Seasonal plasticity of auditory saccular sensitivity in the vocal plainfin midshipman fish, *Porichthys notatus*.

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The plainfin midshipman fish, *Porichthys notatus*, is a seasonally breeding species of marine teleost fish that generates acoustic signals for intraspecific social and reproductive-related communication. Female midshipman use the inner ear saccule as the main acoustic endorgan for hearing to detect and locate vocalizing males that produce multiharmonic advertisement calls during the breeding season. Previous work showed that the frequency sensitivity of midshipman auditory saccular afferents changed seasonally with female reproductive state such that summer reproductive females became better suited than winter non-reproductive females to encode the dominant higher harmonics of the male advertisement calls. The focus of this study was to test the hypothesis that seasonal reproductive-dependent changes in saccular afferent tuning is paralleled by similar changes in saccular sensitivity at the level of the hair cell receptor. Here, I examined the evoked response properties of midshipman saccular hair cells from winter non-reproductive and summer reproductive females to determine if reproductive state affects the frequency response and threshold of the saccule to behaviorally-relevant single tone stimuli. Saccular potentials were recorded from populations of hair cells *in vivo* while sound was presented by an underwater speaker. Results indicate that saccular hair cells from reproductive females had thresholds that were approximately 8 to 13 dB lower than non-reproductive females across a broad range of frequencies that included the dominant higher harmonic components and the fundamental frequency of the male’s advertisement call. These seasonal reproductive-dependent changes in thresholds varied differentially across the three (rostral, middle, and caudal) regions of the saccule. Such reproductive-dependent changes in saccule sensitivity may represent an adaptive plasticity of the midshipman auditory sense to enhance mate detection, recognition and localization during the breeding season.
INTRODUCTION

Recently a novel form of auditory plasticity that is adaptive for encoding social and reproductive-related communication signals was reported for females of the plainfin midshipman fish (*Porichthys notatus*) (Sisneros and Bass 2003, Sisneros et al. 2004a). Females rely greatly on their auditory sense to detect and locate males that "sing" during the breeding season to attract mates. The detection and localization of such vocal signals is essential to the reproductive success of this nocturnal species and can evoke in reproductive females strong phonotactic responses (McKibben and Bass 1998; for recent reviews regarding the neuroethology of this species see Bass 2006, Bass and Ladich 2008, Sisneros 2009). Previous research showed that the frequency sensitivity of auditory saccular afferents from wild-caught females change seasonally with reproductive state such that reproductive females become better suited than non-reproductive females to encode the dominant higher harmonic components of the male’s seasonal advertisement call (Sisneros and Bass 2003). This enhanced sensitivity to the dominant higher harmonics of the advertisement call likely increases signal detection by females because the higher harmonics will propagate further than the call’s fundamental frequency due to environmental and physical constraints of the shallow-water breeding habitat that limit sound transmission (Fine & Lenhardt 1983; Bass & Clark 2003). Furthermore, work by McKibben and Bass (2001) showed that the higher harmonics of the male’s advertisement call can also potentially affect the encoding of the call’s fundamental frequency, which could be important for mate localization when near the sound source.

The seasonal shift in midshipman hearing sensitivity is preceded by a seasonal change in gonadal reproductive state, which leads to elevated levels of circulating gonadal steroids in female midshipman. Approximately 2-3 months before the breeding season, females undergo seasonal recrudescence of the ovaries and then subsequently exhibit brief peaks in circulating plasma levels of testosterone and 17β-estradiol approximately one month before the beginning of the summer spawning season (Sisneros et al 2004b). These seasonal peaks in gonadal steroid hormones have been shown experimentally to induce the female’s reproductive auditory phenotype and enhance the phase-locking accuracy of auditory saccular afferents at higher frequencies that correspond to the dominant frequency content of male advertisement calls (Sisneros et al 2004a). In addition, midshipman-specific estrogen alpha receptor was discovered in the sensory epithelium of the inner ear saccule, the main organ of hearing in the midshipman and most other teleost fish, which provides additional support for a direct steroid effect on the inner ear (Sisneros et al 2004a). This novel form of reproductive-state and steroid-dependent auditory plasticity likely represents an adaptable mechanism that
increases the probability of mate detection and localization by enhancing the frequency coupling between sender and receiver in this vocal-acoustic communication system. A prime candidate site where this novel form of auditory plasticity may occur is at the level of the saccular hair cell.

The major goal of this study was to determine if female reproductive state influences the auditory sensitivity of the sensory hair cell receptors in the saccule and represents the initial steps of ongoing neurophysiological investigations to determine the potential site(s) of action for the related reproductive-state and steroid-dependent plasticity observed in the midshipman peripheral auditory system. Here, I characterize and compare the response properties of auditory evoked saccular potentials from reproductive and non-reproductive females to determine whether there are differences related to reproductive state in the frequency response, dynamic range and auditory threshold of midshipman saccular hair cells to behaviorally-relevant single tone stimuli. An auditory evoked potential recording technique recently described by Sisneros (2007) is used to determine the frequency response of hair cells in the saccule. Saccular potentials in the midshipman, as in other teleost fish, are maximally evoked at twice the auditory stimulus frequency due to the presence of non-linear and opposite oriented hair cell populations in the fish saccule and can be used to characterize the response of saccular hair cells (Hama 1969, Furukawa and Ishii 1967, Fay 1974, Fay and Popper 1974, Sisneros 2007). Using this recording technique, I test the hypothesis that seasonal variation in female reproductive state can modulate the sensitivity of auditory saccular hair cells to the dominant higher harmonic components in male midshipman advertisement calls. The findings are interpreted as they relate to possible auditory adaptations for acoustic communication during the breeding season.

