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## RESEARCH ARTICLE

# Seasonal plasticity of auditory hair cell frequency sensitivity correlates with plasma steroid levels in vocal fish

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**SUMMARY**

**Vertebrates displaying seasonal shifts in reproductive behavior provide the opportunity to investigate bidirectional plasticity in sensory function. The midshipman teleost fish exhibits steroid-dependent plasticity in frequency encoding by eighth nerve auditory afferents. In this study, evoked potentials were recorded *in vivo* from the saccule, the main auditory division of the inner ear of most teleosts, to test the hypothesis that males and females exhibit seasonal changes in hair cell physiology in relation to seasonal changes in plasma levels of steroids. Thresholds across the predominant frequency range of natural vocalizations were significantly less in both sexes in reproductive compared with non-reproductive conditions, with differences greatest at frequencies corresponding to call upper harmonics. A subset of non-reproductive males exhibiting an intermediate saccular phenotype had elevated testosterone levels, supporting the hypothesis that rising steroid levels induce non-reproductive to reproductive transitions in saccular physiology. We propose that elevated levels of steroids act *via* long-term (days to weeks) signaling pathways to upregulate ion channel expression generating higher resonant frequencies characteristic of non-mammalian auditory hair cells, thereby lowering acoustic thresholds.**

Key words: hearing, hormones, hair cell.

## INTRODUCTION

Vertebrate sensory systems undergo ontogenetic changes that can have a profound impact on an organism's ability to hear and communicate acoustically. Mammals show marked decline in auditory sensitivity with age, particularly at higher frequencies within their respective audible ranges (for a review, see Ohlemiller, 2006). Songbirds have been shown to have remarkably stable baseline auditory thresholds throughout ontogeny (Langemann et al., 1999) with species-specific seasonal changes (Lucas et al., 2002; Lucas et al., 2007), whereas studies in fishes have yielded conflicting results (Higgs et al., 2003; Higgs et al., 2002; Iwashita et al., 1999; Kenyon, 1996; Popper, 1971; Sisneros and Bass, 2005). We have investigated peripheral auditory plasticity in a teleost fish, the plainfin midshipman (*Porichthys notatus* Girard 1854; family Batrachoididae), that shows seasonal, reproductive state-dependent plasticity in the ability to encode the upper harmonics of vocalizations (Fig. 1A,B). As females shift from a non-reproductive to a reproductive state, they exhibit a steroid-dependent improvement in frequency encoding by eighth nerve afferents to the saccule (Sisneros and Bass, 2003; Sisneros et al., 2004a), the main auditory division of the inner ear in midshipman and most teleosts (Fig. 1C insert) (McKibben and Bass, 1999; Popper and Fay, 1993). We tested the hypothesis that this plasticity is not sex dependent, with males also exhibiting concurrent shifts in plasma steroid levels and auditory encoding as reflected in frequency sensitivity of the hair cell epithelium of the saccule.

Much of the work on steroid modulation of hearing throughout ontogeny focuses on changes over the entire life span of an animal. For example, human females have enhanced auditory brainstem response performance over males (Don et al., 1993; Jerger and Hall, 1980), but both sexes show marked deficits with increasing age (Jerger and Hall, 1980) that can be slowed with estrogen treatment in postmenopausal women (Kilicdag et al., 2004).

The study reported here is one of a series of midshipman fish studies investigating yearly events as animals transitional between non-reproductive and reproductive states (Sisneros, 2009; Sisneros and Bass, 2003; Sisneros et al., 2004a). So far, this work has focused on females because early behavioral studies showed females exhibiting robust and consistent positive phonotaxis to playbacks mimicking male advertisement 'hums' (Fig. 1A) that females use to localize nesting males (Brantley and Bass, 1994; McKibben and Bass, 1998; McKibben and Bass, 2001). In these studies, males also responded positively to hum playbacks (McKibben and Bass, 1998). In addition, like females, males exhibited increased plasma steroid levels coinciding with gonadal recrudecense prior to onset of reproduction (Brantley et al., 1993b; Knapp et al., 1999; Sisneros et al., 2004b). Lastly, the peripheral auditory system of both sexes expressed androgen and estrogen receptor mRNAs and the steroidogenic enzyme aromatase (also known as estrogen synthase) (Forlano et al., 2005; Forlano et al., 2001; Forlano et al., 2010). Together, these findings strongly suggested that, as in females, peripheral auditory physiology in male midshipman would exhibit seasonal plasticity that parallels changing gonadal status and levels of circulating steroids.

Midshipman fish have two male reproductive morphs. Type I, territorial males build and defend nests, and acoustically court females with hums (Fig. 1A). Type II males sneak or satellite-spawn (fan sperm into the nest) and neither build nests nor engage in acoustic courtship (Brantley and Bass, 1994). Both male morphs and females produce agonistic grunts (e.g. Fig. 1B). Our primary

