just dorsal to the otic capsules, and a 2.5-cm hydrophobic dam was built around the craniotomy to enable submersion without exposing the brain and inner ear to salt water. The cranial cavity was then filled with Teleost Ringer's solution to prevent drying. A subcutaneous injection of 0.25% bupivacaine (approx. $1 \mu\text{g g}^{-1}$ body weight) was administered around the surgical site as an analgesic. During the experiment, the animals were submerged underwater such that the sacculi were approximately 3 cm below the water surface while fresh saltwater was circulated over the gills. The craniotomy was periodically checked visually to verify stable blood flow in the brain and inner ear organs.

Animals were suspended by a custom-built acrylic stereotaxic head-holder that was positioned 10 cm above an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA) in the center of a 40-cm-diameter Nalgene recording tank. The bottom of the tank contained an approximately 4.5 cm layer of small rock substrate in which the speaker was embedded so that only the top 2 cm of the speaker projected upwards into the water column. The water was initially chilled to 12 °C and maintained

between 14 and 15 °C for the duration of the recording session. The distance from the water surface to the surface of the speaker was 13 cm. The tank was positioned on an inflated pneumatic table, inside an acoustic isolation chamber (Industrial Acoustics, New York, NY, USA), and all recording equipment were housed outside this chamber.

Saccular potentials were recorded using glass electrodes (1.0–4.0 M Ω) filled with a 3 M KCl solution. The electrodes were visually positioned in the space between the otolith and sensory maculae in the middle/caudal region of the sacculus. Both left and right sacculi were used in this 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 were presented were of equal amplitude (with ± 1 –2 dB) at a given sound level across all frequencies tested. Electrode signals were band-pass-filtered (130–3000 Hz, Stanford Research Systems SR 650), amplified by 100 \times (Gettling 5A), and then sent to a lock-in amplifier (Stanford Research Systems SR 830) and digitized at 20 kHz (Micro1401 mkII, Cambridge Electronic Design). Data acquisition and stimulus timing were controlled by a custom MATLAB (MathWorks Inc., Natick, MA) script. The output of the lock-in amplifier reflects the relative amplitude of the inner ear sacculus's hair cell response to a tonal stimulus. Because the opposing hair cell orientations within the saccular sensory maculae result in a double-frequency response, the saccular potential is defined here as the amplitude the hair cell response at the second harmonic of the stimulus (Cohen and Winn 1967; Sisneros 2007).

Acoustic stimulus generation and calibration

Single tones were presented at 75–85 Hz in 10 Hz steps and 105–385 Hz in 20 Hz steps. Stimuli were generated by the lock-in amplifier, amplified by an audio amplifier, and then played through the underwater speaker. Stimuli were 750 ms in length and separated by a 500-ms interval. Lock-in measurements were sampled during the last 20 ms of the stimulus. This data collection procedure and use of the lock-in amplifier has been shown to be very sensitive and effective in measuring the saccular potentials at the reported frequencies (Sisneros 2007, 2009b; Rohmann and Bass 2011; Vasconcelos et al. 2011). Each recording session began with control trials (no auditory stimulus) followed by stimulus test trials in which auditory frequency stimuli at a given sound level were presented in a pseudorandom order.

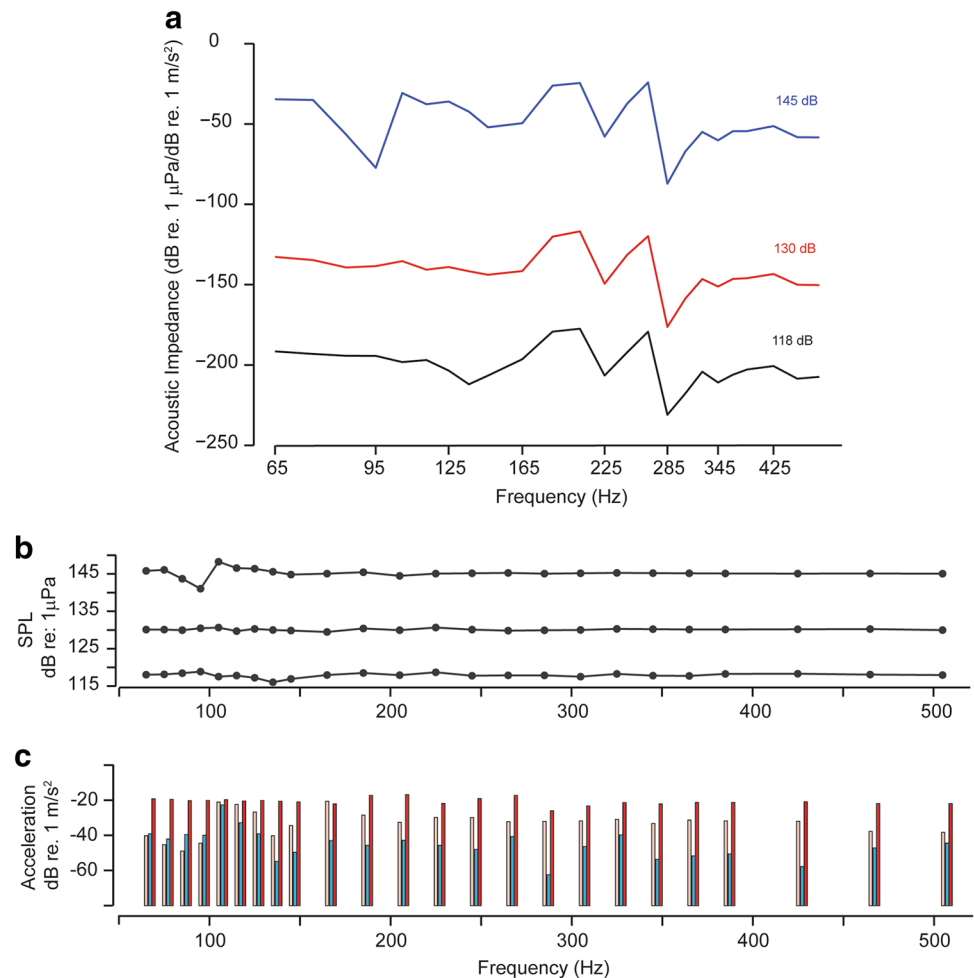
Calibrations were performed before every experiment, after the tank water was initially cooled, but before the fish was placed in the tank. During calibration of the auditory stimuli, the hydrophone and accelerometer were centered