METHODS

Experimental animals

Forty-eight adult female plainfin midshipman fish (P. notatus) with standard lengths (SL) that ranged from 9.0–19.5 cm SL were collected during the years 2006 and 2007 in both the non-reproductive season (January-March) and in the reproductive season (May-July). During the non-reproductive season, females with regressed ovaries that contained only small (<1mm diameter) undeveloped oocytes were collected by otter trawl (R/V Kittiwake, Bio-Marine Enterprises, and the R/V Centennial, Friday Harbor Marine Laboratories) at depths from 60 to 130 m in Puget Sound near Edmonds, WA, USA and in Bellingham Bay, WA, USA. These trawl collected animals showed no visible signs of stress and rapidly adjusted to the sudden change in water depth
while in captivity. During the reproductive season, gravid reproductive females with ovaries that contained relatively large (~ 5 mm diameter) yellow/orange, yolked eggs were collected by hand from the nests of parental (type I) males at low tide from a natural breeding population in the Hood Canal at Seal rock in Brinnon, WA, USA. The reproductive state of the animal was determined by measuring the gonadosomatic index (GSI, defined here as 100 * gonad mass/body mass – gonad mass; according to Tomkins and Simmons 2002). All animals were maintained in saltwater aquaria at 14-16ºC and fed a diet of goldfish every 2-4 days. Saccular potential recordings were performed within 15 days after collection from trawls or nests to avoid any effects of captivity on auditory saccular sensitivity (Sisneros and Bass 2003). All experimental procedures followed National Institutes of Health guidelines for the care and use of animals and were approved by the University of Washington Institutional Animal Care and Use Committee.

Stimulus generation

Acoustic stimuli were generated via the reference output signal of a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA) that was delivered to an audio amplifier and an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA). The frequency response of the underwater speaker in the experimental tank was measured using a mini-hydrophone (8103, Bruel and Kjaer, Norcross, GA, USA) that was positioned 10 cm above the underwater speaker, which is the position normally occupied by the head of the fish during the recordings. Relative sound measurements were then made using the mini-hydrophone and a single channel FFT spectrum analyzer (SR780, Stanford Research Systems, Sunnyvale, CA, USA), calibrated by peak-to-peak voltage measurements on an oscilloscope, and then adjusted with Matlab software so that the sound pressures of the frequencies (75-385 Hz) tested were of equal amplitude within ± 2 dB. Stimulus sound levels were measured and described in terms of sound pressure. Although it is recognized that the midshipman inner ear may be primarily sensitive to particle motion, the determination of sound level in terms of displacement or particle motion is at best difficult. The relationship between particle motion and pressure in small tanks is complex (Parvulescu 1967, Fay and Popper 1980) and the quantification and/or equalization of these two measures is very difficult. However, previous studies have confirmed that the primary axis of particle motion in this type of experimental tank setup is vertical and orthogonal to the surface plane of the underwater speaker (McKibben and Bass 1999) and that the reflections from the tank walls and water surface did not alter the sound pressure waveform of the acoustic signal (Bodnar and Bass 1997, 1999).
Recent evidence indicates that many auditory saccular afferents of the midshipman respond to vertical stimuli or dorsoventral acceleration and that the iso-level response curves based on pressure are similar in shape to iso-intensity curves based on particle motion (Weeg et al. 2002). If the midshipman saccule is indeed primarily particle motion-sensitive, then the two measures of sound (particle motion and pressure) will be proportional but with a different proportionality at each frequency depending on the sound source and tank acoustics. The use of sound pressure to describe the stimulus levels in this study should provide an interpretable measure of sound stimuli that can be used to compare with previous midshipman auditory physiology studies and with other fish species using a similar experimental setup (for extended discussion, see McKibben and Bass 1999, Weeg et al. 2002, also for recent review of underwater sound fields, see Bass and Clark 2003).

Basic auditory stimuli consisted of 8 repetitions of single 500-msec duration tones presented at a rate of one every 1.5 seconds. 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 (frequency) stimulus was randomly selected. To measure iso-level responses, the single tone stimuli were presented at a sound pressure of 130 dB re 1 μPa, which is consistent with sound pressure levels for type I male midshipman calls recorded near their nest (Bass and Clark 2003). To measure threshold tuning responses, single tone stimuli were presented at sound pressure levels from 91 to 154 dB re 1 μPa in incremental steps of 3 dB.

Saccular potential measurements

Recording methods followed those used previously to characterize the evoked potentials from the saccule in the midshipman fish (Sisneros 2007). Midshipman fish were first anesthetized by immersion in a 0.025% ethyl p-aminobenzoate (benzocaine) saltwater bath followed by an intramuscular injection of pancuronium bromide (approximately 0.5 mg/kg) and 0.25% bupivacaine (approximately 1 mg/kg) for immobilization and analgesia, respectively. The saccule was then exposed by a dorsal craniotomy. The cranial cavity was filled with cold teleost Ringer's solution (Cavanaugh 1956) to enhance clarity and prevent drying of the saccule. A 2-cm dam of denture adhesive cream was constructed around the exposed cranial cavity that then allowed the animal to be lowered below the water line in the experimental tank. The fish was positioned in the center of the tank such that the exposed saccule was below the water surface at a distance of 10 cm above the underwater speaker, which was embedded in the sand/gravel on the bottom of a 30 cm diameter, 24 cm high Nalgene tank (similar to that used by Fay 1990). The experimental tank was housed on a vibration
isolation table inside an acoustic isolation chamber (Industrial Acoustics, New York, NY, USA). All the recording and auditory stimulus generation equipment was located outside the chamber. Fish were perfused with chilled seawater (14-15°C) via a small plastic tube that was inserted into the fish’s mouth to provide a continuous stream of recirculated seawater across the gills.

