Behavioral/Systems/Cognitive

# Saccular-Specific Hair Cell Addition Correlates with Reproductive State-Dependent Changes in the Auditory Saccular Sensitivity of a Vocal Fish

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**The plainfin midshipman fish, *Porichthys notatus*, is a seasonal breeding teleost fish for which vocal–acoustic communication is essential for its reproductive success. Female midshipman use the saccule as the primary end organ for hearing to detect and locate "singing" males that produce multiharmonic advertisement calls during the summer breeding season. Previous work has shown that female auditory sensitivity changes seasonally with reproductive state; summer reproductive females become better suited than winter nonreproductive females to detect and encode the dominant higher harmonic components in the male's advertisement call, which are potentially critical for mate selection and localization. Here, we test the hypothesis that these seasonal changes in female auditory sensitivity are concurrent with seasonal increases in saccular hair cell receptors. We show that there is increased hair cell density in reproductive females and that this increase is not dependent on body size since similar changes in hair cell density were not found in the other inner ear end organs. We also observed an increase in the number of small, potentially immature saccular hair bundles in reproductive females. The seasonal increase in saccular hair cell density and smaller hair bundles in reproductive females was paralleled by a dramatic increase in the magnitude of the evoked saccular potentials and a corresponding decrease in the auditory thresholds recorded from the saccule. This demonstration of correlated seasonal plasticity of hair cell addition and auditory sensitivity may in part facilitate the adaptive auditory plasticity of this species to enhance mate detection and localization during breeding.**

## Introduction

Seasonal morphological plasticity of vocal–motor circuits in the adult nervous system is a well established feature of many non-mammalian vertebrate communication systems, particularly in seasonally breeding species where the production of vocal signals is plastic and often under endocrine control (for review, see Ball et al., 2002; Brenowitz, 2004). More recently, seasonal neurophysiological plasticity of the adult auditory system has been documented in several vertebrate species including songbirds (*Poecile carolinensis*, *Baeolophus bicolor*, *Sitta carolinensis*, *Zonotrichia leucophrys gambelii*), the Northern leopard frog (*Rana pipiens pipiens*), and the plainfin midshipman fish (*Porichthys notatus*), suggesting that physiological plasticity of the auditory receiver system is also a widespread vertebrate feature (Lucas et al., 2002, 2007; Sisneros and Bass, 2003; Sisneros et al., 2004a; Goense and Feng, 2005; Henry and Lucas, 2009; Sisneros, 2009a; Caras et al., 2010). However, relatively few studies have investigated the potential concurrent morphological correlates that may occur with known demonstrations of seasonal neurophysiological plasticity in the auditory system (Park et al., 2005; Meitzen et al., 2009). Here, we consider seasonal morphological plasticity of the inner ear associated with seasonal changes in the auditory sensitivity of a seasonally breeding vertebrate for which vocal communication is essential to its reproductive success.

The auditory system of the plainfin midshipman fish provides a highly tractable model for investigating seasonal changes in vocal–acoustic behavior and auditory physiology (Bass, 1996; Bass et al., 1999, McKibben and Bass, 2001; Sisneros, 2009b). These highly vocal fish use acoustic communication for many aspects of their life, particularly reproduction. Previous studies of the midshipman fish have yielded strong evidence for a steroid-dependent, reproductive-state modulation of hearing sensitivity that is thought to enhance coupling of sender and receiver in this acoustic communication system (Sisneros et al., 2004a). The auditory saccular sensitivity of wild-caught females changes seasonally with reproductive state such that reproductive females become better suited than nonreproductive females to detect and perceive the dominant higher harmonic components of the male's seasonal advertisement call, or "hum" (Sisneros and Bass, 2003; Sisneros, 2009a). The hum is a long duration (>1 min) multiharmonic advertisement call with a fundamental frequency

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of ~100 Hz and several prominent harmonics ranging up to 400 Hz (Bass et al., 1999). The seasonal enhanced sensitivity to the hum improves the detection and localization of calling conspecific mates during the breeding season (Sisneros, 2009b).

Here, we test the hypothesis that seasonal changes in auditory saccular sensitivity are concurrent with seasonal increases in auditory hair cell receptors of the saccule, the primary hearing organ in the midshipman and most other teleost species. In general, teleost fish continue to add hair cells throughout most, if not all, of their lifetime (Popper and Hoxter, 1984; Lombarte and Popper, 1994; Lanford et al., 1996). However, only a few studies have examined the relationship between hair cell addition and auditory sensitivity of the fish inner ear, including studies of the cartilaginous thornback ray (*Raja clavata*) and the bony teleost zebrafish (*Danio rerio*) (Corwin, 1983; Higgs et al., 2002). We show evidence of saccular-specific increases in hair cell density that may explain the seasonal increase in auditory saccular sensitivity of reproductive females. We propose that the correlated seasonal increase in saccular-specific hair cells and auditory sensitivity may be induced by the annual peak in gonadal steroids (e.g., 17$\beta$-estradiol) that occurs before the summer reproductive season (Sisneros et al., 2004b).

## Materials and Methods

### *Fish collection and care*

Nonreproductive (winter) females were collected by otter trawl (R/V Kittiwake; Bio-Marine Enterprises) in Puget Sound near Edmonds, Washington at depths of 60–100 m during February 2009 and January 2010. Reproductive (summer) females were collected from a natural breeding population in the Hood Canal at Seal Rock near Brinnon, Washington during June 2008, May 2009, and July 2010. These fish were collected by hand at low tide from the nests of parental (type I) males. To avoid any effects of captivity on auditory saccular sensitivity (Sisneros and Bass, 2003), the summer-collected reproductive females were used within 2 weeks. Fish were housed in saltwater aquaria at 14–16°C at the University of Washington and fed a diet of live goldfish or guppies every 2–4 d. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

Standard length (SL), body mass (BM), and gonad mass were recorded for each fish. The gonadosomatic index (GSI), a measure of the relative reproductive state, was calculated for each fish, defined here according to Tomkins and Simmons (2002) as 100 * gonad mass/(body mass − gonad mass). As it is known that fishes produce new hair cells as they grow (Corwin, 1981, 1983; Popper and Hoxter, 1984; Lombarte and Popper, 1994), only females within a narrow size range (11.4–18.3 cm SL) were used for this study. Initial data analysis examined each season and year separately as well as the pooled data for winter (nonreproductive) and summer (reproductive) animals. All seasonal patterns were evident each year, so the data were pooled by season for the final analysis presented here. It should be noted that some of the winter fish had intermediate GSI values suggestive of some gonadal recrudescence. We therefore examined the dataset using all winter and all summer females, and compared summer females only to winter fish with a GSI $<8$ (representing regressed ovaries). The inclusion of only the subset of fish with low winter GSI values did not change the overall analysis. Some summer females had low GSI values, but in this case low values indicated that the female has just spawned, as these fish were collected from the nests of breeding males and are all considered to be in peak reproductive condition. Therefore, the analysis below includes all females collected during these sampling trips, regardless of GSI.

### *Morphological analysis*

*Tissue processing and fluorescent labeling.* Fish were killed with an overdose of buffered ethyl p-aminobenzoate (benzocaine) followed by decapitation. The bony capsule around the ear was opened and perfused with 4% paraformaldehyde. After 1 h of fixation at room temperature, heads were rinsed in 0.1 M PBS and either processed immediately or stored for 1–3 d at 4°C. The ears were dissected from the head, the saccular otolith was removed, and the saccular, utricular, and lagenar epithelia were carefully trimmed (only saccules were examined from fish collected during June 2008).

Left epithelia from each fish were labeled with an antibody to phosphorylated histone H3 (PH3) to identify cells undergoing mitosis. All solutions were prepared in PBS unless noted otherwise. Briefly, epithelia were rinsed, blocked in 5% normal goat serum with 0.1% Triton-X (Sigma), and incubated overnight in anti-PH3 (Ser28; Imgenex; diluted 1:200 in blocking solution). Tissue was thoroughly rinsed and incubated in secondary antibody (Alexa Fluor 568 goat-anti-mouse, diluted 1:500; Invitrogen). Tissue was then counter-stained with phalloidin (Alexa Fluor 488 phalloidin, diluted 1:100; Invitrogen) to visualize the stereocilia.

Right epithelia (saccule, utricle, and lagena) were processed with a TUNEL assay as a marker of dying cells. Labeling was conducted with an ApopTag fluorescein in situ apoptosis detection kit (Millipore) using the manufacturer's protocol and modification according to Wilkins et al. (2001). All epithelia were mounted whole with Fluoromount-G (Southern Biotech) and coverslipped.

*Fluorescent imaging and analyses.* Two different analyses were conducted to quantify hair bundle density. The first analysis examined density of phalloidin-labeled hair bundles in each inner ear end organ. Images (40×) of each epithelium were taken using a Zeiss Axiovert microscope equipped for epifluorescence. Hair bundle counts were performed in seven nonoverlapping 10,000 $\mu m^2$ regions of each saccule and three 10,000 $\mu m^2$ regions each for the utricle and lagena using ImageJ (v. 1.42q) and a sampling strategy based on Smith et al. (2006) and Oxman et al. (2007) (Fig. 1). The second analysis specifically examined small phalloidin-labeled hair bundles that are not visible in the initial 40× images. Small bundles were defined as those with substantially fewer and shorter stereocilia and smaller cuticular plates than surrounding bundles. While this definition is inherently qualitative, the small bundles counted in the present study were a completely separate morphological population from the normal bundles counted in the first analysis and we are confident that there was no overlap between the bundle categories. These bundles may represent either immature/developing bundles as defined by Schuck and Smith (2009) or a morphologically and perhaps physiologically distinct type of mature hair cell. Small bundles were counted in 15,380 $\mu m^2$ regions on a second set of images taken with a 63× oil-immersion objective from the same seven saccular regions as used for the first set of bundle density counts. Bundle count data for both mature and immature-like bundles were analyzed by two-way ANOVA with epithelial region and season as factors (Graphpad Prism v. 5). Since we planned to compare the same saccular epithelial regions between reproductive and nonreproductive fish, an *a prior t* test was used to determine significant paired comparisons across saccular epithelial regions.