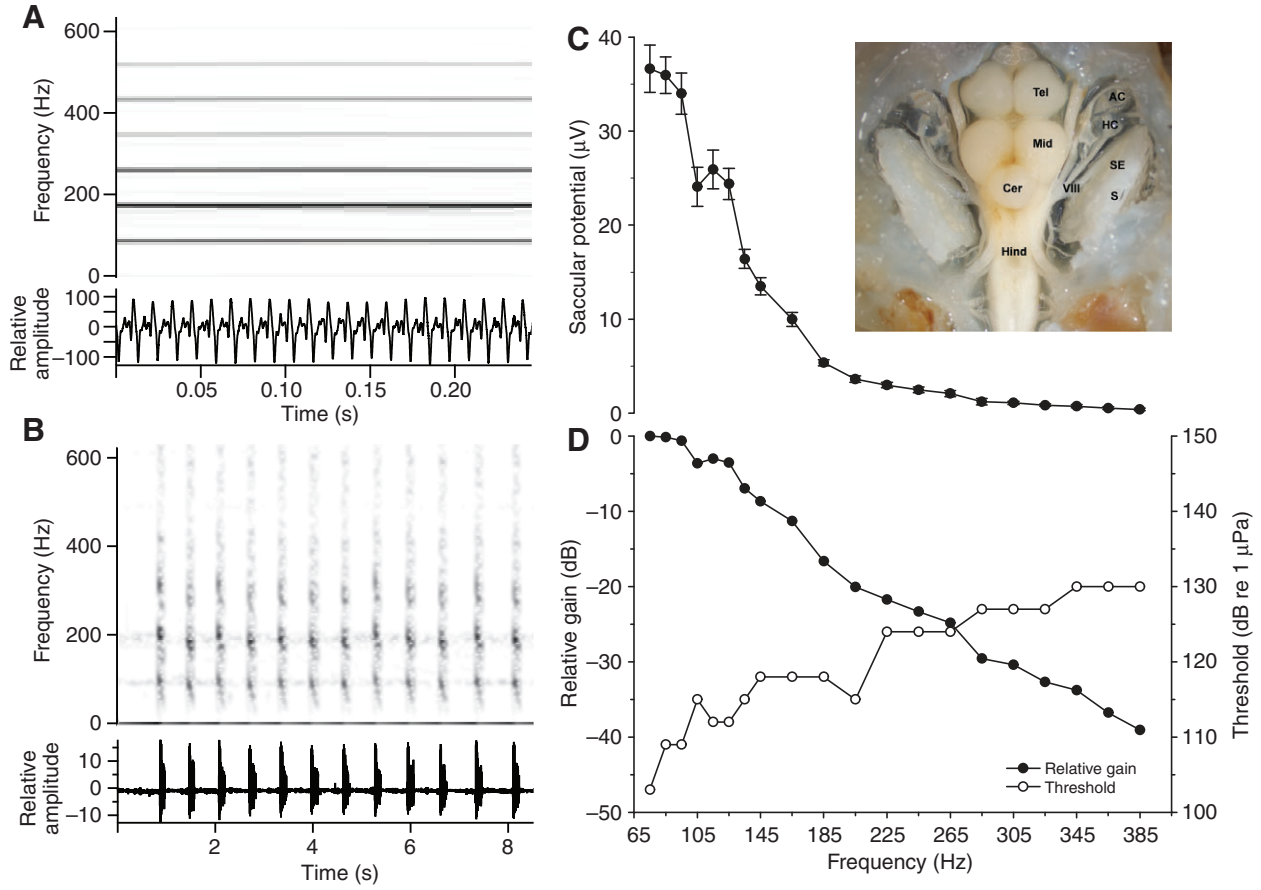


Fig. 1. Plainfin midshipman (*Porichthys notatus*) vocalizations and saccular microphonics. Type I male midshipman vocalizations including a courtship hum (A), and antagonistic 'grunt train' (B) with spectrograms plotted above the waveform for each vocalization. Note the expanded time base in A to show the fine structure of the hum, which lasts for minutes to hours, whereas the complete duration of a series of grunts is visible in B. (C) Representative iso-level response recorded from the saccule in response to single tones at 130 dB. Saccular potentials are plotted as means ± s.d. Inset: dorsal view of midshipman brain and inner ear. AC, anterior canal ampulla; Cer, cerebellum; HC, horizontal canal ampulla; Hind, hindbrain; Mid, midbrain; S, saccule; SE, saccular epithelium; Tel, telencephalon; VIII, eighth cranial nerve. (D) Representative examples of an individual iso-level response curve of evoked saccular potentials and an individual auditory threshold tuning curve obtained from a set of recordings from a reproductive male of which C was a single iso-intensity response. The auditory threshold was defined for each frequency as the stimulus intensity, in dB (re 1 μPa) that evoked a saccular potential ≥2 s.d. above background noise measurements.

goal was to test the hypothesis that type I males exhibit seasonal auditory plasticity at the hair cell level comparable to that recently shown for females (Sisneros, 2009), and that this seasonality correlates with reproductive state and levels of circulating steroid hormones. Type I males were the focus because they show seasonal plasticity in vocal physiology (unknown for type IIs) and wild-caught populations of both non-reproductive and reproductive individuals are readily available (Rubow and Bass, 2009). Our findings are significant in several regards. First, saccular hair cell physiology in males, profiled using auditory evoked potential recordings resembling cochlear microphonics (Furukawa and Ishii, 1967; Sisneros, 2007), showed that seasonal changes in saccular thresholds correlated with fluctuations in circulating steroid levels. Second, we fortuitously collected a subset of non-reproductive males with elevated plasma testosterone levels and a saccular phenotype intermediate between non-reproductive and reproductive conditions. These results were consistent with the dependency of gradual transitions in auditory phenotype in the wild on pre-nesting rises in plasma steroid levels. Additionally, we investigated whether saccular physiology and steroid levels vary as a function of morphometric measures (standard length, relative gonad mass), time in captivity, and time of day. Unlike vocal motor excitability (Rubow and Bass, 2009), we found no measurable diel shifts in saccular hair cell physiology in reproductive males, showing the restriction of this form of acoustic plasticity to comparatively longer-term seasonal patterns.

## MATERIALS AND METHODS

### Animals

This study included 29 type I male and 19 female plainfin midshipman collected during the 2009 non-reproductive (February) and reproductive (May–August) seasons as well as the non-reproductive season of 2010 (December 2009). Reproductive animals were hand collected from nests in the rocky intertidal zone in Tomales Bay, California, USA. Non-reproductive animals were collected by otter trawl off the coasts of California (December 2009) and Washington (February 2009). All animals were shipped to Cornell University and housed in saltwater aquaria at ~16°C and fed a diet of goldfish. During the reproductive season, animals were housed under a 14 h:10 h light:dark cycle with lights out at 18:00 h Eastern Daylight Time whereas non-reproductive animals were housed under a 10 h:14 h light:dark cycle with lights out at 17:00 h

Eastern Standard Time (EST) (Rubow and Bass, 2009). All experiments were conducted during the subjective day except for a subset that were conducted after 18:00 h EST during the subjective night to test the hypothesis that peripheral auditory plasticity has a daily cycle as found in the vocal motor system (Rubow and Bass, 2009).

Type I males caught any time of year are distinguishable from type II males on the basis of multiple somatic traits (Bass, 1996; Brantley and Bass, 1994; Brantley et al., 1993b; Sisneros et al., 2004b). Reproductive state of the animals was confirmed by visual inspection of the gonads (e.g. presence of mature ovarian follicles in gravid reproductive females) as well as by measurement of the gonadosomatic index (GSI; 100 × ratio of gonad mass to body mass – gonad mass) (Tomkins and Simmons, 2002). Additionally, type I male vocal muscle was visually inspected as its enlargement is a prominent secondary sex characteristic induced by elevated plasma androgen levels prior to and during the reproductive season (Brantley et al., 1993a). All methods were approved by the Institutional Animal Care and Use Committee at Cornell University.

### Stimulus generation

Methods were adapted from those previously published (Sisneros, 2007; Sisneros, 2009). Sound stimuli were presented *via* an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA) positioned 10 cm below the fish's head during recordings or hydrophone during calibrations. The speaker was partially embedded in the gravel substrate at the bottom of a 30×24 cm (diameter × height) Nalgene tank; the setup was similar to that used in previous auditory studies of midshipman and other fishes (Bass and McKibben, 2003; Fay, 1990; McKibben and Bass, 1999). Sine wave acoustic stimuli were generated by the reference signal of an SR830 lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA) fed through an audio amplifier that powered the underwater speaker. Custom written MATLAB (MathWorks, Inc., Natick, MA, USA) software was used to control stimulus generation through the lock-in amplifier. Stimuli consisted of 500 ms tones played at 1.5 s intervals repeated eight times. Tones were presented in 10 Hz (75–145 Hz) and 20 Hz increments (165–385 Hz) in random order. For iso-level responses, each frequency was played separately at 130 dB re 1 μPa, the approximate sound pressure level of the fundamental frequency of the male courtship hum (Fig. 1A) measured at a distance (10 cm), which is comparable with that between the fish and the stimulus source in our experimental setup (Bass and Clark, 2003). Stimuli were presented at 3 dB increments above and below 130 dB from 100 to 151 dB re 1 μPa to construct threshold tuning curves (see below).