Evoked potentials from the saccule were recorded with glass microelectrodes filled with 3M KCl (1-7 MΩ). The recording electrode was visually guided into the endolymph of the saccule and positioned approximately 2-4 mm away from the sensory bed of hair cells (macula) in one of the three recorded regions (rostral, middle, and caudal) in either the right or left saccule (see Fig. 1). Analog saccular potentials were amplified (x100) (model 5A, Getting Instruments), inputted into a digital signal processing Lock-in amplifier (SR830, Stanford Research Systems), and then stored on a PC computer running a custom data acquisition Matlab software program. The lock-in amplifier yields a DC voltage output signal that is proportional to the component of the signal whose frequency is exactly locked to the reference frequency. The reference frequency was set to the second harmonic of the stimulation frequency signal (i.e., 2x fundamental frequency) since the maximum evoked potential from the teleost saccule occurs at twice the sound stimulus frequency due to the non-linear response of opposite oriented hair cell populations within the saccule (Zotterman 1943, Furukawa and Ishii 1967, Cohen and Winn 1967, Hama 1969). Noise signals at frequencies other than the reference frequency were rejected by the lock-in amplifier and did not affect the saccular potential measurements.

To measure and compare the evoked iso-level responses of the saccule, the evoked saccular potentials were first measured at 130 dB re 1μPa and then the response profiles were constructed using saccular potential data that was normalized. The magnitude of the evoked saccular potentials varied depending on the distance between recording electrode and the sensory bed of hair cells. In order to control for differences in the absolute magnitude of the evoked saccular potentials recorded from different animals and from different recording regions on the saccular macula (i.e., rostral, middle, and caudal areas of the macula), the signal averaged saccular potential data were normalized and expressed relative to a value of 0 dB that was assigned to the maximum evoked saccular potential recorded at the peak frequency sensitivity or best frequency (BF) for each record. The BF was defined as the stimulus frequency that produced the greatest evoked saccular potential response. Iso-level profiles were recorded from three regions of the saccule: rostral, middle, and caudal (Fig. 1). The response profile of the evoked saccular potentials was determine for each saccular recording region and then summarized
in plots. This analytical method allows for the relative comparisons of response profiles for the three recording regions of the saccule (Sisneros 2007).

Data analysis

Threshold tuning curves were constructed by characterizing the input-output measurements of the evoked saccular potentials over the range of stimulus levels, in incremental steps of 3 dB, from 88 to 154 dB re 1 μPa at the tested stimulus frequencies (see above). In addition, background noise measurements (RMS) were also recorded for 8-10 repetitions of the stimulus interval at each of the tested frequencies from 75 to 385 Hz (see above) with no auditory stimulus present. These background noise measurements were then used to establish the subthreshold saccular potential response levels and determine the auditory threshold at each frequency. The measurement of background noise was performed prior to the recording of each threshold tuning curve. The auditory threshold at each stimulus frequency was designated as the lowest stimulus level that evoked a saccular potential that was at least 2 standard deviations above the background noise measurement. The frequency that evoked the lowest saccular potential threshold was defined as the BF.

Statistical analysis

The effects of reproductive state (non-reproductive vs. reproductive females) and the recording region of the saccule (rostral vs. middle vs. caudal) on the iso-intensity BF of saccular hair cells were determined by a two-way ANOVA. Differences in body size (standard fish length) and response magnitude (relative gain) of the saccular evoked potentials between non-reproductive and reproductive females were determined by t-tests. The association between body size (standard fish length) and the response magnitude (relative gain) of the saccular evoked potentials to iso-level tones of 130 dB (re 1 μPa) among non-reproductive and reproductive females was analyzed using Pearson product-moment correlation. 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 20 frequencies tested (75-385 Hz) as repeats (i.e. within-subject factors) and reproductive-state of the animal as the between-subject factor. In order to determine the effects of reproductive state and stimulus frequency based on saccular recording position, a separate repeated measure ANOVA analysis was performed on data from each recorded region (i.e., rostral, middle and caudal) with the saccular thresholds for each of the 20 frequencies tested (75-385 Hz) as repeats (i.e. within-subject factors) and reproductive-state of the animal as the between-subject factor. In the few cases where there were two positional
recordings from the same individual fish (e.g., rostral threshold measurements from both the left and right saccule), the two threshold tuning curves were then averaged so that a repeated measures ANOVA analysis could be performed. P value corrections were made for all tests of within-subject effects based on the calculated estimates of sphericity (equality of variances of the differences between measurements) (Girden 1992). Since epsilon was < 0.75 in all analyses, the more conservative correction of Greenhouse-Geisser was used to calculate P. The 95% confidence limits (CL) of the mean thresholds (Zar 1999) were calculated and used to determine whether the mean evoked saccular thresholds differed between reproductive and non-reproductive 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 the software programs Statistica for Windows (StatSoft, Inc.) and Systat 7.0 (Systat software Inc).