Dying (TUNEL-labeled) and proliferating (PH3-labeled) cells were counted in entirety for each epithelium using a Zeiss Axiovert microscope and a 63× oil objective. Cell death and proliferation counts were analyzed separately for each epithelium by two-tailed *t* test, as the comparison of interest was a pairwise seasonal comparison within epithelia, rather than between end organs.

Hair bundle orientation was qualitatively mapped in a subset of phalloidin-labeled epithelia from each reproductive group. Bundle orientation was defined as the direction of polarization from the shortest stereocilia to the kinocilium (Flock, 1964; Popper, 1981). Although phalloidin does not label the kinocilium (a tubulin-based structure), the kinocilium position appears as a dark hole at the level of the cuticular plate in the otherwise fluorescent apical surface of the hair cell, allowing the kinocilium position to be definitively identified (Lu and Popper, 1998). Epithelia were viewed on a Zeiss Axiovert microscope using a 63× oil objective and bundle orientation was mapped onto drawings of each epithelium using epithelial landmarks and dissection artifacts as points of reference.

Hair bundle length was also quantified for a subset of phalloidin-labeled saccules. Because our observations suggested that bundle length

varied in both dimensions, images were collected of 14 distinct saccular regions that encompassed both the rostral–caudal and dorsal–ventral axes. Saccules were viewed on an Olympus FV-1000 confocal microscope with a 63× water objective and 4× digital zoom and optical sections taken at 200 nm intervals. Images were collected with Fluoview software (v. 2.1c) and deconvolved with Huygens Professional. Deconvolved images were opened with Fiji (ImageJA 1.45 g) and three to nine hair bundles were measured per image using the Simple Neurite Tracer plugin, with the measurement path following the length of the longest stereocilium of each bundle from the tip to the cuticular plate. Only intact (not splayed) bundles with clearly visible stereocilia were selected for measurement. Hair bundle length was analyzed by two-way ANOVA and an *a prior t* test was used to determine significance of the paired comparisons of the 14 distinct saccular regions between reproductive and nonreproductive females.

*Scanning electron microscopy.* A subset of epithelia from summer 2008 females were also prepared for scanning electron microscopy. Inner ear tissues were fixed in 4% glutaraldehyde in 0.1 M cacodylate with 0.001% $CaCl_2$ 4°C and dissected as described above. Epithelia were postfixed in 1% osmium tetroxide in 0.1 M cacodylate for 1 h, rinsed in cacodylate buffer, and stored in cacodylate buffer at 4°C. Epithelia were then critical-point dried with liquid $CO_2$, mounted on stubs with carbon tape, and sputter-coated gold/palladium to ~20 nm thickness. Samples were viewed with a JSM-6300F field emission scanning electron microscope.

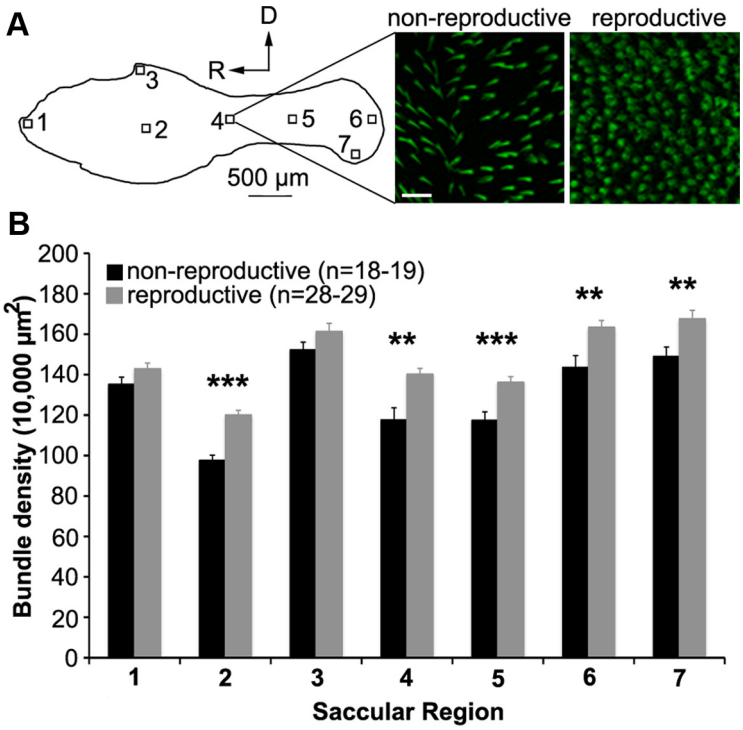


**Figure 1.** Seasonal differences in hair bundle density in the female midshipman saccule. ***A***, Phalloidin-labeled hair bundles were counted in 10,000 $\mu m^2$ areas from seven regions across the saccule, as indicated by the numbered boxes on the line drawing (left). The micrographs (right) show representative images from the middle of the saccule from a nonreproductive female and a reproductive female. Scale bar, 20 $\mu$m. R, Rostral; D, dorsal. ***B***, Hair bundle quantification from these seven saccular regions. Data were pooled over three summer (reproductive) and two winter (nonreproductive) sampling seasons. There was a significant effect of reproductive state ($F_{(1,326)} = 70.77, p < 0.0001$) and saccular region ($F_{(6,326)} = 48.27, p < 0.0001$) but not of the interaction between the two ($F_{(6,326)} = 1.302, p = 0.25$), as determined by two-way ANOVA. Significantly more hair cells were seen in regions 2, 4, 5, 6, and 7 of reproductive females compared with nonreproductive females (*a prior t* tests for paired comparisons were used to determine differences in bundle density between the same regions in nonreproductive and reproductive fish; $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars are mean + 1 SEM. $n = 18–19$ saccules from nonreproductive females, $n = 28–29$ saccules from reproductive females. The variation in sample size occurred because some saccular regions suffered dissection damage and were therefore not used for bundle quantification. Black bars, Nonreproductive females; gray bars, reproductive females.

## *Physiological analysis*

*Acoustic stimulus generation.* Acoustic stimuli were generated using the reference output signal from a lock-in amplifier (SR830; Stanford Research Systems) that was inputted to an audio amplifier and an underwater speaker (UW-30; Telex Communications). The speaker's frequency response was measured using a mini-hydrophone (8103; Bruel and Kjaer) that was positioned below the water line and 10 cm above the underwater speaker, which is the position that is normally occupied by the head of the fish during the saccular potential recordings. Relative sound measurements and speaker calibrations were made using the mini-hydrophone, a single-channel FFT spectrum analyzer (SR780; Standford Research Systems), and an oscilloscope to measure the peak-to-peak voltages of the sound stimuli. The peak-to-peak voltages were then used with a custom Matlab script to adjust the sound pressure levels at all tested frequencies (75–385 Hz) to be of equal amplitude within ±2 dB re 1 $\mu$Pa. Note that the lowest frequency tested in this study was 75 Hz, which is the lowest frequency that could be reliably calibrated using the UW-30 underwater speaker. In addition, we specifically measured the evoked saccular responses to frequencies that are included in the dominant higher harmonic components and the fundamental frequency of the advertisement call, which is produced seasonally by type I singing males to attract reproductive females for courtship and spawning. Although the midshipman fish lacks specialized structures for hearing and is primarily thought to detect acoustic particle motion, we report in this study hearing thresholds in terms of sound pressure for both technical reasons and comparison purposes with previous studies using batrachoidid fish (Sisneros, 2007, 2009a). The goal of the present study was to compare the saccular sensitivity of female *P. notatus* across reproductive states (nonreproductive vs reproductive) under identical experimental conditions. The data presented here in sound pressure levels is used to describe relative hearing sensitivity (i.e., thresholds based on saccular potential measurements) and should not be considered in terms of absolute values, but instead should be used as a means to make reliable and valid comparisons of differences in hearing sensitivity between females that differ in reproductive state. Moreover, the saccular potential recording technique used here and developed by Sisneros (2007) provide data that is comparable to other recently published studies (Rohmann and Bass, 2011; Vasconcelos et al., 2011).

Basic auditory stimuli consisted of eight repetitions of single 500 ms tones presented at a rate of one every 1.5 s. Single tones were presented at 10 Hz increments from 75 to 145 Hz and at 20 Hz increments from 165 to 385 Hz. The presentation order of single tone stimuli was randomly selected. For iso-level responses, single tone stimuli were presented at a sound pressure level of 130 dB re 1 $\mu$Pa, which is consistent with sound pressure levels for type I male midshipman advertisement calls recorded near nest sites (Bass and Clark, 2003). To measure saccular threshold responses across frequencies, single tone stimuli were presented at sound pressure levels from 91 to 154 dB re 1 $\mu$Pa in incremental steps of 3 dB.