10.5 cm above the speaker (2.5 cm below water surface) at the position of the fish's ear. Stimuli were equalized in SPL using an iterative MATLAB script that measured power spectral density for each frequency from recordings taken through a mini-hydrophone (model 8103, Bruel and Kjaer). At each iteration, the voltage signal sent to the speaker was scaled until the measured SPL output at each frequency was within 0.5 dB of the desired amplitude (130 dB re.: 1 μ Pa). This SPL level was chosen for calibration because 130 dB SPL is well above the noise floor for our measurement devices, and it is consistent with sound levels for type I male midshipman calls within and near their nests in the field and therefore within the range of stimulus amplitudes tested here (Bass and Clark 2003). The frequency stimuli used in this study were based on that used in previous studies (Sisneros and Bass 2003; Sisneros 2007, 2009b; Rohmann and Bass 2011). The intention of this study was to compare the saccular sensitivity of type II male *P. notatus* across season (non-reproductive vs. reproductive state) and compare the saccular sensitivity with that of females (Sisneros 2009b) and type I males (Rohmann and Bass 2011) under similar experimental conditions. The data presented

in sound pressure and particle acceleration to describe auditory sensitivities should not be considered in terms of absolute value given the constraints of our experimental tank setup, but instead should be used as a means to make quantifiable comparisons of the relative seasonal, reproductive state-dependent differences in auditory saccular sensitivity across the three sexual phenotypes (females, and type I and II males).

Consistent with previous studies, acceleration measurements were collected in three dimensions relative to fish orientation inside the tank: *x* (anterior/posterior), *y* (left/right) and *z* (dorsal/ventral) (Casper and Mann 2006; Horodysky et al. 2008; Wysocki et al. 2009) using a custom-modified three-dimensional underwater accelerometer (PCB model VW356A12, sensitivity = 100 mV g^{-1} (10.2 mV ms^{-2}) PCB Piezotronics, Depew, NY) that was encased in syntactic foam and epoxy to make it neutrally buoyant. We characterized the acoustic impedance (ratio of sound pressure to particle velocity) of our experimental tank conditions as suggested by Popper and Fay (2011), using the calibration measurements at different three SPLs: 118, 130, and 145 dB (re.: 1 μ Pa). Figure 1a shows the

Fig. 1 Acoustic characterization of the tank used in this study. **a** Acoustic impedance, calculated as the ratio of sound pressure level to particle motion level, measured in the frequency ranges tested at three sound pressure levels (118, 130 and 145 dB re. 1 μ Pa). Note that the three sound pressure levels tested are parallel and show no major resonances at any of the tested frequencies. **b** Amplitude profiles of three sound pressure levels (118, 130 and 145 dB re. 1 μ Pa) are relatively constant and frequency-invariant. **c** Particle acceleration levels measured at 130 dB re. 1 μ Pa at each of the frequencies used in this study in the three Cartesian axes [*x*, *y*, *z*], represented by pink, blue and red, respectively. The majority of the energy is contained in the dorsoventral (“*z*”) axis (red bars)



ratio of sound pressure level to particle velocity for each frequency recorded in our experimental tank setup. The calibrated sound pressure levels were relatively constant across the test frequencies regardless of amplitude (e.g., see Fig. 1b which shows stimulus calibrations at 118, 130 and 145 dB re. 1 μ Pa). For any given sound level, particle acceleration was relatively constant and greatest in the z dimension relative to the dimensions (x and y) orthogonal to speaker motion (Fig. 1c) and particle acceleration in all three dimensions scaled linearly across the SPLs tested.

Data analyses

The acoustic stimuli and the evoked saccular potentials were measured simultaneously and sampled at 20 kHz. During the recordings, the hydrophone and accelerometer were placed approximately 13 cm from the center of the tank (where the fish was positioned) at a depth of 10 cm.

Representative examples of a control (no auditory stimulus) and test trial (75 Hz at 130 dB) are shown in Fig. 2. The middle 700 ms of the recorded signals was used to calculate the relative power of each frequency using the fast Fourier transform (“FFT,” MATLAB version 2007). Note that any acoustic reflections of the stimulus from the tank walls and water surface did not alter the sound pressure waveform of the acoustic signal, even at the lowest frequencies tested (e.g., 75 Hz) as shown in Fig. 2. There was no significant background noise measured by the hydrophone or accelerometer during the control trials (note gray traces in Fig. 2). Saccular potentials contained only relative small peaks of noise at the harmonics of 60 Hz during the recordings of the controls. During test trials, the saccular potentials contained significant energy at the second harmonic of the stimulus frequencies (Fig. 2d). Only the second harmonic component was used by the lock-in amplifier to determine the saccular potential.