A Bruel and Kjaer (Norcross, GA, USA) 8103 mini-hydrophone was positioned 10 cm above the underwater speaker, in the position occupied by the head of the fish during physiological recordings, in order to calibrate the stimuli. Relative sound measures were fed from the hydrophone to a SR780 signal analyzer (Stanford Research Systems) for fast Fourier transform analysis and to an oscilloscope for peak-to-peak voltage measurements. Stimulus amplitudes were then adjusted in MATLAB so that relative sound pressures across all frequencies (75–385 Hz) were within a ±1 dB window. Although all calibrations and data are reported as measures of pressure, previous studies have shown that the midshipman peripheral auditory system is primarily sensitive to particle motion and not pressure (Weeg et al., 2002), like many other fishes that lack specialized adaptations for pressure detection (Popper and Fay, 1993). Despite their sensitivity to particle motion, midshipman auditory afferents respond similarly to both iso-pressure stimuli from sound sources such as those used in the present study and vertical acceleration (Weeg et al., 2002). As has been discussed extensively (McKibben and Bass, 1999; Sisneros, 2009; Sisneros and Bass, 2003; Weeg et al., 2002), sound pressure provides one way to measure stimulus intensity; in the case of this study and others that focus on comparisons of peripheral auditory function between populations using fixed stimuli and recording techniques (Sisneros, 2009; Sisneros and Bass, 2003; Sisneros et al., 2004a) absolute measures of stimulus intensity in terms of particle motion are not necessary to make quantified comparisons of relative differences between groups.

### Saccular potential recordings

Recording methods were adapted from those used previously to record saccular potentials in midshipman (Sisneros, 2007; Sisneros, 2009). Briefly, both saccules were exposed by dorsal craniotomy following anesthetization by immersion in 0.025% ethyl *p*-aminobenzoate (benzocaine; Sigma-Aldrich, St Louis, MO, USA) in saltwater followed by intramuscular injection of pancuronium bromide (~0.5 mg $kg^{-1}$; Tocris, Ellisville, MO, USA) for immobilization and subcutaneous injection of 0.25% bupivacaine (~1 mg $kg^{-1}$; Hopsira, Inc., Lake Forest, IL, USA) with epinephrine (0.1 mg $ml^{-1}$; International Medication Systems, El Monte, CA, USA) for analgesia. A denture cream dam ~2 cm high was constructed around the exposed cranium so that the cranium could be lowed below the water line in the experimental tank. The fish was positioned 10 cm above the stimulus speaker in the chamber described above [see fig. 2 of McKibben and Bass for diagram of experimental setup (McKibben and Bass, 1999)]. The experimental tank was placed on a vibration isolation table within an acoustic isolation chamber (Industrial Acoustics, Bronx, NY, USA), and all hardware for stimulus generation and recording of potentials was positioned outside the acoustic chamber. Fish were perfused through the mouth with recirculated chilled (15–16°C) saltwater providing flow over the gills for respiration.

A glass recording microelectrode (1–7 MΩ) containing 3 mol $l^{-1}$ KCl was inserted into the saccular endolymph approximately 2–4 mm from the sensory epithelium in one of several recording positions along the rostral–caudal axis of the epithelium (rostral, middle, caudal) in either the left or right saccule. Saccular potentials were amplified (Model 5A, Getting Instruments, San Diego, CA, USA), band-pass filtered and further amplified (SR650, Stanford Research Systems), and fed into the lock-in amplifier (mentioned above) for analog to digital conversion and signal processing, and stored on a PC computer using custom-written MATLAB software (Fig. 1C). The lock-in amplifier was configured with a time constant of 100 ms and bandwidth of 12 dB. As discussed in more detail below, saccular potentials are evoked at twice the stimulus frequency in fishes including midshipman (Cohen and Winn, 1967; Fay and Popper, 1974; Furukawa and Ishii, 1967; Hama, 1969; Sisneros, 2007; Sisneros, 2009). The lock-in amplifier uses the stimulus frequency as a reference to output a DC signal with voltage proportional to the signal component at a multiple of the stimulus frequency. Except where noted below, the lock-in amplifier was set to generate outputs at twice the reference (stimulus) frequency in order to isolate evoked responses of saccular hair cells. Iso-level responses were normalized in order to control for variability in distance of electrode from sensory epithelium, with all responses expressed relative to 0 dB assigned to the maximum evoked potential at the peak frequency sensitivity (Fig. 1C,D).

Threshold tuning curves were constructed using methods adapted from those previously published (Fig. 1D) (Sisneros, 2007).

Background noise measurements were made at the beginning of each set of recordings with no sound stimulus, and were used to calculate a response threshold for each frequency. Threshold was set as the minimum amplitude acoustic stimulus needed to elicit a response with amplitude greater than 2 standard deviations (s.d.) above the mean background noise measurement. Threshold tuning curves were constructed by comparing mean responses (average of responses recorded during the eight repetitions of the 500 ms acoustic stimulus at a given frequency) recorded in response to sounds in 3 dB increments above and below the 130 dB reference recording, ranging from 100 to 151 dB re 1 μPa.

### Hormone assays

Plasma testosterone concentration was measured in duplicate 50 μl samples using a solid phase 125I radioimmunoassay (RIA) kit (Siemens, Los Angeles, CA, USA). Cross reaction of the testosterone antibody with 5α-dihydrotestosterone was 3.4%, with 11-keto-testosterone was 16%, and the minimum detectable limit of the assay was 0.04 ng ml$^{-1}$, according to the manufacturer. A pooled sample of plasma from four reproductive type I males was used to validate the testosterone assay. The intra- and inter-assay coefficients of variation were 0.03 ($N$=5 replicates) and 0.11 ($N$=4 replicates), respectively.

### Testis histology

Whole testes from males in the winter 2010 were immersion fixed in 0.4% paraformaldehyde in 0.1 mol l$^{-1}$ phosphate buffer overnight at 4°C, washed several times in 0.1 mol l$^{-1}$ phosphate buffer, and cryoprotected in 30% sucrose overnight at 4°C before sectioning at 30 μm in a cryostat. Sections were dried and mounted onto superfrost slides, stained with Cresyl Violet, dehydrated in an ethanol series, cleared with xylene, covered with Permount and then a coverslip placed on top. The presence of mature and immature sperm was then examined under a light microscope.

### Statistical analysis

All statistical analyses were performed in JMP 8 (SAS Institute Inc., Cary, NC, USA). The effect of reproductive state on thresholds and iso-level responses was determined by a multilevel, repeated measures statistical model with reproductive state as the between-subject factor. Iso-level response or threshold was a response variable of each stimulus frequency. All responses across frequencies for a given recording position were nested within a single trial characterized by recording position (rostral, middle, caudal) and side (left, right), which were, in turn, nested within an individual fish that was characterized according to reproductive state. This model also allowed us to test whether recording position or side had an effect on recordings. Additionally, morphometric data on individual animals including standard length (SL), GSI and plasma testosterone levels were added to determine if they explained any additional variance between groups not already accounted for by reproductive condition. For analyses with more than two groups (e.g. comparison of males from three seasons) Tukey–Kramer HSD *post hoc* tests were used to test for differences between pairs of groups. The same model was used to test the effect of time of day and duration in captivity on thresholds and iso-level responses, replacing reproductive state with each of the aforementioned as the character state assigned to each fish within the model. The effect of season on plasma testosterone levels was determined by ANOVA with Tukey–Kramer HSD *post hoc* comparisons between individual groups. The remaining analyses of relationships between GSI, SL, time in captivity, and plasma testosterone levels were performed using ANOVA on simple linear regressions.