*Saccular potential measurements.* Methods for recording saccular potentials followed those used previously to characterize the evoked potentials from the saccule in midshipman fish (Sisneros, 2007, 2009a). Fish were first anesthetized by immersion in a 0.025% benzocaine saltwater bath followed by an intramuscular injection of pancuronium bromide (~0.5 mg/kg) and 0.25% bupivacaine (~1 mg/kg) for immobilization and analgesia, respectively. The saccule was exposed by a dorsal craniotomy and then cold teleost Ringer solution (Cavanaugh, 1956) was added

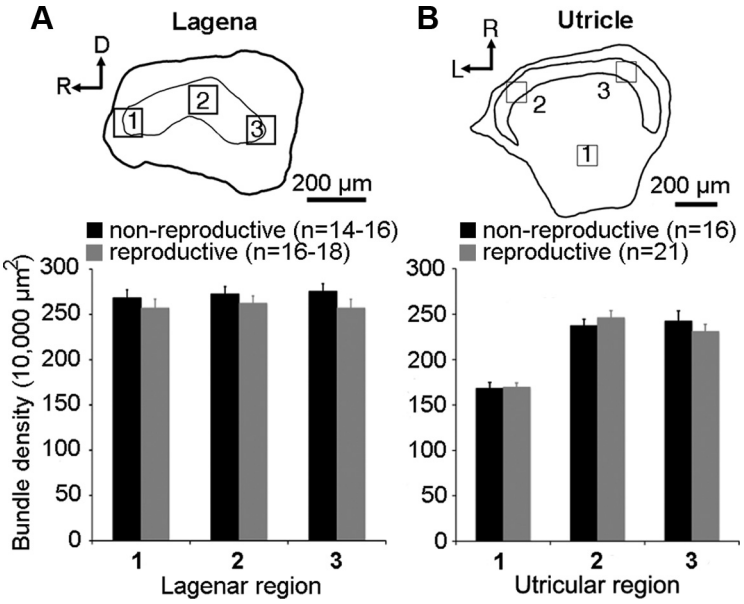


**Figure 2.** ***A***, ***B***, Hair bundle density in the lagena (***A***) and utricle (***B***) does not differ seasonally (two-way ANOVA; $F_{(1,88)} = 3.24$, $p = 0.07$ and $F_{(1,105)} = 0.004$, $p = 0.95$ for the lagena and utricle, respectively). Line drawings (top) depict the regions of each epithelium where hair bundle counts were performed. The outer line represents the edge of the epithelium; the inner line denotes the striolar region of each epithelium, which is an area with larger, morphologically distinct hair cells. The *y*-axis in ***A*** applies to both graphs. Black bars, Nonreproductive females; gray bars, reproductive females. Error bars are mean + 1 SEM. ***A***, $n = 14–16$ lagenae and $n = 16$ utricles from nonreproductive females. ***B***, $n = 16–18$ lagenae and $n = 21$ utricles from reproductive females. The variation in sample size occurred because some lagenar regions suffered dissection damage and were therefore not used for bundle quantification. D, Dorsal; R, rostral; L, lateral.

into the cranial cavity as needed to prevent drying of the saccule. A 2–3 cm dam of denture adhesive cream was constructed around the exposed cranial cavity, which allowed the fish to be lowered below the water surface in the experimental tank. The fish was positioned in the center of the tank such that the exposed saccule was below the water line at a distance of 10 cm above the underwater speaker, which was embedded in gravel on the bottom of a 30 cm diameter, 24 cm high Nalgene tank similar to Fay (1990). During the experiment, chilled seawater (15 ± 1°C) was pumped into the fish's mouth to provide a continuous stream of recirculated seawater across the gills. The experimental tank was located on a vibration isolation table housed inside an acoustic isolation chamber (Industrial Acoustics). All of the recording and auditory stimulus generation equipment was located outside of the isolation chamber.

The evoked potentials from the saccule were recorded with glass microelectrodes filled with 3 M KCl (1–7 MΩ) that were visually guided into the saccular endolymph. Recording electrodes were positioned ~2–4 mm away from the sensory macula in one of three recorded saccular regions (rostral, middle, and caudal). Saccular potentials were preamplified (×10; model 5A; Getting Instruments), band-passed filtered (75–3000 Hz with a digital filter using a gain ×10; SR650; Stanford Research Systems), inputted into a digital signal processing lock-in amplifier (SR830; Stanford Research Systems), and then stored on a PC running a custom data acquisition Matlab software program. The lock-in amplifier converts the saccular potential response [root mean square (RMS)] into a DC voltage output signal that is proportional to the component of the signal whose frequency is exactly locked to the reference frequency. The second harmonic of the stimulation frequency (i.e., 2× stimulation frequency) was set as the reference frequency since the maximum evoked potential from the teleost saccule occurs at twice the stimulus frequency due to the nonlinear response of opposite-oriented hair cell populations within the saccule (Zotterman, 1943; Cohen and Winn, 1967; Furukawa and Ishii, 1967; Hama, 1969). Noise at frequencies other than the reference frequency was rejected by the lock-in amplifier and did not affect the saccular potential measurements.

Threshold response functions were constructed by characterizing the input–output measurements of the evoked saccular potentials over a range of stimulus levels from 88 to 154 dB re 1 μPa in incremental steps of 3 dB at each tested stimulus frequency (see Acoustic stimulus generation, above). In addition, background noise measurements (RMS) were recorded before the threshold measurements and were averaged across eight measurements in the absence of an auditory stimulus. These background noise measurements were used to establish the saccular threshold and were consistently <1–2 μV. The saccular threshold at each stimulus frequency was designated as the lowest stimulus level that evoked a saccular potential that was at least 2 SDs above the background noise measurement. The frequency that evoked the lowest saccular potential threshold was defined as the best frequency (BF).

*Statistical analyses.* Differences in body size (SL and BM), reproductive state (GSI), and BF of the evoked saccular potentials between nonreproductive and reproductive females were determined using a two-tailed *t* test. The overall effects of reproductive state and stimulus frequency on the auditory thresholds of saccular hair cells were analyzed using a repeated-measures ANOVA with thresholds for each of the 17 frequencies tested (75–385 Hz) as repeats (i.e., within-subject factors) and reproductive state of the animal as the between-subject factor. The 95% confidence limits (CL) of the mean thresholds (Zar, 1999) were calculated and were also used to determine whether the mean evoked saccular thresholds differed between reproductive and nonreproductive females at each frequency (i.e., overlapping 95% CL were considered not significantly different). For all statistical analyses, $\alpha$ was set at 0.05. Statistical analyses were performed using Statistica for Windows (StatSoft).

## Results

Morphological analyses were conducted on a total of 20 nonreproductive and 28 reproductive female midshipman. For winter fish, SL = 15.38 ± 2.17 cm (mean ± SEM), BM = 50.17 ± 19.72 g, gonad mass = 3.49 ± 2.50 g, and GSI = 6.23 ± 3.67. For summer females, SL = 16.47 ± 1.18 cm, BM = 54.59 ± 11.68 g, gonad mass = 8.25 ± 5.28 g, and GSI = 16.49 ± 10.51. Summer females were slightly but significantly longer than winter fish (two-tailed *t* test, $p = 0.05$) and had greater gonad mass and GSI (two-tailed *t* test, $p < 0.001$ for both gonad mass and GSI).

### Reproductive females have more saccular hair bundles

We hypothesized that seasonal differences in auditory sensitivity were due to differences in hair cell number and properties. Hair bundle density was quantified in phalloidin-labeled tissue in seven discrete regions of the saccule from 19 nonreproductive and 29 reproductive females (Fig. 1) and three regions each from the utricle and lagena from 16 nonreproductive and 21 reproductive females (Fig. 2). These regions were selected to represent regions of differing hair cell density across each end organ (A. B. Coffin, unpublished observations). The sample size for each specific case (e.g., lagenar region 3 in reproductive females) is sometimes slightly lower than the total sample size collected because some regions of some epithelia were damaged during dissection and therefore hair bundles could not be accurately quantified in the damaged regions.

As shown in Figure 1, there were more hair bundles in the saccules of reproductive females than nonreproductive females,

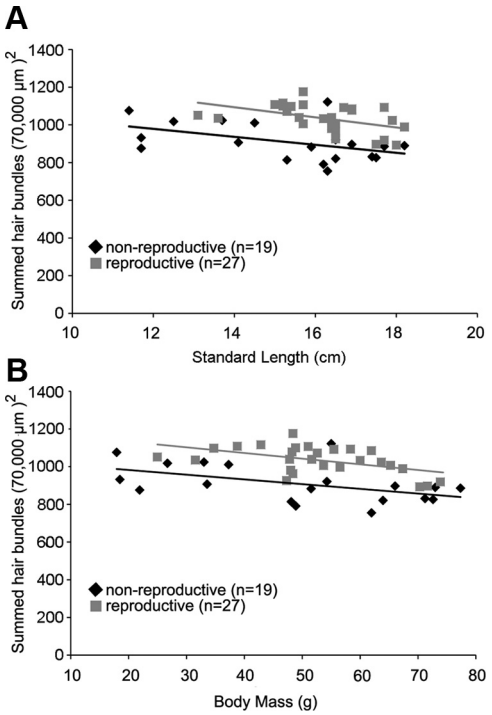


**Figure 3.** Regression of saccular hair bundle number (summed across the seven regions sampled in Fig. 1) on SL (***A***) and BM (***B***) for nonreproductive (black) and reproductive (gray) female midshipman. There is a significant negative relationship between hair bundle number and fish size for both SL (nonreproductive: $F_{(1,17)} = 4.62$, $p = 0.05$; reproductive: $F_{(1,25)} = 6.78$, $p = 0.01$) and BM (nonreproductive: $F_{(1,17)} = 5.43$, $p = 0.03$; reproductive: $F_{(1,25)} = 8.45$, $p = 0.007$). The elevations between the lines are significantly different (SL: $F_{(1,43)} = 38.25$, $p < 0.0001$; BM: $F_{(1,43)} = 35.87$, $p < 0.0001$), again demonstrating that that hair bundle density is significantly greater in saccules from reproductive females. The slopes of the regression lines are not different from one another (SL: $F_{(1,42)} = 0.15$, $p = 0.70$; BM: $F_{(1,42)} = 0.11$, $p = 0.74$).