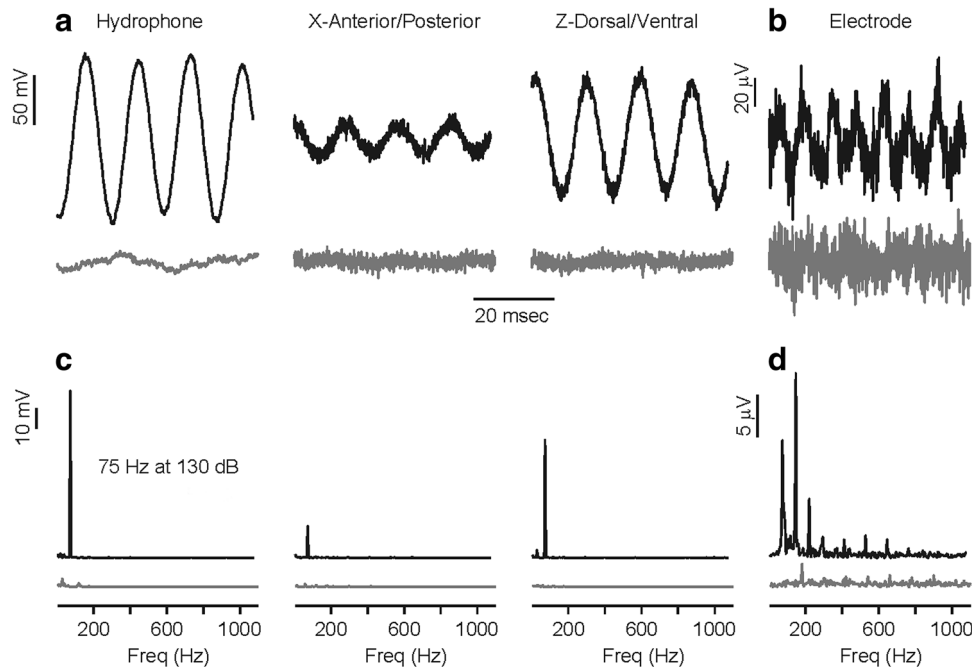


Fig. 2 Representative examples of the acoustic stimulus and the evoked saccular potentials recorded from type II male *P. notatus* that were simultaneously measured during physiology experiments. **a** During the experiments, the fish was positioned in the center of the tank and the hydrophone and/or accelerometer was positioned at a depth of 10 cm and placed horizontally approximately two-third of the distance from the center to the rim of the experimental tank [black trace shows recorded signal during stimulus presentation while gray trace shows the absence of signal during control trial (no auditory stimulus)]; note only two of the three recorded accelerometer axes are shown here— X (anterior/posterior) and Y (dorsal/ventral)]. **b** During stimulus presentation, the saccular potentials were evoked during the duration of stimulation [black trace shows the recorded evoked saccular potential from the recording electrode during a stimulus presentation of 75 Hz at 130 dB re: 1 μ Pa, while the gray trace

shows only background noise during a control (no auditory stimulus) trial]. **c** Power spectrum of the auditory stimulus as measured by the hydrophone and accelerometer shown in **a**. The corresponding power spectra are shown below the measurement for the hydrophone and the recorded X and Z accelerometer axis [black trace is the power spectra for the stimulus frequency of 75 Hz at 130 dB re: 1 μ Pa, while the gray trace shows power spectra for control trial (no auditory stimulus)]. **d** Power spectrum of the recorded evoked saccular potential shown in **b** black trace is the power spectra of the saccular potentials during auditory stimulation while gray trace shows power spectra of saccular potentials during control (no auditory stimulus) trial. Note that the greatest energy in the power spectra of the evoked saccular potentials occurs at the 2nd harmonic of the stimulus frequency (the expected response from opposite oriented hair cells in the sacculus)

The threshold of the evoked saccular response was determined using: (1) the average potential recorded in the absence of a stimulus plus two standard deviations and (2) a continuous estimate of the saccular responses (y_{est}) based on the recorded evoked responses near-threshold levels, which was generated by fitting the data with an exponential function (see Fig. 3, thick fitted line using the equation below). Auditory thresholds were determined using exponential rate-level functions that were fitted to the records of the evoked potentials and then compared to the saccular potential recorded in the absence of an auditory stimulus.