## RESULTS

### Methodological verification: frequency doubling of evoked saccular potentials

As reported previously, in midshipman (Cohen and Winn, 1967; Sisneros, 2007; Sisneros, 2009) and other fishes (Fay and Popper, 1974; Furukawa and Ishii, 1967; Hama, 1969), evoked saccular potentials are usually recorded at twice the stimulus frequency. This has been explained by the presence of two populations of oppositely oriented hair cells in fishes, the potentials for which sum non-linearly (Fay, 1974; Fay and Popper, 1974; Furukawa and Ishii, 1967; Hama, 1969). However, some of these same studies reported locations where the evoked response was primarily at the stimulus frequency and not the second harmonic (Cohen and Winn, 1967; Fay, 1974; Furukawa and Ishii, 1967; Furukawa et al., 1972). Previous studies in midshipman only measured responses at both the fundamental and second harmonic at a single iso-intensity (130 dB re 1 μPa; Fig. 1C,D) (Sisneros, 2007; Sisneros, 2009). We sought to rigorously test whether saccular responses at the fundamental and second harmonic were different across stimulus intensities, in part, because of reports of mosquitoes in which oppositely oriented mechanotransducers in the antennae produce a strikingly similar frequency doubling effect as seen in fishes, but only at lower frequencies (Arthur et al., 2010; Tischner, 1953; Wishart et al., 1962). Similarly, Sisneros showed previously that at 130 dB re 1 μPa there appeared to be a frequency (~145 Hz) above which the response at the fundamental was greater than the response at twice the stimulus frequency (Sisneros, 2007). Unfortunately, the responses at the frequencies in question were at or below threshold at 130 dB re 1 μPa, as determined by the electrical noise of the rig. By examining responses over a wider range of stimulus intensities we sought to more rigorously examine whether frequency doubling is present in the saccular responses of midshipman across all stimulus frequencies.

Unlike recording saccular potentials at twice the stimulus frequency, recordings made at the fundamental using the lock-in amplifier (see Materials and methods) were subject to additional sources of electrical noise, including, but not limited to, physical vibration of the recording electrode and electromagnetic fields induced by the underwater speaker. Because it was not possible to physically isolate the underwater speaker from the recording electrode and the placement of electromagnetic shielding between the underwater speaker and the animal could potentially disturb the underwater sound field, we were unable to completely eliminate these sources of noise. As such, we had to use different criteria for determining the threshold for saccular responses. Background noise recordings were made during acoustic playback after each tuning curve with the electrode placed in the saltwater chamber in the position it had been while recording from inside the saccule. Auditory threshold at each stimulus frequency was defined as the lowest stimulus intensity that evoked a saccular potential greater than the noise reading. This was a rather conservative estimate of threshold as it assumed that the noise and evoked saccular responses summed linearly without any phase cancellation between the two signals. A comparison of the threshold tuning curves recorded at the fundamental and twice the stimulus frequency revealed no notable differences between the two recordings ($N$=2, 2) with thresholds varying by only approximately 3 dB at any given frequency. Such a minor difference between tuning curves was equivalent to one step in sound intensity and was within the range of variability observed between threshold tuning curves collected at twice the stimulus frequency. Although we were limited by the noise present in recordings made at the stimulus frequency that was not present in recordings at twice the stimulus frequency, we were reasonably certain that by recording only the responses at