RESULTS

Iso-level responses of the evoked saccular potentials

The evoked saccular potentials were recorded from a total of 48 adult female midshipman fish: 24 winter non-reproductive females with a size range of 9.0 to 17.5 cm standard length (SL) (mean SL = 12.1 ± 1.9 SD cm, mean body mass (BM) = 22.0 ± 9.3 SD g, mean gonadosomatic index (GSI) = 5.6 ± 5.2 SD) and 24 summer reproductive females with a size range of 12.3 to 19.5 cm SL (mean SL = 16.8 ± 1.6 cm, mean BM = 62.0 ± 17.1 SD g, mean GSI = 18.9 ± 9.8 SD). Iso-level response profiles of the evoked saccular potentials were generated from the presentation of single tone stimuli that ranged from 75 to 385 Hz. Figure 2 shows representative iso-level response curves of evoked saccular potentials to single tones of 130 dB (re 1 $\mu$Pa) recorded from the rostral, middle and caudal regions of the saccule. In general, the evoked saccular potentials were much higher in the middle and caudal regions compared to that of the rostral region of the saccule (Fig. 2). The iso-intensity response curves consisted of profiles that had best frequencies (BF, defined as the frequency that evoked the greatest saccular potential) $\leq$ 85 Hz with the evoked potentials declining rapidly above BF. BFs ranged from 75 Hz to 145 Hz with the majority of BFs at 75 Hz (winter: 72%, summer: 51%), the lowest frequency tested. The mode of BFs (75 Hz) of the evoked potentials to tones of 130 dB (re 1 $\mu$Pa) did not differ across position (rostral, middle, caudal) along the saccule (two-way ANOVA, effect of position, $F = 0.44$, df = 2, 61, $p = 0.64$) or between non-reproductive and reproductive females regardless of the recording position along
the saccule (two-way ANOVA, effect of season, $F = 0.21$, df $= 1$, 61, $p = 0.65$; interaction of reproductive-state and position, $F = 1.36$, df $= 2$, 61, $p = 0.26$).

Because BF did not differ among the rostral, middle, and caudal regions of the saccule, the iso-level saccular potential data were first normalized and expressed relative to a value of 0 dB for the BF in each record and then were pooled for both non-reproductive and reproductive females. The normalized iso-level response data was averaged and then complied to construct a relative gain plot summarized in Fig 3, which shows the relative dynamic range of the saccular potentials evoked from non-reproductive and reproductive females. In general, the recorded iso-level saccular potentials evoked at 130 dB re 1$\mu$Pa were greatest at 75 Hz with a rapid decline in sensitivity (gain) of evoked potentials above 95 Hz to the lowest levels at 305-385 Hz. Although non-reproductive females were smaller than reproductive females ($t$-test, $t= -9.17$, df $= 46$, $p < 0.05$), there was no relationship between female size (SL) and the range of the response magnitude or relative gain of the evoked saccular potentials based on female reproductive state (non-reproductive females, $H_0: \beta = 0$, $t = 0.58$, $p = 0.56$; reproductive females, $H_0: \beta = 0$, $t = -0.44$, $p = 0.96$). However, the magnitude of the evoked saccular potentials were greater in reproductive females compared to the non-reproductive females ($t$-test, $t = 6.64$, df $= 74$, $p < 0.01$). The dynamic range of relative gain from 75-385 Hz was approximately 13 dB greater in reproductive females (dynamic range = 44 dB) than in non-reproductive females (dynamic range = 31 dB) (Fig. 3). Based on saccular region, this seasonal difference in dynamic range of relative sensitivity (gain) from 75-385 Hz between reproductive and non-reproductive females was approximately 16 dB, 18 dB, and 7 dB for the rostral, middle, and caudal positions, respectively (Fig. 4).

Seasonal differences in auditory saccular sensitivity

Auditory threshold tuning curves were determined for whole populations of hair cells in the rostral, middle, and caudal regions of the saccule in both non-reproductive and reproductive female fish. Figure 5 shows representative tuning curves recorded from the three different regions of the saccule. Threshold tuning curves for the saccular potentials generally consisted of profiles with lowest thresholds at frequencies $< 145$ Hz that steadily increased to the highest thresholds at frequencies $> 305$Hz (Fig. 5). Best frequencies (BFs, defined as the frequency that evoked the lowest saccular potential threshold) ranged from 75 Hz to 145 Hz for non-reproductive females and 75 Hz to 135 Hz for reproductive females with a mode of BFs occurring at 75 Hz for both non-reproductive and reproductive females. The distribution of BFs based on the threshold tuning profiles...
did not differ by recording position (rostral, middle, caudal) along the saccule (two-way ANOVA, effect of
position, $F = 0.99$, df = 2, 61, $p = 0.37$) nor by reproductive-state of the animal regardless of recording position
along the saccule (two way ANOVA, effect of reproductive-state, $F = 1.82$, df = 1, 61, $p = 0.18$; interaction of
season/reproductive-state and saccular position, $F = 1.58$, df = 2, 61, $p = 0.21$).

Although there was no seasonal difference in BF, there were significant threshold differences at the
tested frequencies between non-reproductive female and reproductive female midshipman. In general, auditory
thresholds were significantly lower at every frequency (75-385 Hz) in reproductive females than in non-
reproductive females (repeated measures ANOVA: between-subject factor ‘reproductive-state’: $F = 117.20$, df =
1,65, $P < 0.001$) with no significant interaction of frequency between females of different reproductive states
(repeated measure ANOVA: within-subject contrast ‘frequency*reproductive-state’: $F = 1.96$, df = 19,1235, $P =
0.058$; assumptions of homogeneity of variances were met for each of the tested frequencies: Levene’s test, $P$
values $> 0.09$). The threshold tuning curves for non-reproductive and reproductive females are summarized in
Fig. 6. In addition, there were also significant threshold differences at various frequencies of the tuning curves
from the three saccular regions that varied by reproductive-state of the animal and saccular position. In general,
the threshold tuning curves for each region of the saccule were similar in shape with lowest thresholds at 75 and
85 Hz that rapidly increased to highest thresholds at 365 and 385 Hz (Fig. 7). Auditory thresholds recorded from
the rostral region of the saccule were lower in reproductive females at 165 Hz, 185 Hz, 205 Hz and 225 Hz
compared to non-reproductive females (repeated measures ANOVA: between-subject factor ‘reproductive-
state’: $F = 17.48$, df = 1, 14, $P < 0.001$; within-subject contrast ‘frequency*reproductive-state’: $F = 0.68$, df = 19,
266, $P = 0.69$). In contrast, the threshold recorded from the middle saccular region were lower in reproductive
dfemales at every frequency except 95 Hz, 125 Hz, 345 Hz, and 365 Hz compared to non-reproductive females
(repeated measure ANOVA: between-subject factor ‘reproductive-state’: $F = 26.48$, df = 1, 19, $P < 0.001$; within-
subject contrast ‘frequency*reproductive-state’: $F = 1.30$, df = 19, 361, $P = 0.25$). Similarly, thresholds recorded
from the caudal saccular region were lower in reproductive females at every frequency except 75 Hz compared
to non-reproductive females (repeated measure ANOVA: between-subject factor ‘reproductive-state’: $F = 79.82$,
df = 1, 28, $P < 0.001$; within-subject contrast ‘frequency*reproductive-state’: $F = 1.03$, df = 19, 532, $P = 0.41$).