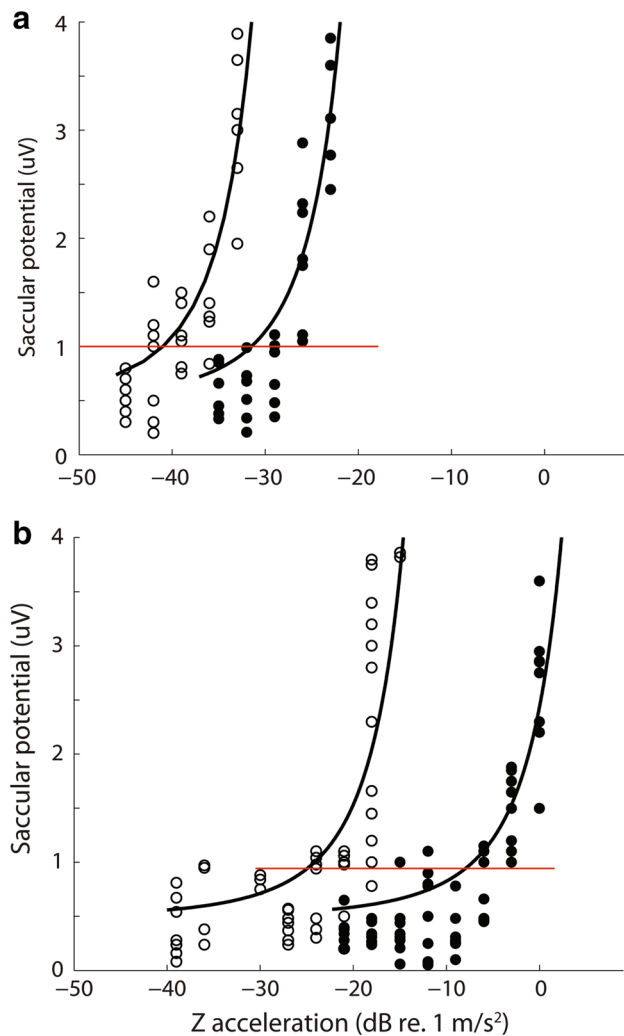


Fig. 3 Illustrated examples of how the evoked saccular potential thresholds were determined at the tested frequencies. *Figure* shows examples at the lowest frequency tested (i.e., 75 Hz) (**a**) and the highest frequency tested (i.e., 385 Hz) (**b**) for reproductive (open circles) and non-reproductive fish (filled circles). Each data set was fitted with a best-fit model curve (black lines) and thresholds were interpolated from the best-fit curve (see detailed description in “Materials and methods”). Auditory threshold level was calculated as two standard deviations above baseline and is represented in figure as the horizontal red line

Figure 3 shows two illustrated examples of threshold determination using the rate-level function near threshold at the minimum and maximum frequencies tested. Auditory threshold was defined as the minimum stimulus level above which the estimated saccular response (y_{est}) exceeded 2 standard deviations above the recorded evoked potential in the absence of a stimulus (Fig. 3 horizontal line). Exponentials were fit using data points from near-threshold trials (10–15 dB above and below threshold) using the following equation:

$$y_{\text{est}} = \frac{e^{x \cdot b_1}}{1 + e^{x \cdot b_2}},$$

where x is the stimulus amplitude, y_{est} is the estimated saccular response, and b_1 and b_2 are the fitted variables defining the curve. Particle motion thresholds were derived from sound pressure thresholds using best-fit linear coefficients derived from calibrations. Because there was a linear relationship between pressure and particle motion at all frequencies tested, best-fit linear transformations were derived for each frequency using pressure calibration measurements and the equivalent particle motion measurements in the x , y and z axes. After deriving thresholds in the x , y and z dimensions, particle motion threshold reported as the combined magnitude vector, calculated as $20 \log (\sqrt{x^2 + y^2 + z^2})$ similar to Wysocki et al. (2009) and Vasconcelos et al. (2011).

The overall effect of reproductive state on the auditory saccular thresholds based on sound pressure and particle acceleration in non-reproductive and reproductive type II male midshipman was analyzed using a repeated measures ANOVA with thresholds for each of the 17 frequencies tested (75–385 Hz) as the repeats (i.e., within-subject factors) and reproductive state of the animal as the between-subject factor followed by the HSD test with unequal N for post hoc planned comparisons of thresholds at each stimulus frequency. For all statistical tests, α was set at 0.05. Statistical analyses were performed using Statistica for Windows (StatSoft).

Results

Evoked saccular potentials were recorded from a total of 32 adult type II male midshipman: 5 non-reproductive type II males with a size range of 10.2–16.0 cm SL [mean SL = 13.5 ± 1.8 cm SD, mean body mass (BM) = 35.2 ± 17.1 g SD, and mean GSI = 4.4 ± 3.1 SD] and 32 reproductive type II males with a size range of 8.0–10.5 cm SL [mean SL = 9.5 ± 0.7 cm SD, mean body mass (BM) = 10.4 ± 2.3 g SD, and mean GSI = 13.5 ± 4.5 SD]. The evoked saccular potentials were recorded from all type II males within 26 days of field collection, with

the exception of two non-reproductive type II males that were recorded approximately 5–7 months after collection; however, both of these males were maintained on a short-day “winter” photoperiod. No relationship was observed between GSI and the number of days that the animal were kept in captivity within 26 days of collection ($r^2=0.047$, $p>0.2$).

Auditory thresholds based on sound pressure and particle acceleration were determined for whole populations of hair cells in the saccule of non-reproductive and reproductive type II males. Threshold tuning curves were constructed using an exponential rate-level function that was fitted to the records of evoked saccular potentials and compared to the evoked potentials recorded in the absence of a stimulus (background noise measurement) (Fig. 3). The auditory threshold at each stimulus frequency was designated as the lowest stimulus level that evoked a saccular potential greater than 2 SD above the background noise measurement (Fig. 3, red line). The auditory thresholds for the recorded saccular hair cells based on sound pressure and particle acceleration are summarized in Fig. 4.

The threshold tuning curves based on sound pressure generally consisted of tuning profiles with lowest thresholds at 75 and 85 Hz that gradually increased to highest thresholds at frequencies ≥ 205 Hz (Fig. 4a). Significant pressure threshold differences between non-reproductive and reproductive type II males were observed at every tested frequency except 75 Hz (repeated measures ANOVA: between-subject factor reproductive state, HSD test with unequal n : $F_{(17,21)}=4.94$, $p<0.001$). The auditory thresholds of the saccular hair cells from reproductive male type II were ~ 8 –20 dB (re. $1 \mu\text{Pa}$) lower than non-reproductive type II males at frequencies from 85 to 385 Hz (Fig. 4a).