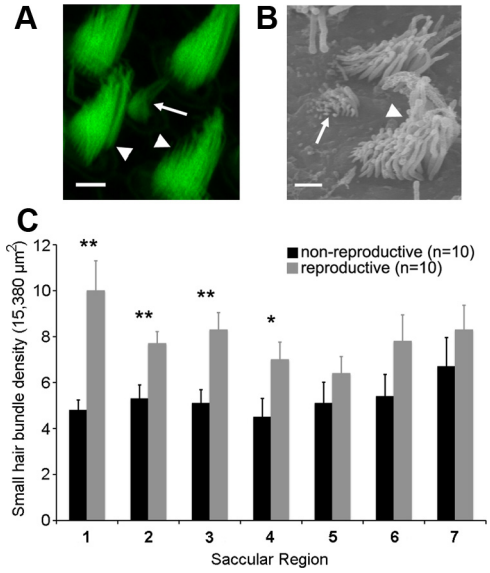


**Figure 4.** Seasonal differences in the number of small hair bundles in the female midshipman saccule. ***A***, ***B***, Confocal brightest-point projection (***A***) and scanning electron micrograph (***B***) showing a small, immature-like hair bundle (arrows) surrounded by larger, mature hair bundles (arrowheads). Scale bars: ***A***, 2 $\mu$m; ***B***, 1 $\mu$m. ***C***, Small hair bundles were counted in seven 15,380 $\mu m^2$ regions of 10 saccules from each reproductive group. Black bars, Winter females; gray bars, summer females. There were significantly more small hair bundles in the summer saccules (two-way ANOVA, $F_{(1,126)} = 31.62$, $p < 0.0001$) but there were not significant differences across saccular region (two-way ANOVA, $F_{(6,126)} = 1.24$, $p = 0.29$). *A prior t* tests for paired comparisons were used to determine differences in the number of small hair bundles between the same saccular regions in nonreproductive and reproductive fish, demonstrating significant pairwise differences in the rostral half of the saccule (regions 1–4, $^*p < 0.05$, $^{**}p < 0.01$). Error bars are mean + 1 SEM.

with an average of 13% more hair bundles in sampled regions of saccules from reproductive females. These differences manifested as a significant main effect of reproductive state in the two-way ANOVA ($p < 0.0001$). Differences were seen in all regions except the rostral-most tip and what we term here as the rostral "thumb," which is a small rostrodorsal extension with high hair bundle density in females from either reproductive condition. The greatest hair bundle increase was seen in the midrostral region, where saccules from reproductive females had 23% more hair bundles. There was also a significant main effect of saccular region ($p < 0.0001$), demonstrating that hair bundle density was not constant across saccular regions. In contrast, data presented in Figure 2 show that hair bundle density in the lagena and utricle did not differ seasonally ($p = 0.07$ and $p = 0.95$, respectively). These data suggest that there is seasonal, saccular-specific hair cell addition in female midshipman.

Given that summer females used in this study were slightly larger than winter females, we next asked whether increased hair bundle density was correlated with increased fish size (Fig. 3). This analysis used all saccular epithelia where hair bundle counts were obtained in each of the seven sample regions ($n = 19$ nonreproductive, $n = 27$ reproductive fish). For both winter and summer populations, we found a significant negative relationship between hair bundle density (represented as the sum of the seven saccular regions analyzed in Fig. 1) and SL or BM ($p \leq 0.05$). This result suggests that larger fish have lower bundle density overall, although the total number of hair cells may be the same since the saccule grows in proportion to the fish. However, the slopes of the regression lines were not different from one another, showing that the relationship between fish size and hair bundle density is constant across reproductive state. These data show that increased hair bundle density is not correlated with increased fish size and that the increase in hair bundle density seen in summer females is consistent across the range of fish sizes sampled in the present study.

In addition to an overall increase in hair cells in the saccule of summer females, we also detected a significant increase in the number of small hair bundles ($p < 0.0001$; Fig. 4). Small hair bundles were quantified in the seven saccular sampling regions from 10 nonreproductive and 10 reproductive females. While the number of small bundles was very low in all saccular regions, there were between 25 and 100% more small hair bundles (mean = 52%) in saccules from reproductive females compared with nonreproductive females. These bundles, which may represent either immature hair cells or a physiologically distinct subtype of mature hair cells, were not counted in the overall bundle counts, as they were too small to discern clearly in the 40× images used for initial bundle density quantification. If these small bundles represent developing hair cells, it suggests that more hair cells are added to the saccules of summer females, consistent with our finding of increased hair bundle density in this end organ. In addition, there was no significant main effect of saccular region ($p = 0.29$), suggesting that new hair cells may be added in all saccular regions.

### Decreased cell death in saccules from reproductive females

We next examined cell death and proliferation in all three epithelia to determine the source of additional hair cells identified in

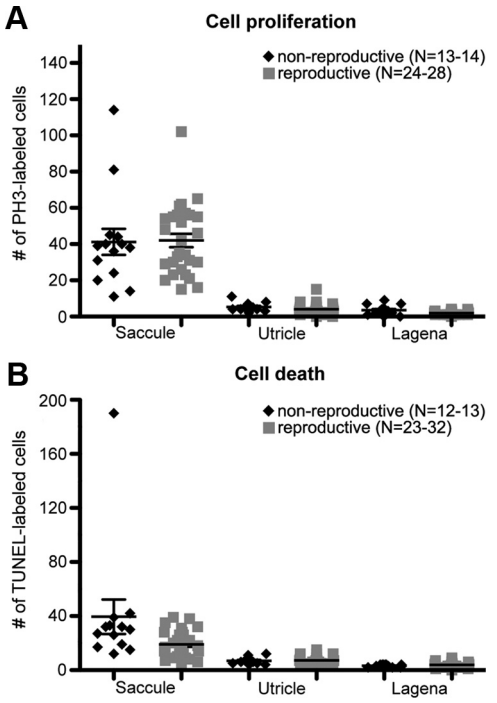


**Figure 5.** ***A***, ***B***, Cell proliferation (***A***) and cell death (***B***) in inner ear epithelia. There were no significant differences in cell proliferation (two-tailed *t* test, $p \geq 0.06$) or cell death ($p \geq 0.14$) in any end organ. Scatter plots were used so that the contribution of saccular outliers could be visually assessed. Cell proliferation sample sizes: nonreproductive saccule, $n = 14$; lagena, $n = 14$; utricle, $n = 13$; reproductive: saccule, $n = 28$; lagena, $n = 24$; utricle, $n = 27$. Cell death sample sizes: nonreproductive: saccule, $n = 13$; lagena, $n = 12$; utricle, $n = 12$; reproductive: saccule, $n = 32$; lagena, $n = 23$; utricle, $n = 23$. Data are presented as mean ± 1 SEM. Black, Nonreproductive females; gray, reproductive females.

saccules from summer females. Figure 5 shows quantification of cell proliferation and cell death in all three epithelia, as determined by counts of PH3- and TUNEL-labeled cells, respectively. Cells were counted for each complete epithelium in 12–14 epithelia from nonreproductive females and 23–32 epithelia from reproductive females. Exact samples sizes for each epithelium and label type are shown in Figure 5.

Pairwise *t* tests were used to analyze cell counts here, as the comparisons of interest were within epithelium rather than across end organs. When the entire dataset was analyzed, there were no significant seasonal differences in cell proliferation ($p \geq 0.06$) or cell death ($p \geq 0.14$) in any end organ. However, there was one nonreproductive animal with unusually high numbers of both proliferating and dying cells (six times more dying cells than seen in other animals), as shown in the scatter plots in Figure 5. The high level of cell death and proliferation suggests that the saccule in this animal may have been damaged before dissection and that recent trauma may have triggered a regeneration response. When this animal is removed from the dataset, the seasonal difference in cell death becomes statistically significant ($p = 0.02$). These results suggest that there may be seasonal differences in cell death in the saccule of female midshipman.