Thus, auditory saccular thresholds were lower in reproductive females compared to non-reproductive females
with thresholds varying differentially across the three (rostral, middle, and caudal) regions of the saccule.
DISCUSSION

The goal of this study was to determine if seasonal variation in gonadal reproductive state can modulate
the sensitivity of auditory saccular hair cells to behaviorally-relevant single tone stimuli in female midshipman
fish. The results from this evoked potential recording study indicate that the auditory saccular hair cells from
reproductive females had lower tuning thresholds and were more sensitive to all the frequencies tested (75-385
Hz) compared to that of saccular hair cells from non-reproductive females. This study represents the first
attempt to determine the potential site of action for the related reproductive-state and steroid-dependent
plasticity observed in the previous saccular afferent studies (Sisneros and Bass 2003, Sisneros et al. 2004a). In
this discussion, I interpret the results as they relate to the encoding of vocal communication signals for the
midshipman fish and discuss possible adaptations for the seasonal plasticity of auditory tuning and how this
seasonal shift in tuning may facilitate acoustic communication during the midshipman breeding season.

Seasonal changes in the iso-level response of the evoked saccular potentials

In general, the iso-level saccular potential records indicate that hair cells from the midshipman saccule
were most sensitive to frequencies ≤ 145 Hz at the behaviorally relevant sound level of 130 dB re 1 μPa, a level
that is consistent with known sound levels for midshipman advertisement calls recorded near the entrance of
type I male nests (Bass and Clark 2003). These iso-level responses of midshipman saccular hair cells were
characterized to pure tones at 130 dB re 1 μPa so that they could be compared to the responses of primary
auditory afferents characterized at similar levels in previous studies (McKibben and Bass 1999, Sisneros and
Bass 2003). Results indicate that the iso-level response profiles of the saccular potentials (Fig. 2) were similar to
the profiles plotted for auditory afferent spike rate at the same sound level in a previous study (see figure 3 in
Sisneros and Bass 2003). McKibben and Bass (1999) and Sisneros and Bass (2003) previously reported a
secondary mode of BFs at approximately 140 Hz for auditory afferents in midshipman fish which is congruent
with the iso-level profiles of the saccule in non-reproductive and reproductive females reported here that show a
secondary peak around 145 Hz (Fig. 3, but also see individual representative profiles in Fig. 2). Remarkably the
distribution of BFs for the midshipman saccule reported here and in a previous study (Sisneros 2007) is similar
to the distribution of BFs for the saccule in the toadfish (Opsanus tau), which has bimodal peaks at 74 Hz and
140 Hz (Fay and Edds-Walton 1997a). In O. tau, there is no evidence for regional differences in frequency
sensitivity along the saccule (Fay and Edds-Walton 1997b), which is also consistent with the frequency
sensitivity data reported here that shows no difference in peak sensitivity (75 Hz) along the three recorded
regions of the saccule for females in a given reproductive state (i.e., non-reproductive vs. reproductive).

The iso-level response profiles for saccular potentials evoked at 130 dB re 1μPa indicate that saccular
hair cells from reproductive females had a greater magnitude and relative gain of evoked potentials compared to
that of non-reproductive females. Reproductive females had a dynamic range (expressed in relative gain or
sensitivity) that was 14 dB greater than the range of evoked saccular potentials from non-reproductive females
(Fig. 3). Although there was no relationship between female size (SL) and the range of the response magnitude
or relative gain of the evoked saccular potentials based on female reproductive state, there was considerable
variation in the range of the evoked saccular potentials from both non-reproductive and reproductive females
(see supplementary figure). Nevertheless, this seasonal increase in the sensitivity of the evoked saccular
response was found to occur across the entire saccule in reproductive females having a range that was 16 dB,
18 dB and 9 dB greater than that of non-reproductive females for the rostral, middle and caudal saccular
positions, respectively. This difference in seasonal sensitivity could be potentially due to changes in hair cell
responsiveness to stimuli in the vertical axis. Although the hair cell orientation patterns of the saccule are not
known for *P. notatus*, many auditory primary afferent neurons that innervate midshipman saccular hair cells are
known to respond to vertical stimuli or dorsoventral acceleration (Weeg et al. 2002, Sisneros and Bass 2003).
Based on the evoked potentials recorded from each of the three saccular regions (rostral, middle and caudal), it
is likely that the hair cells found in each of the three regions have hair cell orientation patterns that respond to
stimuli in the vertical axis. In the closely related toadfish *Opsanus tau*, the hair cell orientation pattern in the
saccule gradually changes from patches of rostrally oriented hair cells in the rostral region to primarily vertical
oriented hair cells in the middle region to caudal oriented hair cells in the caudal region (Edds-Walton and
Popper 1995). In this study, the evoked potentials recorded from the rostral region of the saccule were the
lowest in magnitude and more variable compared to evoked potentials from the middle and caudal regions of
the saccule. Such differences are likely related to hair cell orientation patterns found in the midshipman saccule.

It would be interesting to know in future work whether hair cells found in the middle and caudal saccular regions
are oriented to receive particle motion stimuli from the vibrations of the fish’s swim bladder produced by the
pressure component of the sound during sound reception. Future studies that examine the distribution,
morphology and orientation patterns of saccular hair cells in the midshipman will be insightful in determining
how hair cell orientation patterns relate to the evoked saccular potentials recorded from different regions of the saccule.