The threshold tuning curves based on particle acceleration were similar in shape to the pressure threshold profiles with lowest thresholds at 75 and 85 Hz that gradually increased to highest thresholds at frequencies ≥ 185 Hz (Fig. 4b). Significant threshold differences based on acceleration between non-reproductive and reproductive type II males were observed at every frequency except 75 Hz (repeated measures ANOVA: between-subject factor reproductive state, HSD test with unequal n : $F_{(17,21)}=4.95$, $p<0.001$). The auditory acceleration thresholds of the saccular hair cells from reproductive male type II were ~ 8 –20 dB re. 1 m s^{-2} lower than non-reproductive type II males at frequencies from 85 to 385 Hz (Fig. 4b).

Discussion

The goal of this study was to characterize the evoked saccular potentials of type II male midshipman to determine whether auditory threshold and frequency response of

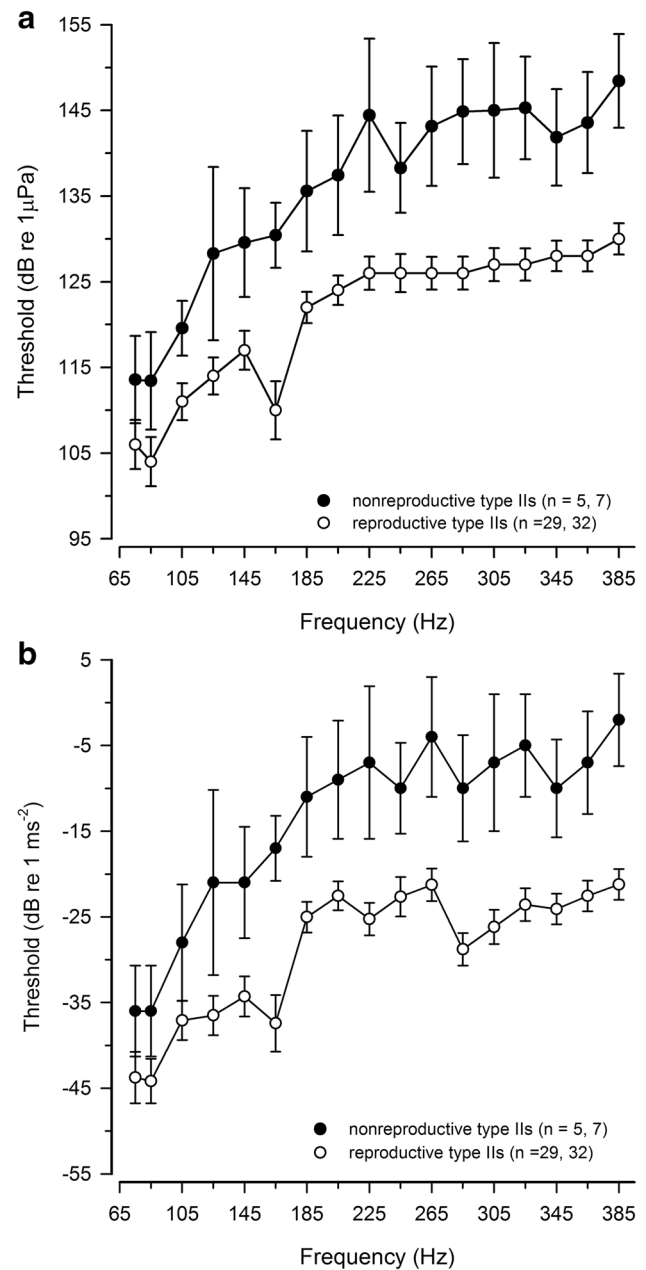


Fig. 4 Seasonal changes in saccular sensitivity-based sound pressure and particle acceleration measurements in the plainfin midshipman fish (*Porichthys notatus*). **a** Auditory threshold tuning curves for non-reproductive and reproductive type II males based on saccular potential recordings and measurements of sound pressure. All data are plotted as mean \pm 95% confidence limit. The number of animals and records are indicated in parentheses. **b** Auditory threshold tuning curves for non-reproductive and reproductive type II males based on saccular potential recordings and measurements of acoustic particle acceleration. All data are plotted as mean \pm 95% confidence limit. The number of animals and records are indicated in parentheses

saccular hair cells change seasonally to behaviorally relevant auditory stimuli. Our results indicate that the summer reproductive type II males were more sensitive than winter

non-reproductive type II males across a range of frequencies tested (85–385 Hz). Our data support the hypothesis that the saccular sensitivity of reproductive type II males is similar in sensitivity to the other reproductive sexual phenotypes (females and type I males) and better adapted than that of non-reproductive type II males to detect the dominant frequency content of the type I male vocalizations. In this discussion, we also interpret our results as they relate to the reception of social acoustic signals based on sound pressure and particle motion and how the changes in auditory sensitivity of type II males may facilitate the localization and assessment of midshipman social acoustic signals during the breeding season.

Appropriate stimuli

There has been much discussion regarding the appropriate sound stimuli to measure in fish hearing studies (Popper and Fay 1993, 2011; Fay and Popper 2012). Sound can be measured in terms of both acoustic particle motion and pressure. Fish have evolved at least two modes of hearing: an inertial (particle motion) mode and a pressure-mediated mode. All teleost fishes are thought to be capable of detecting a particle motion vector, such as acoustic particle acceleration, via their inner otolithic end organs which essentially act as acceleration detectors that respond directly to displacement of the fish by particle motion (De Vries 1950; Hawkins 1993; Sisneros and Rogers 2016). In contrast, the pressure-mediated mode of fish hearing requires the use of specialized morphological adaptations that enable fish to transduce the pressure-induced vibrations of the swim bladder to the otolithic end organs to detect sound pressure. Otophysan fish, such as the goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*), possess skeletal adaptations known as Weberian ossicles that link the swim bladder to the inner ear for enhanced pressure detection while other fishes such the Hawaiian squirrelfish (*Myripristis kuntee*) and the West African ladyfish (*Elops lacerta*) have no specialized connections between the swim bladder and inner ear, but do exhibit increased pressure sensitivity simply due to the close proximity of these gas-filled structures to the inner ear (Greenwood 1970; Coombs and Popper 1979; Braun and Grande 2008; Tricas and Boyle 2015). Additional studies have shown that natural or artificial gas-filled bladders without direct apposition to the inner ear can also significantly enhance hearing sensitivity in fishes (Chapman and Sand 1974; Jerko et al. 1989). Given that the swim bladder in female and type II male midshipman extends anteriorly via “horn-like” extensions of the swim bladder to caudal regions of the otic capsule (Whitchurch et al. 2012), and that this caudal region of otic capsule has been proposed to be an “acoustic channel” to the saccule through the neurocranium (Edds-Walton et al. 2015), the