**Seasonal differences in hair bundle height but not orientation**

Having established that there is a saccular-specific increase in hair cells in summer females, we then asked which characteristics of these additional hair cells might contribute to the increase in auditory sensitivity seen in summer females. For the present study, we focused on hair bundle orientation and bundle length differences across the saccule, two morphological features with potential physiological consequences (Popper and Coombs, 1982; Platt and Popper, 1984; Sugihara and Furukawa, 1989; Popper and Fay, 1999). There is considerable variation across species in the number and directionality of bundle orientation groups in fish saccules and different orientation group patterns have been observed in the saccules of fish with differing auditory sensitivity (Popper, 1977, 1981; for review, see Popper and Fay, 1993; Popper and Lu, 2000). Lesser variation has been noted in the other end organs, although species-specific patterns do exist, particularly in species with specialized utricles (for review, see Popper and Coombs, 1982; Popper and Fay, 1993). The functional consequences of these orientation pattern differences remain unknown.

As shown in Figure 6, we mapped hair bundle orientation patterns for all three end organs in both summer and winter female midshipman to determine the overall orientation pattern and to identify possible seasonal variation ($n \geq 4$ fish from each season). There are four main orientation groups in the saccule, with rostral bundles oriented either rostrally or caudally and caudal bundles oriented laterally and in opposition to one another. There is some variability in this four-quadrant pattern, particularly in the caudal-most end of the saccule. This pattern closely resembled the standard four-quadrant pattern noted in many fish species (Popper and Coombs, 1982). Bundles in the lagena and utricle are also oriented similarly to other fish species. These bundles group into two main orientation classes for each end organ, with groups of opposite polarity meeting in the striolar region of each epithelium, which is a region with larger, morphologically distinct hair cells that have differing immunoreactivity and innervation profiles when compared with surrounding (extrastriolar) hair cells (Flock, 1964, Saidel et al., 1990a, 1990b; Chang et al., 1992). We did not observe any seasonal difference in orientation patterns for any end organ.

We quantified hair bundle length for 14 regions of the saccule in five winter and five summer females (Fig. 7*A*). We selected regions that encompassed both the rostral–caudal and dorsal–ventral axes of the saccule, as preliminary observations suggested that bundles in the center of the saccule were shorter than those on the perimeter. As seen in Figure 7, saccules from reproductive females have significantly shorter hair bundles than those in nonreproductive females ($p = 0.01$). These differences, however, are small, with the greatest difference of 3.25 $\mu$m seen in the ventral portion of the caudal region (Fig. 7, region 6V). Confirming our previous qualitative observations, we also noted significant variation in bundle height across saccular region, with shorter bundles located more rostrally.

**Seasonal reproductive state-dependent changes in auditory saccular sensitivity**

Auditory evoked saccular potentials were recorded from a total of 24 adult female fish: 12 winter nonreproductive females with a size range of 11.7–17.1 cm SL (mean SL = 14.0 ± 2.0 cm, mean BM = 35.5 ± 15.3 g, mean GSI = 2.7 ± 2.6) and 12 summer reproductive females with a size range of 12.3–17.0 cm SL (mean SL = 15.4 ± 1.5 cm, mean BM = 47.0 ± 11.8 g, mean GSI = 14.6 ± 9.9). Although there was no difference in SL between nonreproductive and reproductive females (*t* test, $t = 1.95$, df = 22, $p = 0.06$), there was a trend in differences of BM (*t* test, $t = 2.07$, df = 22, $p = 0.05$) and a significant difference in GSI (*t* test, $t = 4.05$, df = 22, $p < 0.01$), as expected, with reproductive females being heavier and gravid (full of ripe eggs, high GSI). In general, the recorded evoked saccular potentials were greatest at

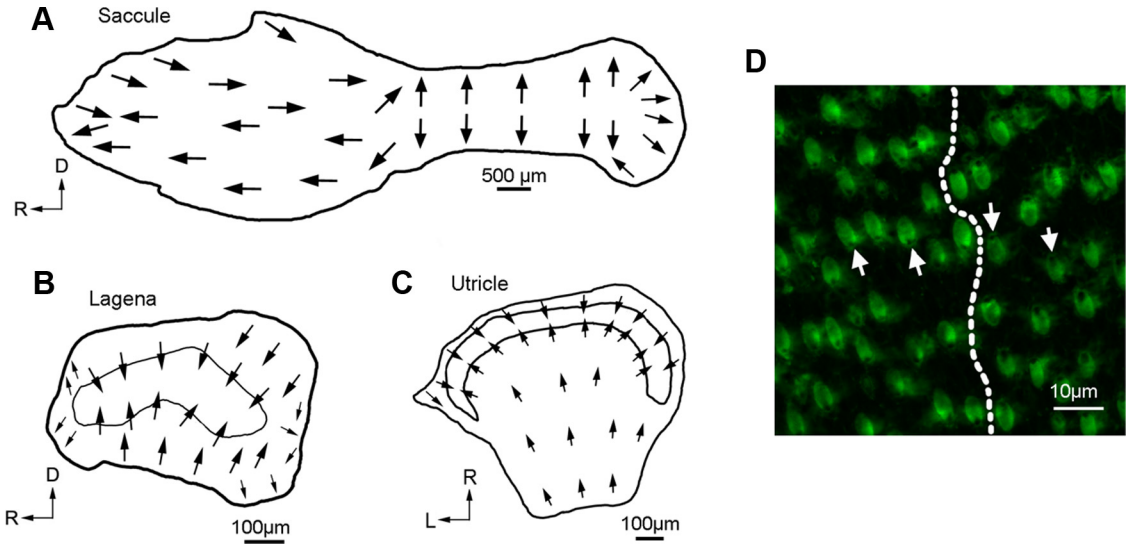


**Figure 6.** ***A–C***, Hair bundle orientation maps for female midshipman saccule (***A***), lagena (***B***), and utricle (***C***). Arrows indicate the polarizing direction of hair bundles in a given region of each epithelium. Thin black inner lines denote the striolar region of the lagena (***B***) and utricle (***C***). Maps are representative of both winter ($n = 4$ fish) and summer ($n = 7$ fish) epithelia (saccules: $n = 2$ summer fish for utricles and lagenae). D, Dorsal; R, rostral; L, lateral. ***D***, Single plane confocal image from the midrostral region of the saccule, taken at the level of the cuticular plates (labeled with phalloidin). Arrows indicate kinocilia insertions into the cuticular plates of a few example hair cells. The white dotted line divides bundles into two distinct orientation groups, one oriented rostrally and the other caudally, as shown in ***A***.

75 Hz with a rapid decline in magnitude at frequencies >85 Hz at most stimulus levels. In addition, the evoked saccular potentials were greater in reproductive females than in nonreproductive females. For example, at a stimulus level of 130 dB re 1 $\mu$Pa, the mean saccular potential evoked at 75 Hz from reproductive females (mean = 97 $\mu$V) was ~4.5× greater than that evoked from nonreproductive female (mean = 21 $\mu$V) (Fig. 8).

Auditory thresholds were determined for whole populations of hair cells in the saccule of nonreproductive and reproductive female fish. Threshold tuning curves were constructed by characterizing the input–output measurements of the evoked saccular potentials generated from the presentation of single tone stimuli at frequencies from 75 (the lowest frequency tested) to 385 Hz over a range of stimulus levels. Figure 9 shows representative input-out measurements of evoked saccular potentials for both nonreproductive and reproductive females. The threshold tuning curves for the saccular potentials generally consisted of profiles with lowest thresholds at 75 and 85 Hz that gradually increased to highest thresholds at frequencies ≥305 Hz. BFs ranged from 75 to 105 Hz for nonreproductive females and 75 to 85 Hz for reproductive females, with the majority of BFs occurring at 75 and 85 Hz for both nonreproductive and reproductive females. Mean BF did not differ between nonreproductive and reproductive females (*t* test, $t = 0.59$, $p = 0.56$).

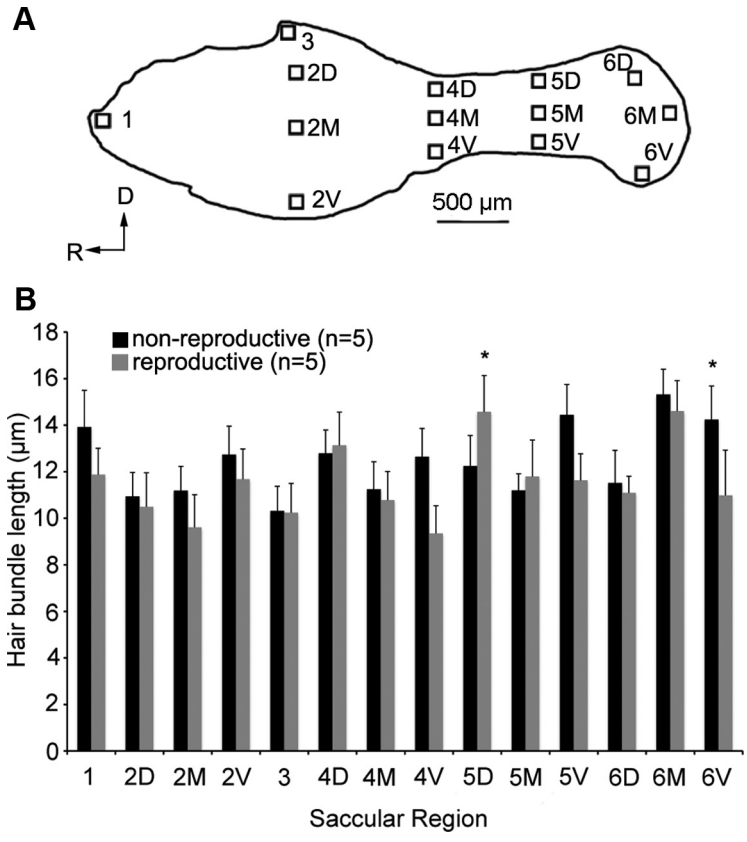


**Figure 7.** Hair bundle height in female saccules differs seasonally. Hair bundle length, defined here as the distance between the tallest stereocilium and the cuticular plate, was measured in five saccules from each reproductive condition, with four to nine bundles measured for each region of each saccule. Images were collected at the 14 regions shown in ***A*** (D, Dorsal; M, middle; V, ventral) for most rostral–caudal regions. ***B***, There was a significant effect of reproductive state ($F_{(1,105)} = 6.9$, $p = 0.01$) and saccular region ($F_{(13,105)} = 4.35$, $p < 0.0001$), as determined by two-way ANOVA. *A prior t* tests for planned comparisons show individual regional differences in the saccule (*$p < 0.05$). Error bars are mean + 1 SEM.