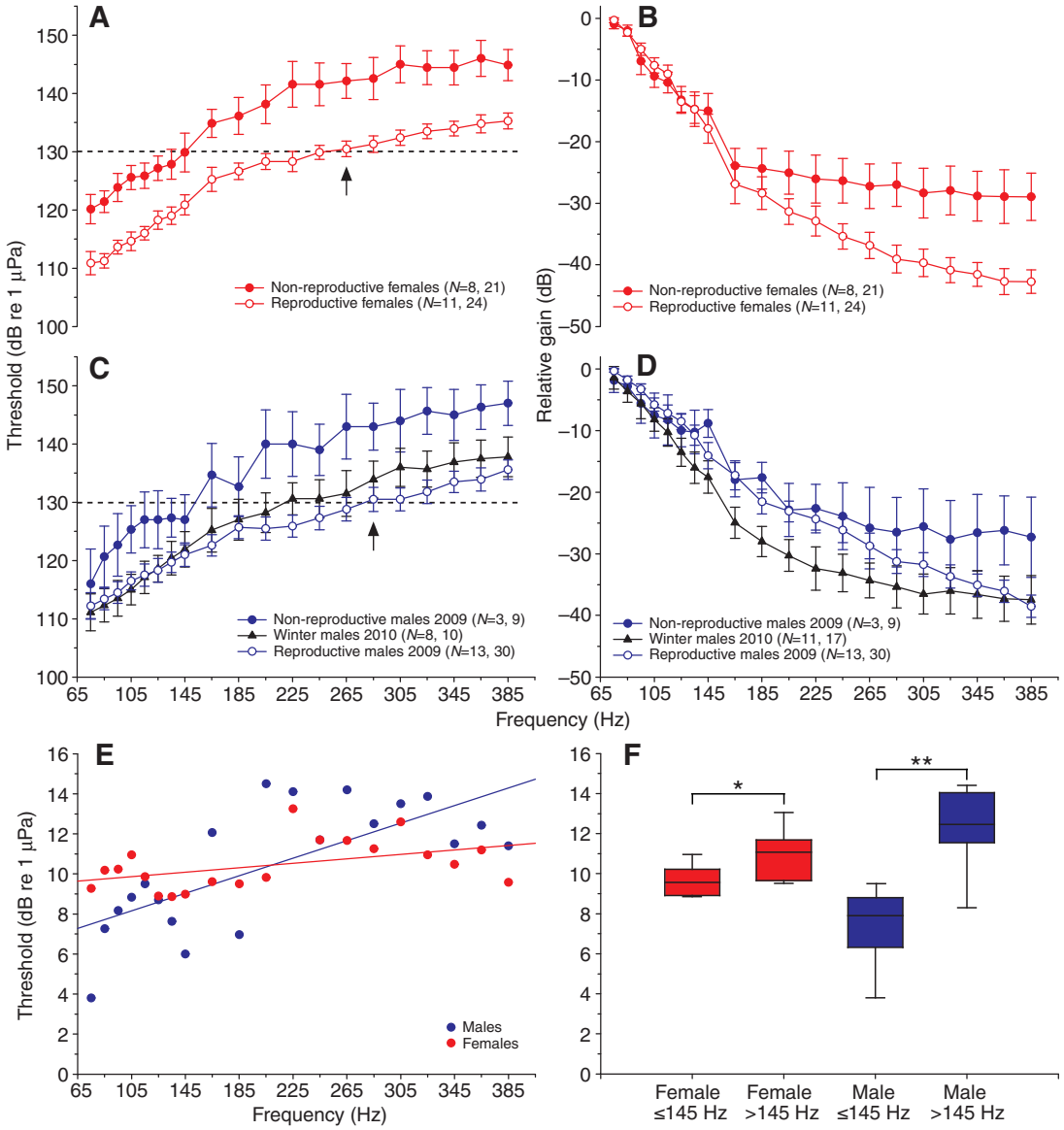


Fig. 2. Saccular thresholds show frequency-dependent seasonal plasticity. (A,C) Auditory threshold tuning curves for non-reproductive and reproductive female (A) and male (C) midshipman based on evoked saccular potentials. Vertical arrows mark frequencies above which threshold is greater than the 130 dB stimulus level used in iso-level responses shown in B and D. (B,D) Evoked potentials recorded from the saccule of non-reproductive and reproductive female (B) and male (D) midshipman in response to iso-level tones of 130 dB (re 1 µPa). Data were normalized with a relative gain value of 0 dB assigned to the peak response for each recording, with all other responses expressed as relative dB re the frequency with the largest response. In A–D data are plotted as means ± 95% confidence limit (CL) with the number of animals and records indicated in parentheses. (E) Change in mean threshold between reproductive and non-reproductive seasons in females (see A) and males (see C) as a function of frequency. (F) Box plot of change in mean threshold between reproductive and non-reproductive seasons in females and males for each frequency or >145 Hz. Asterisks indicate statistically significant difference (*$P$<0.05; **$P$<0.0001) between groups.

twice the stimulus frequency we were not overlooking potentials evoked at the stimulus frequency, as both recording methods yielded similar results. We chose to continue recording saccular responses at twice the stimulus frequency and to use the criteria for determining threshold of responses used previously (Sisneros, 2007; Sisneros, 2009) for all other results reported in this study.

### Seasonal plasticity of saccular sensitivity in females

Given the potential for differences in recording setups and hence acoustics, we first replicated previous studies of seasonal saccular plasticity in female midshipman (Sisneros, 2009) to both determine consistency of the plasticity and test the null hypothesis that, for the same wild-caught population of fish, males would not exhibit seasonal changes in auditory phenotype like females. Evoked saccular potentials were recorded from 19 adult midshipman females: eight non-reproductive females with a size range of 9.7–12.3 cm (SL mean ± s.d.=11.2±0.9 cm) collected in February 2009 and housed in captivity for 14–30 days; 11 reproductive females, 11.6–15.6 cm (SL=13.1±1.1 cm), collected between May and August 2009 and captive for 3–15 days. Auditory thresholds were significantly lower in reproductive than non-reproductive females (multi-level repeated-measures model; between-subject factor reproductive state $P$<0.0001; Fig. 2A) with a significant interaction of frequency and reproductive state ($P$=0.04; see Fig. 2E).

Because there was no significant effect of either side (i.e. left or right saccule; $P$=0.48) or electrode recording position (rostral, middle, caudal; $P$=0.92) on threshold, all recordings were pooled for analysis, as summarized in Fig. 2A. Additionally, there was no effect of SL on threshold ($P$=0.34).

Similarly, iso-level responses of evoked saccular potentials revealed reproductive females had a greater relative gain than non-reproductive females (multi-level repeated-measures model: between-subject factor reproductive state $P$=0.0006; Fig. 2B) with a significant interaction of frequency and reproductive state ($P$<0.0001). Range of relative gain (responses normalized relative to 0 dB assigned to the maximum evoked potential at the peak frequency sensitivity) showed similar differences between reproductive (43 dB) and non-reproductive (29 dB) animals as reported previously (44 and 31 dB, respectively) (Sisneros, 2009). As with threshold tuning curves, there was no significant effect of either side ($P$=0.53) or electrode recording position ($P$=0.61) so all recordings were pooled for analysis and summarized in Fig. 2B.

These results demonstrated that seasonal plasticity of the female saccular phenotype was highly stable; animals collected and tested during 2006 and 2007 (Sisneros, 2009) exhibited similar saccular responses to those in 2009 (current study). These results also confirmed the consistency of recording techniques developed by Sisneros for midshipman and Furukawa for goldfish, and that transporting and maintaining animals at Cornell University did not have any noticeable effect on saccular physiology (Sisneros, 2007; Sisneros, 2009; Furukawa and Ishii, 1967). Thus, we were confident in making comparisons to possible seasonal changes in male auditory phenotypes.

### Seasonal plasticity of saccular sensitivity in males

Evoked saccular potentials were recorded from 16 type I male midshipman: three non-reproductive males with a size range of 10.4–10.8 cm (SL=10.6±0.2 cm) collected during February 2009; 13 reproductive males, 12.2–17.7 cm (SL=14.4±1.9 cm) collected between May and August 2009. There were significant threshold differences between non-reproductive and reproductive males collected in 2009 (multi-level repeated-measures model: between-subject factor reproductive state $P$=0.0069; Fig. 2C) with a significant interaction of frequency and reproductive state ($P$<0.0001; see Fig. 2E). Examination of threshold tuning curves (Fig. 2C) suggested an increase in seasonal differences at frequencies >145 Hz. Seasonal differences in mean threshold were significantly greater at frequencies >145 Hz than at frequencies ≤145 Hz in both males ($P$<0.0001, ANOVA) and females ($P$=0.02, ANOVA; Fig. 2F). Generally, reproductive males had lower thresholds than non-reproductive animals (Fig. 2C). Recordings were pooled between sides ($P$=0.10) and recording position ($P$=0.84). There was no significant effect of SL on thresholds ($P$=0.19). There were also no statistical differences in thresholds between sexes in either reproductive (Tukey–Kramer HSD, $P$=0.99) or non-reproductive (Tukey–Kramer HSD, $P$=0.95) animals.

Unlike females, there was no significant difference in iso-level responses of evoked saccular potentials between reproductive and non-reproductive males collected in 2009 (multi-level repeated-measures model: between-subject factor reproductive state $P$=0.31; Fig. 2D). However, males followed the same trend as females with non-reproductive animals having a smaller range of relative gain of responses (27.3 dB) in the saccule compared with reproductive animals (38.5 dB). Recordings were pooled between sides ($P$=0.47) and recording position ($P$=0.53). The Discussion provides a detailed analysis of discrepancies between threshold and iso-level response data.

Although type I males and females showed similar seasonal plasticity of saccular thresholds between reproductive and non-reproductive seasons in 2009, males collected during the 2010 non-reproductive season (December 2009) showed an intermediate phenotype. Evoked saccular potentials were recorded from 11 type I males with a size range of 13.0–18.8 cm (SL=15.1±1.9 cm). There was an overall effect of collection period on thresholds among the three collecting seasons (multi-level repeated-measures model: between-subject factor collecting season $P$=0.01; Fig. 2C) with a significant interaction of frequency with collecting season ($P$<0.0001). Thresholds were not significantly different between 2010 animals and either non-reproductive (Tukey–Kramer HSD, $P$=0.07) or reproductive (Tukey–Kramer HSD, $P$=0.60) males from 2009 (Fig. 2C). Recordings were pooled between sides ($P$=0.13) and recording position ($P$=0.66). Iso-level responses of evoked saccular potentials were not significantly different between these three groups (multi-level repeated-measures model: between-subject factor collecting season $P$=0.12; Fig. 2D).