midshipman is very likely sensitive to both sound pressure and particle motion. Thus, in the current study we characterized the midshipman auditory thresholds in terms of both sound pressure and particle motion. As suggested by Popper and Fay (2011), we also report the impedance (ratio of pressure to particle velocity) of our test tank (see Fig. 1a) so that the acoustic impedance of the tank conditions in this study can be used in comparison with that of future fish hearing studies.

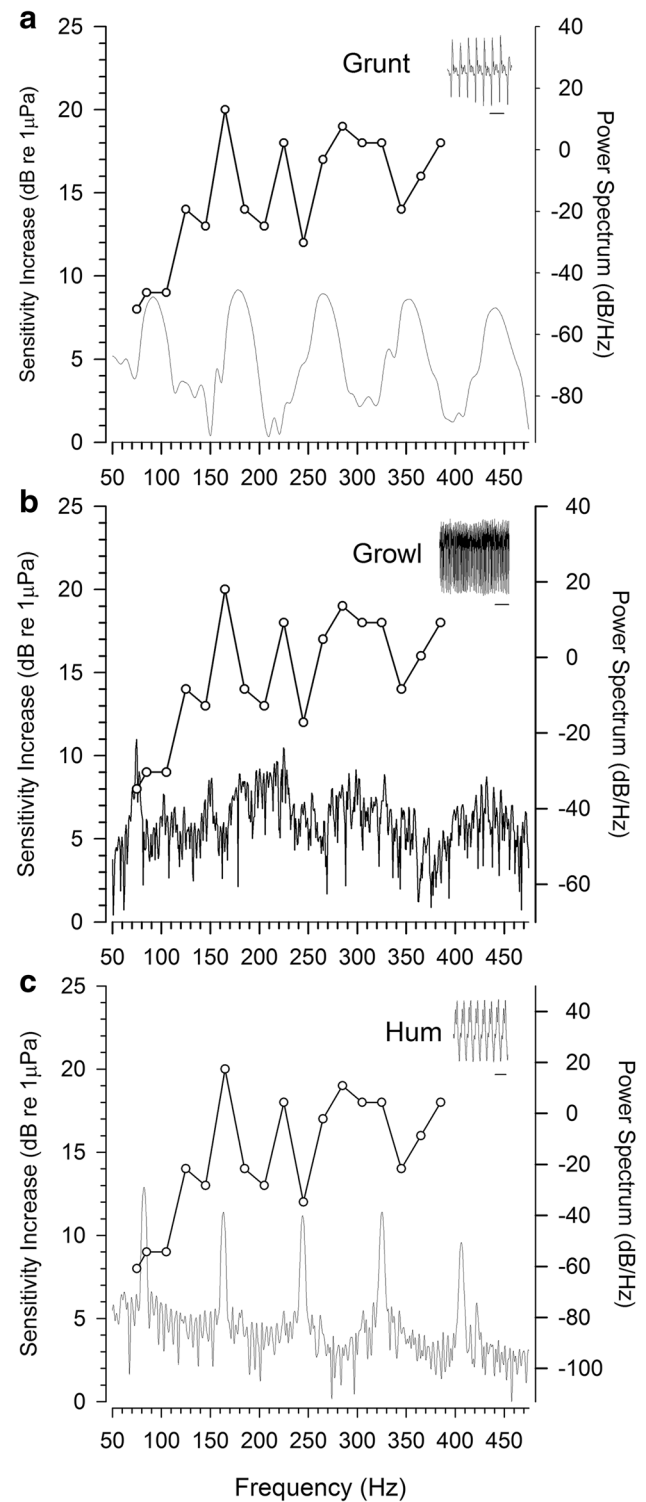
Reproductive state-dependent changes in auditory saccular sensitivity

We observed dramatic reproductive state-dependent differences in the sound pressure and particle acceleration thresholds of the saccule in type II males. The relative differences in sensitivity between reproductive and non-reproductive type II males for both pressure and acceleration (re. 1 μPa and 1 ms^{-2} , respectively) varied between 8 and 21 dB at frequencies from 75 to 385 Hz (Fig. 4). Furthermore, the saccular thresholds of reproductive type II males were at least 13 dB lower (an enhanced sensitivity equivalent to 4.5 times greater) than non-reproductive type II males at frequencies >185 Hz, which corresponds to the higher frequency components with the majority of energy in type I male vocalizations (Fig. 5). Nesting type I males produce three types of acoustic signals that include agonistic grunts and growls and advertisement calls known as hums (Bass et al. 1999; Forlano et al. 2015). Reproductive type I males often produce trains of broadband, short-duration (50–200 ms) grunts (Fig. 5a) to fend or ward off potential nest intruders (Brantley and Bass 1994). Grunts trains produced by type I males can have a repetition rate of about 2.5 Hz with grunt intervals of approximately 400 ms (Bass et al. 1999). Growls are relatively long-duration (>1 s), multiharmonic signals (Fig. 5b) with fundamental frequencies that gradually change over the duration of the call from 116 to 59 Hz (Bass et al. 1999). In comparison, the hum is a long-duration, multiharmonic signal with a fundamental frequency that can range from 80 to 102 Hz (personal observation, JAS; also see Bass et al. 1999). In addition to the fundamental frequency, the hum also contains a number of prominent harmonics that range up to 400 Hz (Fig. 5c) with additional lower amplitude harmonics ranging up to 800 Hz and beyond (Bass et al. 1999). In the context of signal recognition and preference, type II males have been observed to exhibit positive phonotaxis to the playback of simulated hums (McKibben and Bass 1998). Our results suggest that the saccular sensitivity of reproductive type II males is well suited to encode frequencies ≥ 165 Hz which include the dominant higher frequency components in type I male vocalizations including hums (Fig. 5). This enhanced saccular sensitivity may be adaptive and function