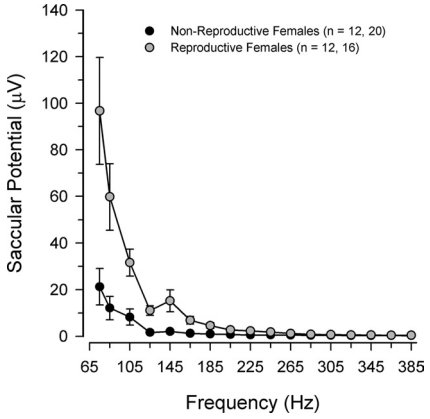


**Figure 8.** Representative examples of response curves of the evoked saccular potentials recorded from nonreproductive and reproductive female midshipman in response to single tones at 130 dB (re 1 $\mu$Pa). Error bars are mean + 1 SEM.

Although there was no reproductive state-dependent difference in BF, there was a highly marked threshold difference between nonreproductive and reproductive female midshipman. The auditory thresholds of saccular hair cells from reproductive females were ~8–15 dB lower than nonreproductive females at frequencies from 75 to 385 Hz (Fig. 10). A two-way ANOVA confirmed this interpretation with highly significant main effects for reproductive state and frequency ($F_{(1,569)} = 302.04$, $p < 0.001$ and $F_{(16,569)} = 55.25$, $p < 0.001$, respectively).

## Discussion

The goal of this study was to determine whether seasonal changes in auditory sensitivity are concurrent with seasonal increases in hair cell receptors in the saccule of the vocal plainfin midshipman fish. We show that saccules in reproductive females have significantly greater hair bundle density, suggesting that saccular-specific hair cell addition occurs in reproductive females. Quantification of dying and proliferating cells suggests that there is a modest level of new hair cell addition in saccules from both reproductive and nonreproductive females but that the level of cell death decreases during the reproductive season, leading to a net increase in overall hair cell numbers in reproductive fish. This increase in hair cell density is not simply a size-dependent effect due to overall increased somatic growth since similar changes in hair cell density were not detected in the other end organs. The seasonal increase in saccular hair cell density in reproductive females was paralleled by an increase in the evoked magnitude of saccular potentials and in the corresponding decrease in auditory thresholds of saccular hair cells in reproductive females compared with that of nonreproductive females. To our knowledge, this study is the first to demonstrate concurrent seasonal plasticity of receptor density and auditory sensitivity in the inner ear from a natural population of vertebrates.

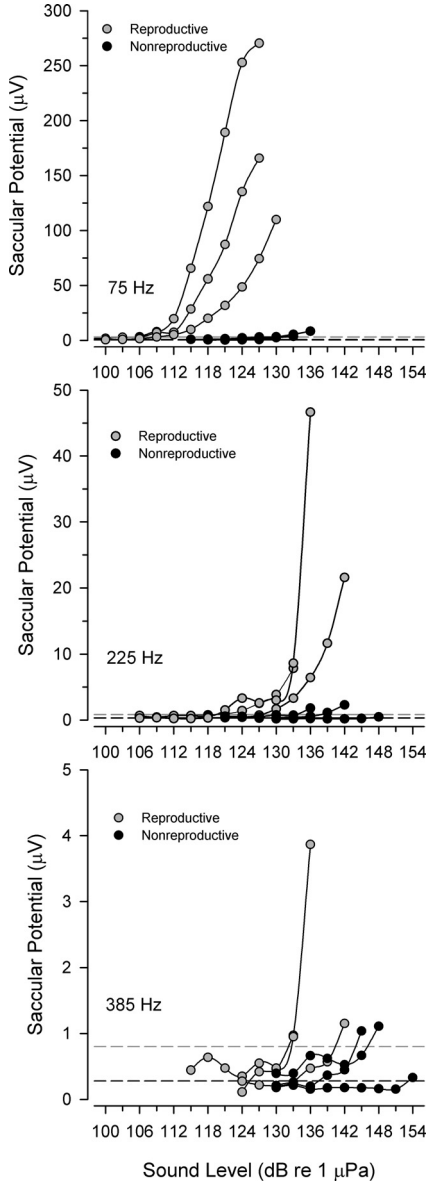


**Figure 9.** Representative input–output measurements of evoked saccular potentials at different stimulus levels recorded from three reproductive and three nonreproductive female midshipman fish. The input-out response curves are plotted at three different frequencies: 75 (top), 225 (middle), and 385 (bottom) Hz. Auditory threshold at each stimulus frequency was determined as the lowest stimulus intensity in decibels (re 1 $\mu$Pa) that evoked a saccular potential that was at least 2 SD above the background noise measurement. The upper and lower ranges of auditory thresholds for the six recordings in each plot are noted by the horizontal dashed lines. Note that the scales of the *y*-axes for the input–output response plots are different and emphasize the reduction in the magnitude of evoked saccular potentials at higher stimulus frequencies.

### Seasonal plasticity of saccular hair cell receptors and auditory sensitivity

We show that there is a seasonal increase in hair cell density across the entire saccule (rostral, middle, and caudal regions) in reproductive females (Fig. 1). In contrast, we did not find evidence for increased hair cell density in the other two end organs, the lagena and utricle (Fig. 2). As reproductive females were slightly larger than their nonreproductive counterparts, one might expect that the saccular-specific increase in hair cell density would be related to fish size, since fish add significant numbers of postembryonic hair cells throughout much of their lifetime (Corwin, 1981; Lombarte and Popper, 1994). However, our study shows that hair cell density is negatively correlated with fish size (Fig. 3), which suggests that if seasonal variation in hair bundle density was a size-dependent phenomenon we would expect a lower saccular bundle density in our slightly larger reproductive females. Based on our current results, we conclude that the increased saccular hair bundle density in summer females is linked to reproductive state rather than size.

In addition to the seasonal increase in saccular hair bundle density, we also observed an increase in the number of small saccular hair bundles in reproductive females than in nonrepro-

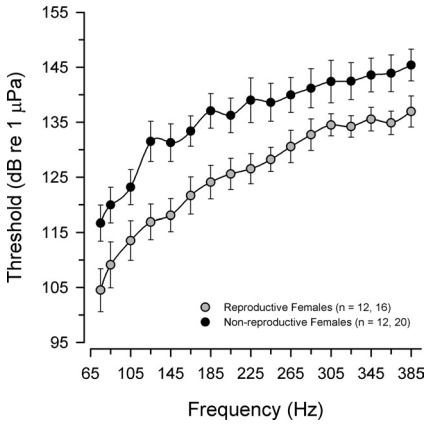


**Figure 10.** Auditory threshold tuning curves for nonreproductive and reproductive female fish based on the evoked potentials recorded from the midshipman saccule. All data are plotted as mean ± 95% CL and the number of animals and recordings are indicated in parentheses. Auditory threshold at each stimulus frequency was determined as the lowest stimulus intensity in decibels (re 1 $\mu$Pa) that evoked a saccular potential that was at least 2 SD above the background noise measurement.

ductive females (Fig. 4). We hypothesize that these small bundles could either represent immature hair cells or a morphologically distinct subtype of mature hair bundle. Given that we see a seasonal increase in hair bundle density, it would be logical to expect more developing saccular hair cells in reproductive females than in nonreproductive females. However, we did not observe any seasonal differences in saccular cell proliferation. One possible explanation is that the smaller hair bundles in reproductive females represent immature hair cells that resulted from increased proliferation that occurred earlier during the spring pre-nesting season (Sisneros et al., 2004b). The timing of hair bundle maturation in fishes is unknown, but studies in developing chick and rodent inner ears suggest that morphological maturation of hair bundles can take 10 d or more after hair bundles are first visible (Goodyear et al., 2006). This timing is consistent with our hypothesis of increased hair cell proliferation before nesting. Future studies examining hair cell proliferation during other time periods of the female reproductive cycle, especially during the spring pre-nesting phase, are needed to determine the developmental state of small hair bundles observed in reproductive females.

We show that there is a length difference in the typical, mature-appearing hair bundles observed throughout the saccule, with saccules from reproductive females having significantly shorter hair bundles than those from nonreproductive animals (Fig. 6). A structure–function link between hair bundle length and frequency selectivity of hair cells has been previously reported in other vertebrates (Holton and Hudspeth, 1983; Simmons et al., 1994). In the goldfish saccule, hair cells with longer bundles are located in the caudal region and are more responsive to low frequencies, while rostral cells have shorter bundles and respond better to higher frequencies (<500 Hz) (Furukawa and Ishii, 1967; Fay, 1978; Platt and Popper, 1984). Similar mechanisms related to hair bundle length and frequency tuning may also contribute to the increased saccular sensitivity seen in reproductive female midshipman.