Unlike previous analyses, there was a significant effect of SL on threshold ($P$=0.03). Further analyses revealed an absence of any significant interaction between SL and collecting season ($P$=0.74) resulting in loss of significance in the effect of SL on threshold ($P$=0.73). This reflected the overall size difference between non-reproductive (winter; smaller) and reproductive (summer) animals, but indicated the effect of collecting season on thresholds is not due to differences in SL between seasons.

Together, the results demonstrated that males, like females, show frequency-dependent seasonal plasticity in saccular thresholds.

### Daily rhythms in saccular physiology

Type I males show diel and seasonal shifts in vocal motor excitability: compared with non-reproductive males tested any time of day and reproductive males tested during the day, nocturnal reproductive type I males show both a marked increase in duration of, and decrease in threshold to elicit, the vocal motor volley that directly sets natural call duration and frequency (Rubow and Bass, 2009). Because the vocal system has direct input to central auditory circuitry (Bass et al., 1994; Chagnaud et al., 2009; Weeg et al., 2005), a subset of reproductive type I males ($N$=4 animals, nine tuning curves) were tested during the subjective night. Recordings of evoked saccular potentials from animals at night were not markedly different from those recordings made during the day. Thresholds recorded at night (not shown) were not significantly different from those recorded during the day (multi-level repeated-measures model: between-subject factor time of day $P$=0.36) and were within the range of variability seen between individuals recorded during the day and were thus included in the larger data set.

### Plasma testosterone levels

A subset of type I males collected during the non-reproductive (winter) season of 2010 had large, vascularized vocal muscles and/or enlarged testes, traits associated with transitioning from a non-reproductive to reproductive state (Bass, 1996; Brantley and Bass, 1994; Sisneros et al., 2004b). Sections through testes of a subset of winter 2010 males showed the presence of mature and immature sperm (not shown) (see Sisneros et al., 2004b), consistent with earlier studies of midshipman showing an increase in the number of mature sperm during the pre-nesting period leading up to the reproductive season. Male testes collected during the winter 2009 were not sectioned as there was no indication of transitioning from non-

reproductive to reproductive states as described above for winter 2010 animals (i.e. gonads and vocal muscle were small and poorly vascularized). Our previous study showed that the testes of non-reproductive males contain no mature sperm (Sisneros et al., 2004b).

Circulating steroids, including testosterone, increase in both male and female midshipman during the transition from non-reproductive to reproductive states (Sisneros et al., 2004b). Plasma testosterone levels were measured using RIA to determine whether winter 2010 animals were undergoing this transition. Samples were collected following saccular recordings from 40 type I males across three seasons ($N$=29 reproductive, summer 2009; $N$=3 non-reproductive, winter 2009; $N$=8 non-reproductive, winter 2010). Owing to limited plasma volume, several samples were tested only once instead of in duplicate ($N$=5 summer 2009; $N$=3 winter 2009; $N$=3 winter 2010). Plasma testosterone levels were log transformed for statistical analyses because of unequal variance between populations, due, at least in part, to the relatively small sample sizes for winter animals. Log-transformed plasma testosterone levels were significantly different in the three groups of males (ANOVA $P$<0.05; Fig. 3A) with non-reproductive, winter 2010 animals having elevated levels compared with the 2009 reproductive, summer animals (Tukey–Kramer HSD $P$=0.04). Thus, unlike non-reproductive, winter 2009 animals, non-reproductive, winter 2010 males had apparently begun to transition to a reproductive state with gonadal recrudescence and increased testosterone synthesis, consistent with the prevalence of mature sperm and enlarged, vascularized vocal muscle in these males (see above). These differences were the basis for separating the physiological data collected from winter males into two populations based on the year in which they were collected. The threshold data for all three study populations (Fig. 2C) revealed that log plasma testosterone levels had no additional significant effect on threshold ($P$=0.14). Thus, plasma testosterone levels did not account for any additional variance not already accounted for by collecting seasons.

We further examined the relationship between plasma testosterone levels and time in captivity. There was a significant decrease in levels with increased time in captivity in reproductive males (ANOVA $P$=0.02, $R^2$=0.19; Fig. 3B), but no significant change in levels over time in captivity in non-reproductive males pooled across 2009 and 2010 (ANOVA, $P$=0.57, $R^2$=0.04; Fig. 3C). Based on the male reproductive-related decrease in testosterone levels during captivity, the decline in primary afferent tuning in reproductive females after more than 2 weeks in captivity (Sisneros and Bass, 2003), and evidence for decreased reproductive vocal excitability after more than 2 weeks in captivity (A.H.B. and T. Rubow, unpublished observations), we divided the 2009 reproductive male population into two groups: those kept in captivity for less than 15 days (mean for the group of 7±2 days) and those kept for more than 15 days (mean 21.6±5.0 days). Evoked saccular potentials were recorded from 16 reproductive type I males of 12.2–17.6 cm SL (mean, 14.4±1.6 cm) that had been in captivity for longer than 15 days. There was no significant difference in either thresholds (multi-level repeated-measures model: between-subject factor captive duration $P$=0.49; Fig. 4A) or iso-level responses of evoked saccular potentials (multi-level repeated-measures model: between-subject factor captive duration $P$=0.41; Fig. 4B) between reproductive males kept in captivity less than or more than 15 days.

Plasma testosterone levels in non-reproductive (winter) and reproductive (summer) males were further analyzed to see if they correlated with either SL or GSI (see Materials and methods for calculation). GSI levels peak during the pre-reproductive period and decline during the reproductive season (Sisneros et al., 2008). For non-reproductive animals, GSI values were log transformed for statistical analysis because of unequal variances, partly as a result of a relatively small sample size. In males collected during the non-reproductive, winter seasons of 2009 and 2010 there was a significant positive correlation between GSI and plasma testosterone (ANOVA, $P$=0.03, $R^2$=0.44; Fig. 5A), but no significant correlation between either SL and plasma testosterone (ANOVA, $P$=0.60, $R^2$=0.03; Fig. 5B) or SL and GSI (ANOVA, $P$=0.32, $R^2$=0.11; Fig. 5C). Like winter males, reproductive males showed a significant positive correlation between GSI and plasma testosterone (ANOVA, $P$=0.006, $R^2$=0.08; Fig. 5D) that was independent of time in captivity ($P$=0.53). Furthermore, there was no significant relationship between SL and plasma testosterone in reproductive males (ANOVA, $P$=0.79, $R^2$=0.03; Fig. 5E) but a significant interaction between SL