Fig. 5 Comparison between the vocal characteristics of type I male vocalizations and the change in auditory saccular tuning of type II males based on seasonal reproductive state. **a** Sensitivity increase in auditory saccular thresholds (left y axis) of reproductive type II males compared to non-reproductive type II males at each frequency tested and the power spectrum of a type I male grunt (right y axis, in relative dB values), inset: the temporal waveform of a type I male grunt recorded at 13.5 °C in a laboratory setting (scale bar 20 ms). **b** Sensitivity increase in auditory saccular thresholds (left y axis) of reproductive type II males compared to non-reproductive type II males at each frequency tested and the power spectrum of a type I male growl (right y axis, in relative dB values), inset: the temporal waveform of a type I male growl recorded at 13.5 °C (scale bar 20 ms). **c** Sensitivity increase in auditory saccular thresholds (left y axis) of reproductive type II males compared to non-reproductive type II males at each frequency tested and the power spectrum of a nesting type I male advertisement call or “hum” (right y axis, in relative dB values), inset: the temporal waveform of a type I male hum recorded at 13.5 °C at Seal Rock, WA, in a nesting site (scale bar 20 ms)

to increase the probability of signal detection by sneaker males in shallow water environments where the propagation of acoustic signals is greatly affected by substrate composition and water depth (Rogers and Cox 1988; Bass and Clark 2003; Maruska and Sisneros 2015). The relative high frequencies (approximately those greater than 180 Hz) contained within type I vocalizations will propagate further than the calls' fundamental frequencies in shallow water due to the inverse relationship between water depth and the cutoff frequency of sound transmission (Fine and Lenhardt 1983; Rogers and Cox 1988; Bass and Clark 2003). In addition, the substrate composition (e.g., the rocky substrate of the intertidal zone where midshipman breed) can greatly influence the speed of sound in the bottom substrate and affect the cutoff frequency of signal propagation which can attenuate the transmission of low-frequency acoustic signals (e.g., the fundamental frequencies of type I male vocalizations) (Rogers and Cox 1988). Thus, the enhanced sensitivity of type II males to the dominant higher frequencies within type I male vocalizations may potentially facilitate eavesdropping by sneaker males for the assessment of type I male vocalizations and the selection of cuckoldry sites during the breeding season.

The observed seasonal, reproductive state-dependent changes in auditory saccular sensitivity based on sound pressure for type II males were remarkably similar to that reported for female and type I male midshipman. Previously, seasonal and reproductive state-dependent changes in auditory saccular sensitivity based on sound pressure have been reported for both females and type I males across the same range of frequencies from 75 to 385 Hz (Sisneros 2009b; Rohmann and Bass 2011). Results from this study revealed that the difference in saccular sensitivity based on sound pressure between reproductive and non-reproductive type II males ranged from 9 to 21 dB at 75 to 385 Hz, respectively (Fig. 4), which is similar to the reproductive



state-dependent differences observed for females and type I males (Fig. 6). Although the sound pressure tuning curves were similar for all three sexual phenotypes, there appear to be slight seasonal differences in the mean threshold profiles between the reproductive and non-reproductive sexual phenotypes. Here, we show that reproductive state-dependent

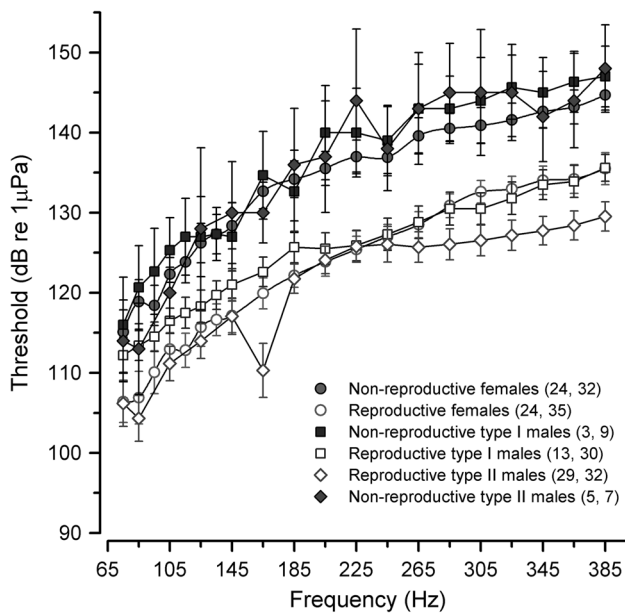


Fig. 6 Frequency sensitivity of saccular hair cells in the plainfin midshipman fish (*Porichthys notatus*). Shown here is a plot of the auditory saccular tuning curves based on evoked potential recordings in all three adult sexual phenotypes (females, type I and type II males) in reproductive and non-reproductive conditions. All data are plotted as mean \pm 95% confidence limit. The number of animals and records are indicated in parentheses. Auditory saccular tuning data for females is adapted from Sisneros (2009b), and from Rohmann and Bass (2011) for type I males