Concurrent with seasonal increases in hair bundle density and differences in hair bundle length, we also observed a dramatic increase in the magnitude of the evoked potentials from the saccule of reproductive females. The response profiles for saccular potentials evoked at 130 dB re 1 $\mu$Pa indicate that evoked magnitudes were ~4.5 times greater at 75 Hz for saccular hair cells from reproductive females compared with nonreproductive females (Fig. 8). In addition, the auditory thresholds of saccular hair cells from reproductive females were ~8–15 dB lower than nonreproductive females at frequencies from 75 to 385 Hz (Fig. 10). Such seasonal differences in the magnitude and threshold of the evoked saccular potentials are consistent with increases in the relative number of saccular hair cells. In addition to differences in hair cell density, seasonal differences in saccular physiology may be due to reproductive state-dependent effects on central input to the hindbrain efferent nucleus and the efferent neurons that innervate the inner ear of the midshipman (Bass et al., 1994). In the closely related oyster toadfish (*Opsanus tau*), saccular efferents provide inhibitory inputs to the hair cells and activation of these efferent neurons generally reduces the sensitivity (gain) of the receptor potentials during stimulation (Holstein et al., 2004; Boyle et al., 2009). In addition, work by Xiao and Suga (2002) demonstrates that neurons in the mammalian auditory cortex of the mustache bat (*Pteronotus parnellii*) can also modulate the frequency sensitivity of cochlear hair cells in the auditory periphery. Therefore, future studies investigating potential reproductive state-dependent changes of these inhibitory efferent inputs to saccular hair cells may reveal novel central processing mechanisms that contribute to seasonal plasticity of saccular sensitivity in female midshipman.

### Functional significance of reproductive-dependent changes in hair cell number and sensitivity

The female reproductive state-dependent auditory plasticity reported in this study is consistent with earlier findings of seasonal changes in saccular thresholds (Sisneros, 2009a). The threshold shift in reproductive females occurs across a broad range of frequencies that include the dominant higher harmonic components and the fundamental frequency of the males' seasonal advertisement call (Sisneros et al., 2004a; Sisneros, 2009b). In the present study, we show that the auditory thresholds of saccular hair cells from reproductive females were at least 9 dB lower (a sensitivity increase equal to ~3 times greater) than nonreproductive females at frequencies that corresponded to the fundamental frequency (~100 Hz) and the dominant second harmonic (~200 Hz) component of the male advertisement call. This seasonal shift in auditory saccular sensitivity may be adaptive for reproductive females to enhance mate detection and localization in the shallow water environments where midshipman fish court and spawn. The dominant harmonics of the male's advertisement call (≤400 Hz) are hypothesized to be important for the detection and localization of the calling male because the higher harmonics will propagate further than the fundamental frequency due to the inverse relationship between water depth and the cutoff frequency of sound transmission (Fine and Lenhardt, 1983; Roger and Cox, 1988; Bass and Clark, 2003). Thus, the concurrent seasonal changes in saccular hair cell density and auditory threshold may function to increase the probability of conspecific mate recognition, detection, and localization during the summer breeding season.

As demonstrated for the auditory saccular afferents, the mechanism responsible for the observed changes in auditory saccular sensitivity is most likely due to the seasonal fluctuation in circulating steroid hormones (Sisneros et al., 2004b). Nonreproductive females treated with either testosterone or 17$\beta$-estradiol exhibit a dramatic increase in the inner ear's frequency sensitivity that mimics the reproductive female's auditory phenotype and leads to an increased detection of the male's advertisement call (Sisneros et al., 2004a). Estrogen ($E_2$) may also potentially induce

morphological changes in saccular hair cell density and bundle morphology in reproductive females. Midshipman-specific estrogen receptor (ER) $\alpha$ is expressed in the saccular epithelium of reproductive females (Sisneros et al., 2004a; Forlano et al., 2005), consistent with a possible role in cell protection and proliferation in this end organ. ER signaling in the saccular epithelium could influence hair cell survival, a hypothesis supported by the present finding of decreased cell death in the summer saccule compared with the winter condition and by previous studies showing protective effects of estrogens in the CNS (for review, see McCullough and Hurn, 2003; Arnold and Beyer, 2009). Similarly, $E_2$ is known to play a role in neurogenesis (for review, see Barha and Galea, 2010; Le Page et al., 2010) and saccular supporting cells could potentially respond to proliferative estrogenic signaling. In addition, steroid hormones such testosterone and $E_2$ may also affect the ion channel current kinetics of auditory hair cells and influence hair cell tuning by upregulating the differential expression and alternate splicing of calcium-activated potassium (BK) channels, as proposed for similar steroid-related changes in the tuning of electroreceptors (Keller et al., 1986; Art and Fettiplace, 1987; Meyer et al., 1987; Zakon, 1987; Ramanathan et al., 1999, 2000; Miranda-Rottmann et al., 2010). At least one BK channel subunit is expressed in the midshipman saccule (Rohmann et al., 2009). Future studies that examine the potential effects of estrogen on hair cell death and proliferation and in the regulation of BK channel expression in saccular hair cells will be instrumental in understanding the cellular basis of how peripheral auditory sensitivity is modulated in the midshipman fish.

## References


Arnold S, Beyer C (2009) Neuroprotection by estrogen in the brain: the mitochondrial compartment as presumed therapeutic target. J Neurochem 110:1–11.

Art JJ, Fettiplace R (1987) Variation of membrane properties in hair cells isolated from the turtle cochlea. J Physiol 385:207–242.

Ball GF, Riters LV, Balthazart J (2002) Neuroendocrinology of song behavior and avian brain plasticity: multiple sites of action of sex steroids. Front Neuroendocrinol 23:137–178.

Barha CK, Galea LA (2010) Influence of different estrogens on neuroplasticity and cognition in the hippocampus. Biochim Biophys Acta 1800:1056–1067.

Bass AH (1996) Shaping brain sexuality. Am Sci 84:352–363.

Bass AH, Clark CW (2003) The physical acoustics of underwater sound communication. In: Springer Handbook of Auditory Research (Simmons AM, Fay RR, Popper A, eds), pp. 1–64. New York: Springer.

Bass AH, Marchaterre MA, Baker R (1994) Vocal-acoustic pathways in a teleost fish. J Neurosci 14:4025–4039.

Bass AH, Bodnar D, Marchaterre MA (1999) Complementary explanations for existing phenotypes in an acoustic communication system. In: The design of animal communication (Hauser MD, Konishi M, eds.), pp 493–514. Cambridge, MA: MIT.

Boyle R, Rabbitt RD, Highstein SM (2009) Efferent control of hair cell and afferent responses in the semicircular canals. J Neurophysiol 102:1513–1525.

Brenowitz EA (2004) Plasticity of the adult avian song control system. Ann N Y Acad Sci 1016:560–585.

Caras ML, Brenowitz E, Rubel EW (2010) Peripheral auditory processing changes seasonally in Gambel's white-crowned sparrow. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 196:581–599.

Cavanaugh GM (1956) Formulae and methods VI of the Marine Biological Laboratory. Woods Hole, MA: The Marine Biological Laboratory.

Chang JS, Popper AN, Saidel WM (1992) Heterogeneity of sensory hair cells in a fish ear. J Comp Neurol 324:621–640.

Cohen MJ, Winn HE (1967) Electrophysiological observations on hearing and sound production in the fish, *Porichthys notatus.* J Exp Zool 165:355–369.

Corwin JT (1981) Postembryonic production and aging in inner ear hair cells in sharks. J Comp Neurol 201:541–553.

Corwin JT (1983) Postembryonic growth of the macula neglecta auditory detector in the ray, *Raja clavata*: continual increases in hair cell number, neural convergence and physiological sensitivity. J Comp Neurol 217:345–356.

Fay RR (1978) Coding of information in single auditory-nerve fibers of the goldfish. J Acoust Soc Am 63:136–146.

Fay RR (1990) Suppression and excitation in auditory nerve fibers of the goldfish, *Carassius auratus.* Hear Res 48:93–109.

Fine ML, Lenhardt ML (1983) Shallow-water propagation of the toadfish mating call. Comp Biochem Physiol A 76:225–231.

Flock A (1964) Structure of the macula utriculi with special reference to directional interplay of sensory responses as revealed by morphological polarization. J Cell Biol 22:413–431.

Forlano PM, Deitcher DL, Bass AH (2005) Distribution of estrogen receptor alpha mRNA in the brain and inner ear of a vocal fish with comparisons to sites of aromatase expression. J Comp Neurol 483:91–113.

Furukawa T, Ishii Y (1967) Neurophysiological studies on hearing in goldfish. J Neurophysiol 30:1377–1403.

Goense JB, Feng AS (2005) Seasonal changes in frequency tuning and temporal processing in single neurons in the frog auditory midbrain. J Neurobiol 65:22–36.

Goodyear RJ, Kros CJ, Richardson GP (2006) The development of hair cells in the inner ear. In: Vertebrate hair cells (Eatock RA, Fay RR, Popper AN, eds), pp 20–94. New York: Springer.

Hama K (1969) A study on the fine structure of the saccular macula of the goldfish. Z Zellforsch Mikrosk Anat 94:155–171.