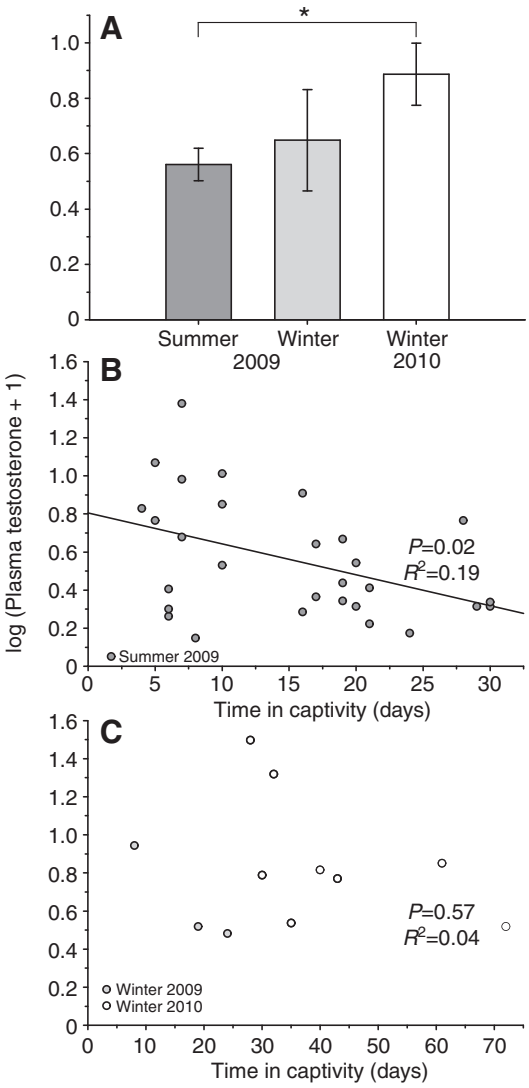


Fig. 3. Plasma testosterone levels (ng ml$^{-1}$) vary with saccular physiology. (A) Log-transformed plasma testosterone levels in male midshipman from the reproductive, summer season of 2009 and the non-reproductive, winter seasons of 2009 and 2010. Data are plotted as means ± s.e.m. *, statistically significant ($P$<0.05) difference between groups. (B,C) Log-transformed plasma testosterone levels in males as a function of time spent in captivity in (B) summer 2009 and (C) winter 2009 and 2010.

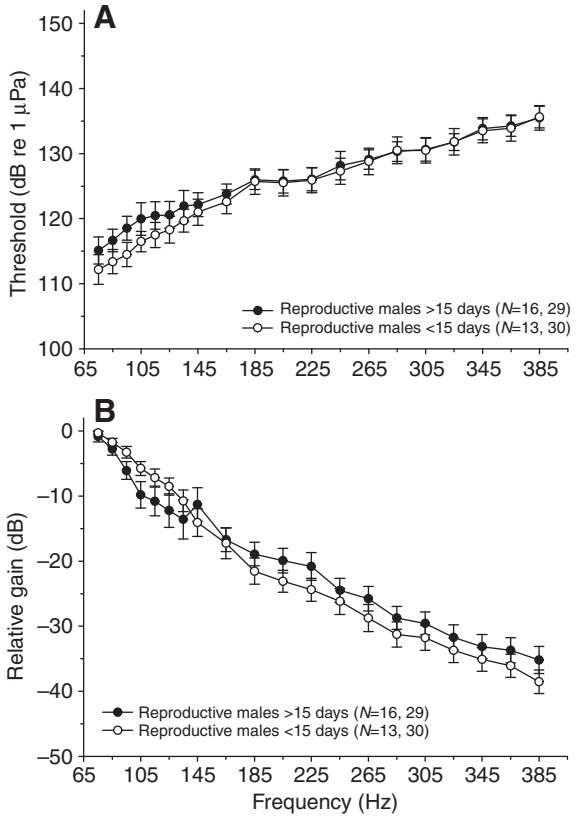


Fig. 4. Reproductive saccular physiology is stable in captivity. (A) Auditory threshold tuning curves for reproductive male midshipman kept in captivity for either less than or more than 15 days, based on evoked saccular potentials. (B) Evoked potentials recorded from the saccule of reproductive males kept in captivity for either less than or more than 15 days in response to iso-level tones of 130 dB (re 1 μPa). Data were normalized with a relative gain value of 0 dB assigned to the peak response for each recording with all other responses expressed as relative dB re the frequency with the largest response. For both A and B, data are plotted as means ± 95% CL and the number of animals and records are indicated in parentheses.

and time in captivity ($P$=0.004). This interaction resulted in a negative correlation between SL and plasma testosterone levels in animals in captivity for less than 15 days. Animals in captivity for more than 15 days showed a positive correlation between SL and plasma testosterone levels. There was no significant relationship between SL and GSI in reproductive males (ANOVA, $P$=0.69, $R^2$=0.01; Fig. 5F). Despite the positive correlation between GSI and plasma testosterone, there was no significant difference in GSI between the three collecting seasons (ANOVA, $P$=0.08). Similar to plasma testosterone levels, GSI had no significant effect on thresholds ($P$=0.65), indicating GSI did no account for any variance not already accounted for by collecting season.

## DISCUSSION

The primary goal of this study was to test the hypothesis that male midshipman fish, like females, undergo seasonal changes in frequency sensitivity of auditory saccular hair cells concurrent with seasonal changes in gonadal state and circulating levels of steroid hormones. We report two significant new findings. First, type I males, who build nests in the intertidal zone from where they acoustically court females, undergo seasonal changes in saccular physiology that are statistically indistinguishable from those of females. This is consistent with evidence from underwater playbacks showing both type I males and females using the type I male hum to localize nests (McKibben and Bass, 1998). Thus, seasonal auditory plasticity is apparently a species rather than a sex-typical trait; we expect type II satellite-spawning males to exhibit the same phenotype because they too show positive phonotaxis to acoustic playbacks of advertisement calls (McKibben and Bass, 1998). Second, we unexpectedly collected a population of type I males from offshore habitats during non-nesting months that were in transition from a non-reproductive to a reproductive state, characterized by elevated plasma testosterone levels and testes with both mature and immature sperm (Sisneros et al., 2004b). These males had saccular hair cell thresholds intermediate between non-reproductive and reproductive males. This provides essential evidence tying naturally occurring seasonal plasticity in saccular physiology to changing reproductive status, including gonadal recrudescence and circulating steroid levels.

### Iso-level responses

As with thresholds, iso-level responses of evoked saccular potentials in female midshipman showed reproductive-state-dependent variation. The consistency of the results with those previously reported (Sisneros, 2009) demonstrates the robustness of seasonal auditory plasticity as well as the reliability of the recording technique by multiple users in different laboratory settings using similar hardware configurations. Although seasonal plasticity in thresholds is consistent between males and females, the relationship is lacking for iso-level response data. We propose that iso-level responses are not valid for seasonal comparisons. Iso-level responses were recorded in this and the earlier study of females (Sisneros, 2009) only at 130 dB re 1 μPa. For non-reproductive animals, frequencies above 145 Hz had thresholds above 130 dB, whereas in reproductive animals frequencies greater than 265–285 Hz fell below the same cut-off (vertical arrows, Fig. 2A,C). Thus, iso-level responses recorded at 130 dB in non-reproductive and reproductive animals are sub-threshold above 145 and 265–285 Hz, respectively. It is at frequencies greater than 145 Hz that iso-level responses at 130 dB begin to diverge between non-reproductive and reproductive females. When using iso-level responses of evoked saccular potentials to quantitatively distinguish auditory function between seasons, one should either record responses at a stimulus amplitude sufficient to elicit supra-threshold responses at all frequencies or limit the analysis to frequencies with supra-threshold responses when only using 130 dB stimuli. We suggest that threshold data are the more robust quantitative measure of saccular hair cell responses because threshold response inherently limits itself to analysis of supra-threshold responses and is internally referenced to the background noise of a given animal and recording site, reducing variability between individuals and/or recordings.