changes in type II male saccular sensitivity were frequency dependent with seasonal threshold differences greatest at frequencies ≥ 125 Hz (~ 12 – 20 dB) and lowest threshold differences at frequencies < 125 Hz (~ 8 – 9 dB). Similarly, type I males also exhibited reproductive state-dependent changes in saccular sensitivity that were frequency dependent with greatest threshold differences at frequencies > 145 Hz (~ 12 – 15 dB) and lowest threshold differences at frequencies ≤ 145 Hz (~ 4 – 9 dB; see Rohmann and Bass 2011). In contrast, seasonal differences in female saccular sensitivity were not frequency dependent but were instead relatively constant (~ 8 – 13 dB) across the same range of tested frequencies from 75 to 385 Hz (Sisneros 2009b). Consistent across phenotypes is the observation that reproductive midshipman showed significantly lower saccular thresholds than non-reproductive midshipman (Fig. 6). In sum, the results from this study now confirm that seasonal, reproductive state-dependent changes in auditory saccular sensitivity are a species-typical trait found in all midshipman sexual phenotypes.

Although no previous study has examined seasonal differences in acoustic acceleration sensitivity of the sacculus in type II males, our results suggest that the saccular acceleration sensitivity of non-reproductive type II males is at least similar to that of non-reproductive type I males

(Weeg et al. 2002). Estimates for particle motion sensitivity in non-reproductive type I male saccular afferents range from -15 to 26 dB (re. 1 nm), which corresponds to a displacement of 0.8 – 20 nm, whereas our estimate for particle acceleration threshold at the most sensitive frequency tested (85 Hz) is -45 dB \pm 7 dB (re. 1 m s $^{-2}$). Numerical integration of the mean acceleration threshold value at 85 Hz provides a rough estimate of a displacement threshold of 17 nm, which is within the displacement sensitivity range reported for type I males by Weeg et al. (2002). Furthermore, the study by Weeg et al. (2002) demonstrated that type I male saccular afferents responded best at 50 and 100 Hz. Although we did not test the same frequencies, we do show that non-reproductive type II males share a similar particle acceleration sensitivity to that of type I males with lowest acceleration thresholds reported at frequencies < 100 Hz. However, it should be noted that the published results on type I males (Weeg et al. 2002) were obtained using a shaker table system which primarily only delivers particle acceleration stimuli, whereas in our study we used a speaker, which delivers both particle motion and pressure stimuli. Further tests will need to be conducted using a shaker system to more accurately compare the particle acceleration sensitivities for the three midshipman sexual phenotypes.

Potential mechanisms for the plasticity of saccular sensitivity

The underlying mechanism(s) responsible for the seasonal reproductive-dependent changes in saccular sensitivity of type II males is likely similar to that proposed for females and type I males. Previous studies by Rohmann and Bass (2011) and Rohmann et al. (2013) showed that the seasonal variation in the frequency sensitivity of saccular hair cells in type I males could be explained by seasonal changes in the abundance of calcium-activated potassium BK channels, which are known to be responsible for a large conductance, outward current that produces electrical receptor oscillations along the hair cell epithelium (Lewis and Hudspeth 1983; Roberts et al. 1988; Fettiplace and Fuchs 1999). The electrical resonance that arises from the ion-channel current kinetics of hair cells is thought to be the major contributing factor that influences low-frequency (< 1 kHz) hair cell tuning in reptiles and birds (Fettiplace and Fuchs 1999) and in fish (Steinacker and Romero 1991, 1992). Rohmann et al. (2013) showed that by using the BK channel antagonist iberiotoxin to reduce BK channel availability they could effectively induce a saccular neurophysiological phenotype similar to that found in non-reproductive type I males. Thus, the BK channels of hair cells are thought to be a primary mechanism that can account for the seasonal changes in the frequency selectivity of hair cells in males

and females. Another potential mechanism responsible for seasonal changes in saccular sensitivity is the reproductive state-dependent increases in saccular hair cell density and decreases in hair cell death observed in females by Coffin et al. (2012). Such seasonal increases in saccular hair cell density were independent of body size (age) and not observed in the other inner ear end organs, the lagena and utricle. In addition, Coffin et al. (2012) also observed in reproductive females that the increase in saccular hair cell density was concurrent with increases in overall magnitude of the evoked saccular potentials and decreases in auditory saccular thresholds (i.e., increased auditory sensitivity). The above-mentioned mechanisms potentially responsible for the observed seasonal changes in saccular sensitivity have been proposed to be induced by estrogens and androgens acting via long term signaling pathways to upregulate BK channels and modulate hair cell proliferation and death to ultimately enhance the hearing sensitivity in this species (Coffin et al. 2012; Forlano et al. 2015, 2016). However, this steroid-mediated mechanism hypothesis remains to be tested.

In summary, our results indicate that the seasonal plasticity of auditory sensitivity previously reported for females and type I males also occurs in type II male midshipman. This form of reproductive state-dependent plasticity of auditory sensitivity provides an adaptive mechanism that enhances hearing sensitivity in type II males during the reproductive season. Such an increase in auditory sensitivity should increase the probability of conspecific detection by type II sneaker males and facilitate eavesdropping of type I male vocalizations for the assessment of type I male rivals and the selection of cuckoldry sites during the breeding season.

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