Henry KS, Lucas JR (2009) Vocally correlated seasonal auditory variation in the house sparrow (*Passer domesticus*). J Exp Biol 212:3817–3822.

Higgs DM, Souza MJ, Wilkins HR, Presson JC, Popper AN (2002) Age- and size-related changes in the inner ear and hearing ability of the adult zebrafish (*Danio rerio*). J Assoc Res Otolaryngol 3:174–184.

Holstein GR, Rabbitt RD, Martinelli GP, Friedrich VL Jr, Boyle RD, Highstein SM (2004) Convergence of excitatory and inhibitory hair cell transmitters shapes vestibular afferent responses. Proc Natl Acad Sci U S A 101:15766–15771.

Holton T, Hudspeth AJ (1983) A micromechanical contribution to cochlear tuning and tonotopic organization. Science 222:508–510.

Keller CH, Zakon HH, Sanchez DY (1986) Evidence for a direct effect of androgens upon electroreceptor tuning. J Comp Physiol A 158:301–310.

Lanford PJ, Presson JC, Popper AN (1996) Cell proliferation and hair cell addition in the ear of the goldfish, *Carassuis auratus.* Hear Res 100:1–9.

Le Page Y, Diotel N, Vaillant C, Pellegrini E, Anglade I, Mérot Y, Kah O (2010) Aromatase, brain sexualization and plasticity: the fish paradigm. Eur J Neurosci 32:2105–2115.

Lombarte A, Popper AN (1994) Quantitative analyses of postembryonic hair cell addition in the otothic endorgans of the inner ear of the European hake, *Merluccius merluccius* (Gadiformes, Teleostei). J Comp Neurol 345:419–428.

Lu Z, Popper AN (1998) Morphological polarizations of sensory hair cells in the three otolithic organs of a teleost fish: fluorescent imaging of ciliary bundles. Hear Res 126:47–57.

Lucas JR, Freeberg TM, Krishnan A, Long GR (2002) A comparative study of avian auditory brainstem responses: correlations with phylogeny and vocal complexity, and seasonal effects. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 188:981–992.

Lucas JR, Freeberg TM, Long GR, Krishnan A (2007) Seasonal variation in avian auditory evoked responses to tones: a comparative analysis of Carolina chickadees, tufted titmice, and white-breasted nuthatches. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 193:201–215.

McCullough LD, Hurn PD (2003) Estrogen and ischemic neuroprotection: an integrated view. Trends Endocrinol Metab 14:228–235.

McKibben JR, Bass AH (2001) Peripheral encoding of behaviorally relevant acoustic signals in a vocal fish: harmonic and beat stimuli. J Comp Physiol A 187:271–285.

Meitzen J, Weaver AL, Brenowitz EA, Perkel DJ (2009) Plastic and stable electrophysiological properties of adult avian forebrain song-control neurons across changing breeding conditions. J Neurosci 29:6558–6567.

Meyer JH, Leong M, Keller CH (1987) Hormone-induced and maturational changes in electric organ discharges and electroreceptor tuning in the weakly electric fish Apteronotus. J Comp Physiol A 160:385–394.

Miranda-Rottmann S, Kozlov AS, Hudspeth AJ (2010) Highly specific alternative splicing of transcripts encoding BK channels in the chicken's co-

chlea is a minor determinant of the tonotopic gradient. Mol Cell Biol 30:3646–3660.
Oxman DS, Barnett-Johnson R, Smith ME, Coffin AB, Miller DD, Josephson R, Popper AN (2007) The effect of vaterite deposition on otolith morphology, sound reception and inner ear sensory epithelia in hatchery-reared Chinook salmon (*Oncorhynchus tshawytscha*). Can J Fish Aquat Sci 64:1469–1478.
Park KH, Meitzen J, Moore IT, Brenowitz EA, Perkel DJ (2005) Seasonal-like plasticity of spontaneous firing rate in a songbird pre-motor nucleus. J Neurobiol 64:181–191.
Platt C, Popper AN (1984) Variation in lengths of ciliary bundles on hair cells along the macula of the sacculus in two species of teleost fishes. Scan Electron Microsc 1984:1915–1924.
Popper AN (1977) A scanning electron microscopic study of the sacculus and lagena in the ears of fifteen species of teleost fishes. J Morph 153:397–418.
Popper AN (1981) Comparative scanning electron microscopic investigations of the sensory epithelia in the teleost sacculus and lagena. J Comp Neurol 200:357–374.
Popper AN, Coombs S (1982) The morphology and evolution of the ear in Actinopterygian fishes. Am Zool 22:311–328.
Popper AN, Fay RR (1993) Sound detection and processing by fish: critical review and major research questions. Brain Behav Evol 41:14–38.
Popper AN, Fay RR (1999) The auditory periphery in fishes. In: Comparative hearing: fish and amphibians (Fay RR, Popper AN, eds.) pp 43–100. New York: Springer.
Popper AN, Hoxter B (1984) Growth of a fish ear. 1. Quantitative analysis of sensory hair cell and ganglion cell proliferation. Hear Res 15:133–142.
Popper AN, Lu Z (2000) Structure–function relationships in fish otolith organs. Fisheries Res 46:15–25.
Ramanathan K, Michael TH, Jiang GJ, Hiel H, Fuchs PA (1999) A molecular mechanism for electrical tuning of cochlear hair cells. Science 283:215–217.
Ramanathan K, Michael TH, Fuchs PA (2000) Beta subunits modulate alternatively spliced, large conductance, calcium-activated potassium channels of avian hair cells. J Neurosci 20:1675–1684.
Roger PH, Cox M (1988) Underwater sound as a biological stimulus. In: Sensory biology of aquatic animals (Atema J, Fay RR, Popper AN, Tavolga WN, eds.), pp 130–149. New York: Springer.
Rohmann KN, Bass AH (2011) Seasonal plasticity of auditory hair cell frequency sensitivity correlates with plasma steroid levels in vocal fish. J Exp Biol 214:1931–1942.
Rohmann KN, Deitcher DL, Bass AH (2009) Calcium-activated potassium (BK) channels are encoded by duplicate slo1 genes in teleost fishes. Mol Biol Evol 26:1509–1521.
Saidel WM, Popper AN, Chang JS (1990a) Spatial and morphological differentiation of trigger zones in afferent fibers to the teleost utricle. J Comp Neurol 302:629–642.
Saidel WM, Presson JC, Chang JS (1990b) S-100 immunoreactivity identifies a subset of hair cells in the utricle and saccule of a fish. Hear Res 47:139–146.
Schuck JB, Smith ME (2009) Cell proliferation follows acoustically-induced hair cell bundle loss in the zebrafish saccule. Hear Res 253:67–76.
Simmons DD, Bertolotto C, Narins PM (1994) Morphological gradients in sensory hair cells of the amphibian papilla of the frog, *Rana pipiens* pipiens. Hear Res 80:71–78.
Sisneros JA (2007) Saccular potentials of the vocal plainfin midshipman fish, *Porichthys notatus.* J Comp Physiol A Neuroethol Sens Neural Behav Physiol 193:413–424.
Sisneros JA (2009a) Seasonal plasticity of auditory saccular sensitivity in the vocal plainfin midshipman fish, *Porichthys notatus.* J Neurophysiol 102:1121–1131.
Sisneros JA (2009b) Adaptive hearing in the vocal plainfin midshipman fish: getting in tune for the breeding season and implications for acoustic communication. Integr Zool 4:33–42.
Sisneros JA, Bass AH (2003) Seasonal plasticity of peripheral auditory frequency sensitivity. J Neurosci 23:1049–1058.
Sisneros JA, Forlano PM, Deitcher DL, Bass AH (2004a) Steroid-dependent auditory plasticity leads to adaptive coupling of sender and receiver. Science 305:404–407.
Sisneros JA, Forlano PM, Knapp R, Bass AH (2004b) Seasonal variation of steroid hormone levels in an intertidal-nesting fish, the vocal plainfin midshipman. Gen Comp Endocrinol 136:101–116.
Smith ME, Coffin AB, Miller DL, Popper AN (2006) Anatomical and functional recovery of the goldfish (*Carassius auratus*) ear following noise exposure. J Exp Biol 209:4193–4202.
Sugihara I, Furukawa T (1989) Morphological and functional aspects of two different types of hair cells in the goldfish sacculus. J Neurophysiol 62:1330–1343.
Tomkins JL, Simmons LW (2002) Measuring relative investment: a case study of testes investment in species with alternative male reproductive tactics. Anim Behav 63:1009–1016.
Vasconcelos RO, Sisneros JA, Amorim MC, Fonseca PJ (2011) Auditory saccular sensitivity of the vocal Lusitanian toadfish: low frequency tuning allows acoustic communication throughout the year. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 197:903–913.
Wilkins HR, Presson JC, Popper AN, Ryals BM, Dooling RJ (2001) Hair cell death in a hearing-deficient canary. J Assoc Res Otolaryngol 2:79–86.
Xiao Z, Suga N (2002) Modulation of cochlear hair cells by the auditory cortex in the mustached bat. Nat Neurosci 5:57–63.
Zakon HH (1987) Hormone-mediated plasticity in the electrosensory system of weakly electric fish. Trend Neurosci 10:416–421.
Zar JH (1999) Biostatistical analysis, 4th edition. pp 98–104. New Jersey: Prentice Hall.
Zotterman Y (1943) The microphonic effect of teleost labyrinths and its biological significance. J Physiol 102:313–318.