One possible explanation to account for such seasonal differences in the magnitude and range of the evoked potentials could be due to a differential seasonal increase in the relative number of saccular hair cells that response to vertical axis stimulation in reproductive females compared to that of non-reproductive females.

A number of studies have demonstrated that fish continue to add hair cells for a number of years post-embryonically (Platt 1977, Corwin 1983, Lombarte and Popper 1984, Popper and Hoxter 1984), but only a few studies have examined the relationship between hair cell addition and auditory sensitivity of the fish inner ear. Based on multiunit recordings of primary afferents from the macula neglecta in the skate *Raja clavata*, Corwin (1983) demonstrated that an increase in auditory sensitivity was correlated with an increase in the number of hair cells innervated by individual auditory afferents. Similarly, Sento and Furukawa (1987) also found that the sensitivity of auditory saccular afferents in the goldfish, *Carassius auratus*, was correlated with the number of hair cells innervated by individual afferent neurons. In addition, age/size-related increases in behavioral auditory threshold sensitivity have been reported using behavioral conditioning techniques for two species of teleost fish, the damselfish (*Pomacentrus* sp.) and the Red Sea bream *Pagrus major* (Kenyon 1996, Iwashita et al. 1999).

In contrast to previous findings, Higgs et al. (2002, 2003) using the auditory brainstem recording technique showed that auditory sensitivity did not increase with age and size of the zebrafish, *Danio rerio*. Similarly, Popper (1971) showed that behavioral auditory sensitivity in the goldfish (*C. auratus*) did not change with age/size (and presumably with hair cell addition which is correlated with age/size) between two subadult groups of goldfish. Although the study by Popper (1971) did not directly test the effects of hair cell addition on auditory sensitivity of the goldfish saccule, the results are congruent with one model of fish hearing that predicts hair cell addition with fish growth will maintain sound detection and processing capabilities as the relative sizes and positions of different structures associated with fish hearing change during ontogeny (Popper et al. 1988, Rogers et al. 1988).

Alternatively, the seasonal differences in the magnitude and relative gain (sensitivity) of the evoked midshipman saccular potentials could be related to reproductive-state dependent effects on the central input from the hindbrain efferent nucleus and the efferent neurons that innervate the inner ear of the midshipman and other teleost fishes (Bass et al. 1994). Efferents to the saccule provide inhibitory inputs to saccular hair cells and can directly modulate auditory sensitivity (gain) to auditory stimuli (Furukawa and Matsura 1978, Lin and Faber...
1988). Recent evidence suggests that auditory efferent feedback can modulate the signal to noise ratio of auditory responses from saccule to brain in teleost fish such that the efferent regulatory effects can be either excitatory or inhibitory depending on the level of background noise (Tomchik and Lu 2006). Future studies that investigate the seasonal addition of hair cells in the saccule and the potential seasonal effects of efferent activation and modulation of auditory hair cell sensitivity will be needed in order to determine the mechanism for the observed seasonal differences in relative sensitivity (gain) of saccular potentials found between reproductive and non-reproductive female midshipman fish.

Seasonal plasticity of auditory saccular sensitivity and its functional significance

Comparison of the tuning curves revealed a dramatic seasonal difference in the auditory thresholds of saccular hair cells between reproductive female and non-reproductive females. The auditory thresholds of saccular hair cells from reproductive females were approximately 8 to 13 dB lower than non-reproductive females at frequencies from 75 to 385 Hz compared to (Figs. 6 and 8). Furthermore, the auditory thresholds of saccular hair cells from reproductive females were at least 9.5 dB or lower (a sensitivity increase equal to 3X or greater) than non-reproductive females at frequencies that corresponded to the fundamental frequency (~ 100 Hz) and the dominant second harmonic (~ 200 Hz) component of the male’s advertisement call (Fig. 8). It is important to note the differences of the evoked saccular responses reported in Figures 3 and 6. The iso-level gain data (Fig. 3) illustrated that the evoked saccular potentials of reproductive females stimulated at 130 dB re 1μPa showed progressively more relative gain starting at 200 Hz up to 385 Hz compared to non-reproductive females. These results suggest that the plasticity may potentially be frequency dependent, which would be consistent with previous single unit data of saccular afferents that showed an increase in phase-locking accuracy within the same frequency range (Sisneros and Bass 2003). In contrast, the threshold data (Fig. 6) showed a near parallel shift in sensitivity with reproductive females having a greater sensitivity over the same frequency range compared to non-reproductive females, which suggests an overall increase in sensitivity that is not frequency dependent. This apparent discrepancy is noteworthy and should be investigated in future work. An analysis of the general receptor potential dynamics with a description of the slopes from the input-output functions of the evoked saccular potentials would be very informative in future work in determining any possible frequency dependent effects that could not be resolved in this study.
An important finding reported in this study was that the seasonal change in auditory threshold occurred across a broad range of frequencies that included the dominant higher harmonic components and the fundamental frequency of the male’s advertisement call. This seasonal change in auditory threshold may function to increase the probability of conspecific mate detection and localization during the summer breeding season as previously proposed by Sisneros and Bass (2003). The seasonal shift in auditory saccular sensitivity may be adaptive for females to enhance the acquisition of auditory information needed for mate detection and localization when both far and near the sound source during the breeding season, especially in shallow water environments like those where midshipman fish court and spawn. The dominant harmonics of the advertisement call that range up to 400 Hz often contain as much or more spectral energy than the fundamental frequency and are hypothesized to be important for the detection and sound source localization of the advertisement call by females during the midshipman breeding season. The mate call’s harmonics likely increase signal detection by the receiver because the higher frequency harmonics will propagate further than the mate call’s 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). According to this relationship, as water depth decreases the frequency below which sound transmission is negligible (cutoff frequency) increases. Thus, in very shallow water (< 5m) low frequency signal components such as the mate call’s fundamental frequency should attenuate rapidly while the higher frequency harmonics that are above the cutoff frequency should propagate more readily away from the sound source. However, the enhanced detection of the fundamental frequency (~100 Hz) would also be equal important in localizing the advertisement call when the female receiver is very close or near the sound source. In addition, the dominant higher harmonics of the advertisement call may also affect the encoding of the call’s fundamental frequency when near the sound source. Previously McKibben and Bass (2001) showed that the encoding of the advertisement call’s fundamental frequency was enhanced when harmonics were added to tonal stimuli that were similar to the fundamental frequencies of the male’s advertisement call. Interestingly, recent evidence also suggests that the lateral line system of the midshipman fish is also sensitive to the fundamental frequency component of the male’s advertisement call (Weeg and Bass 2002) with supporting neuroanatomical evidence for the central integration of lateral line and auditory information (Weeg and Bass 2000, Edds-Walton and Fay 2003). In sum, the seasonal increase in auditory saccular sensitivity may represent an adaptive plasticity of the midshipman auditory sense for the enhancement of mate detection and localization during the breeding season.
Currently it is not known whether the observed peripheral auditory plasticity also extends to the male morphs (types I and II) of the plainfin midshipman fish. Type I males build nests, acoustically court females, and provide parental care for fertilized eggs during the breeding season, whereas type II males neither build nests nor acoustically court females, but instead sneak or satellite spawn to steal fertilizations from type I males (Brantley and Bass 1994). There is no *a priori* reason to expect that the auditory plasticity of peripheral frequency sensitivity be limited to only females. The seasonal enhancement of frequency sensitivity for conspecific detection and localization would also be adaptive for type I or nesting males during mate competition in the establishment of nest sites and in the case of type II or sneaker males in the selection of cuckoldry sites for sneak or satellite spawning. Thus, future studies will be needed to determine whether seasonal reproductive-state and/or steroid-dependent plasticity occurs in the male peripheral auditory system. In addition, similar mechanisms of auditory plasticity may also be operative in other vertebrate groups were studies have suggested either seasonal or steroid-related changes in audition, which includes recent studies of birds (Lucas et al. 2002, Lucas et al. 2007), amphibians (Penna et al. 1992, Goense and Feng 2005, Gordon and Gerhardt 2009) and humans (Guimaraes et al. 2006, Hultcrantz et al. 2006).

**Mechanisms for the plasticity of saccular sensitivity**

As demonstrated for the auditory saccular afferents, the mechanism responsible for the observed changes in auditory sensitivity of midshipman saccular hair cells is most likely dependent on seasonal changes in circulating levels of gonadal steroids, specifically testosterone (T) and 17β-estradiol (E2). Approximately 1 month before the beginning of breeding season, female midshipman fish exhibit annual seasonal peaks in circulating levels of T and E2 (Sisneros et al. 2004b). Experimental implants of T and E2 in female midshipman confirmed a steroid-dependent effect and induced an increased phase-locking accuracy of the auditory saccular afferents at higher frequencies within the midshipman’s hearing range, especially at frequencies that corresponded to the dominant frequency content of the male’s advertisement call (Sisneros et al. 2004a). Concurrent with these findings, midshipman-specific estrogen receptor alpha was demonstrated to be expressed in the saccular epithelium and in the saccular afferent branches that were proximal to the saccular epithelium (Sisneros et al. 2004a, Forlano et al. 2005). It was the discovery of the estrogen receptor alpha in the midshipman saccule that was impetus to first determine in this study if related reproductive-state dependent changes in auditory sensitivity occur at the level of the hair cell in female midshipman fish. Results from this
study now show that seasonal changes in auditory tuning do occur at the level of the saccular hair cell and thus
will now be used to guide future studies that examine the potential role of T and E\(_2\) in modulating the response
properties of midshipman saccular hair cells.

The potential cellular mechanism(s) responsible for the steroid-dependent plasticity of peripheral
auditory frequency sensitivity in the plainfin midshipman is still unknown. One proposed mechanism for the
observed changes in midshipman is the action that steroid hormones may have on the ion-channel current
kinetics of auditory hair cells by genomically up regulating the differential expression ion channels such as
calciump-dependent BK or Kv channel types (and/or their related subunits), which then in turn could potentially
influence the biophysical properties of hair cells and their electrical resonance. Such a mechanism has been
posited for similar steroid-related changes in the frequency sensitivity of electroreceptors in weakly electric
fishes (Zakon 1987, Zakon et al. 1991). The electrical resonance that arises from ion-channel current kinetics of
the basolateral membrane of auditory hair cells is thought to be the major contributing factor that establishes
hair cell low frequency (< 1kHz) tuning in non-mammal auditory systems (Fettiplace and Fuch 1999) including
the toadfish, *Opsanus tau* (Steinacker and Romero 1991, 1992). Hair cell electrical resonance originates from
the interaction between inward calcium and outward Ca+-dependent K+ currents that produce an electrical
oscillation of the receptor potential along the hair cell epithelium (Lewis and Hudspeth 1983, Roberts et al.
1988). Perhaps the observed changes in auditory threshold in reproductive females is due changes in the
electrical tuning properties of hair cells that result in the electrical “detuning” of the saccular hair cells to the
effect that widens the restricted frequency response of hair cells observed in non-reproductive females (Fig. 3)
via changes in non-linear input-out functions of the hair cells. Thus, the characterization of the hair cell ion-
channel current kinetics in non-reproductive and reproductive females would help to elucidate the potential role
female reproductive plays in affecting the electrical tuning properties of saccular hair cell.

Alternative sites of action for steroid hormones and their effects on the response properties of the
midshipman auditory system include the auditory saccular afferents and hindbrain efferent nuclei. Gonadal
steroids may have direct effects on the saccular afferents that innervate hair cells in the saccule. Forlano et al.
(2005) shows evidence for aromatase-ir ganglion cells in the saccular afferent branches of the VIII auditory
nerve, which reveals potential sites for the conversion of T into E\(_2\) in areas of the midshipman auditory nerve
that are contiguous to hair cells in the saccular epithelium. Other steroid sensitive sites that may potentially
affect peripheral auditory processing include hindbrain efferent nuclei and the efferents that directly innervate
the saccule in the midshipman inner ear (Bass et al. 1994). Efferents from the hindbrain nuclei provide inhibitory
input from the CNS to the auditory periphery and modulate the gain or sensitivity of hair cells in the saccule
(Furukawa and Matsura 1978). Work by Xiao and Suga (2002) more recently showed that neurons in the
mammalian auditory cortex modulate the frequency sensitivity of cochlear hair cells via olivocochlear efferents in
the corticofugal (descending) auditory system. Future studies that examine the potential cellular effects of
steroid hormones on auditory saccular afferent neurons and the potential seasonal reproductive-state and
steroid-dependent effects on the efferent activation and modulation of saccular hair cell sensitivity will be
instrumental in the understanding of how peripheral auditory sensitivity is modulated in the midshipman fish.

In summary, the results reported in this study indicate that seasonal plasticity of auditory sensitivity
previously reported in the female midshipman fish does occur at the level of the saccular hair cell. This novel
form of seasonal reproductive-state dependent plasticity of auditory saccular sensitivity provides an adaptive
mechanism that enhances the coupling between sender and receiver in this communication system and should
enhance the probability of mate detection and localization by female midshipman during the breeding season.
This adaptive plasticity of the female midshipman’s auditory system may act to increase the probability of mate
detection/localization and potentially enhance the acquisition of auditory information needed for mate choice
decisions during the breeding season.

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Figure 1. Dorsal view of the brain and inner ear of the plainfin midshipman fish, *Porichthys notatus*. The inner ear saccule (S) is marked by a dotted line that defines the border and indicates the region of the saccule (rostral, middle and caudal) that was used to record the evoked saccular potentials. Note that in this photo the saccule has been deflected laterally to expose the dorsal view of the nerves. C cerebellum, M midbrain, T telencephalon.

Figure 2. Representative examples of iso-level curves of the evoked saccular potentials recorded from the rostral, middle and caudal regions of the saccule in response to single tones at 130 dB (re 1 µPa) for non-reproductive and reproductive female midshipman. Note that the scale of the y-axis for the representative evoked saccular potentials in the plots are different to emphasis the overall relative shape of the iso-level response curves. All saccular potentials are plotted as mean ± 1 SD and that most of the SD bars are obscured by the symbols.

Figure 3. The evoked potentials recorded from the saccule of non-reproductive and reproductive female midshipman fish based on the response to iso-level tones of 130 dB (re 1 µPa). Peak frequency sensitivity of 75 Hz was the lowest frequency tested. In order to control for absolute sensitivity of the saccule from different recording positions and compare across different animals, the iso-level response data were normalized to a relative value of 0 dB assigned to the peak response for each record and then expressed in relative dB re Best Frequency Sensitivity. All data are plotted as mean ± 1 SD. Note the number of animals and records per group (reproductive vs. non-reproductive) are indicated in parentheses.

Figure 4. Iso-level response curves of the evoked saccular potentials recorded from the rostral, middle and caudal regions of the saccule in response to single tones at 130 dB (re 1 µPa) for non-reproductive (NR) and reproductive (R) female midshipman. Iso-intensity response data were normalized to a relative value of 0 dB assigned to the peak response for each record and then expressed in relative dB re Best Frequency Sensitivity. All data are plotted as mean ± 1 SD. Note the number animals and records are indicated in parentheses.
Figure 5. Representative examples of individual auditory threshold tuning curves for non-reproductive and reproductive females based on the evoked potentials recorded from the rostral, middle and caudal regions of the midshipman saccule. The auditory threshold at each stimulus frequency was determined as the lowest stimulus intensity in dB (re 1 μPa) that evoked a saccular potential that was at least 2 SD above the background noise measurement.

Figure 6. Auditory threshold tuning curves for non-reproductive and reproductive female midshipman based on the evoked potentials recorded from the sacculus. All data are plotted as mean ± 95% confidence limit (CL) and the number of animals and records are indicated in parentheses. Auditory threshold at each stimulus frequency was determined as the lowest stimulus intensity in dB (re 1 μPa) that evoked a saccular potential that was at least 2 SD above the background noise measurement.

Figure 7. Auditory threshold tuning curves for non-reproductive and reproductive female fish based on the evoked potentials recorded from the rostral, middle and caudal regions of the midshipman sacculus. All data are plotted as mean ± 95% CL and the number of animals and records are indicated in parentheses. Auditory threshold at each stimulus frequency was determined as the lowest stimulus intensity in dB (re 1 μPa) that evoked a saccular potential that was at least 2 SD above the background noise measurement.

Figure 8. Comparison between the vocal characteristics of the male advertisement call and the change in auditory threshold saccular tuning in female midshipman fish based on seasonal reproductive state. Shown here is a combined plot of the difference in auditory saccular thresholds between non-reproductive and reproductive females at each frequency tests (Δ Threshold, left y axis) and the power spectrum of the male midshipman advertisement call showing the first 4 harmonics (right y axis, in relative dB values); inset show the temporal waveform of the advertisement call from a nesting male midshipman fish recorded at 16°C at a nest site (scale bar = 50 ms).