The above conclusion regarding the validity of 130 dB iso-level responses goes some way towards resolving a paradox previously raised for females, namely frequency-dependent seasonal plasticity of iso-level responses, but frequency-independent plasticity in saccular thresholds (Sisneros, 2009). The current study helps resolve this quandary in two ways. First, we propose that the frequency dependence of iso-level response plasticity is an artifact of the recording technique as described above. Furthermore, whereas a prior study (Sisneros, 2009) reported no significant interaction between frequency and reproductive state when examining thresholds, the present study showed a significant interaction for both females and type I males (Fig. 2E). This interaction was more

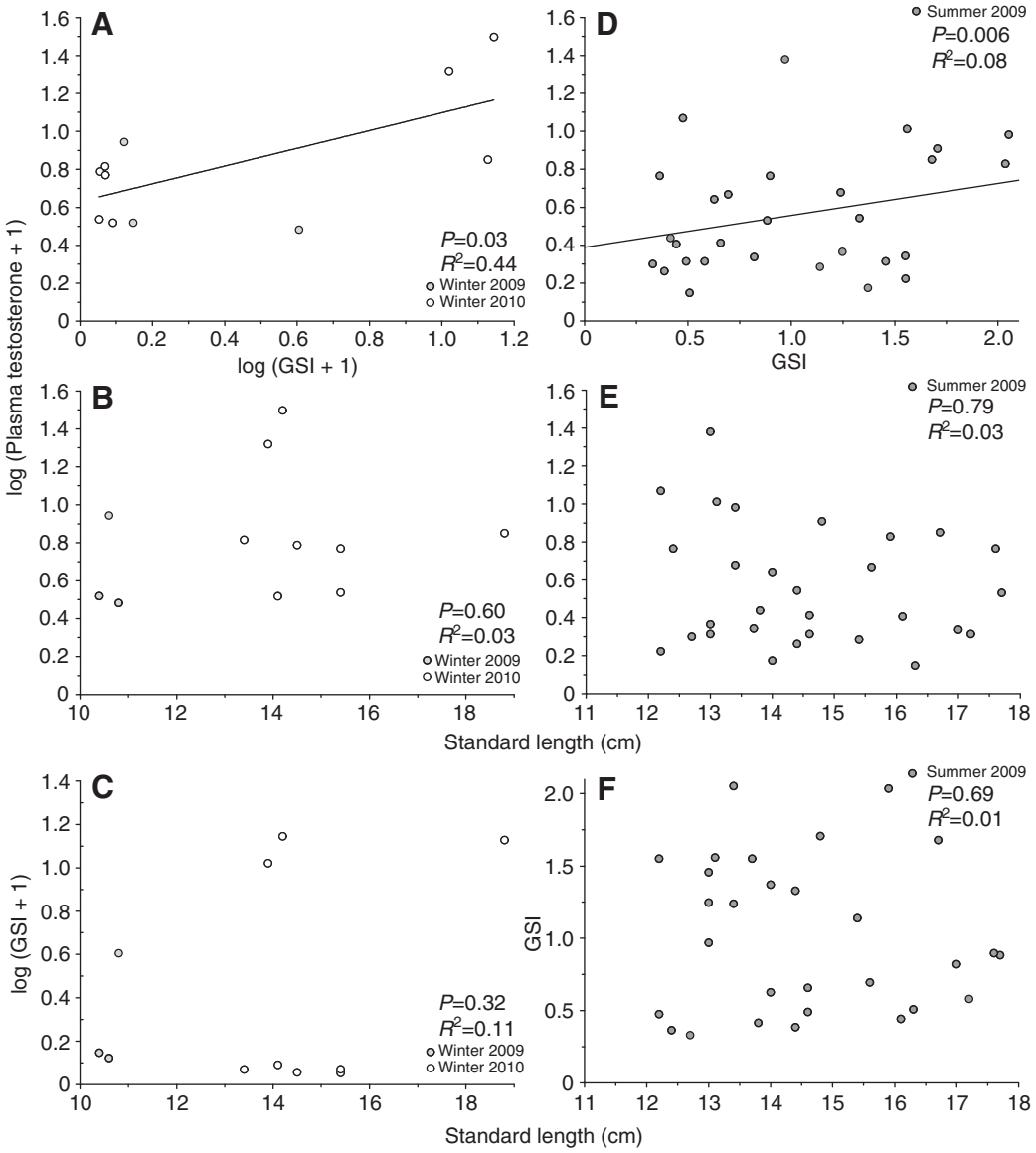


Fig. 5. Plasma testosterone levels (ng ml$^{-1}$) correlate with relative gonad mass. (A,B) Log-transformed plasma testosterone levels in non-reproductive (winter 2009 and 2010) male midshipman *versus* (A) log-transformed gonadosomatic index (GSI) and (B) standard length (SL). (C) Log-transformed GSI of winter male midshipman *versus* standard length. (D,E) Log-transformed plasma testosterone levels in reproductive (summer) male midshipman *versus* (D) GSI and (E) SL. (E) GSI of reproductive (summer) male midshipman *versus* SL.

apparent in male threshold data (Fig. 2C) at frequencies >145 Hz, where seasonal differences are greater. Taken together, these lines of evidence suggest that regardless of the validity of 130 dB iso-level response data, there is a frequency dependence of seasonal plasticity in saccular thresholds.

Resolving this previous conflict in saccular physiology data shows that, similar to primary auditory afferents (Sisneros and Bass, 2003; Sisneros et al., 2004a), saccular hair cells show frequency dependence in their seasonal plasticity, with greater differences occurring at frequencies overlapping the upper harmonics of male calls. This is consistent with the proposed behavioral role of seasonal plasticity in peripheral auditory function enabling detection of higher harmonics (see Fig. 1A,B) in shallow water (Bass and Clark, 2003) (also see Fine and Lenhardt, 1983; Sisneros and Bass, 2003). Similar to hair cell recordings (Fig. 2A,C), auditory afferents in non-reproductive females had weak responses at 130 dB re 1 µPa at frequencies above 140 Hz with the greatest enhancement among reproductive females at frequencies >140 Hz and up to ~340 Hz (Sisneros and Bass, 2003). Hence, the frequency range over which auditory afferent plasticity is greatest, namely >145 Hz, parallels that for saccular thresholds (Fig. 2E,F). We propose that seasonal changes in afferent physiology, specifically the frequency dependence of differences >145 Hz, largely, if not entirely, reflect plasticity in auditory hair cell function.

### Daily rhythms of vocal-acoustic communication

Unlike the vocal motor system (Rubow and Bass, 2009), we found no photoperiod-dependent sensitivity in any measures of evoked saccular potentials in reproductive type I males. The results may reflect the divergent demands on vocal and auditory systems. Although reproductive type I males only produce long-duration (minutes to >1 h) vocalizations at night (Bass et al., 1999; Brantley and Bass, 1994), they produce brief grunts (see Fig. 1B) at all times of the day (Brantley and Bass, 1994; Cohen and Winn, 1967). Thus, although midshipman tend to be more quiescent in general during the light part of the photoperiod (photophase) (Bass, 1996), the auditory system is probably subjected to a similar set of constant demands, resulting in a lack of daily rhythm in its sensitivity. Although our results argue against the presence of daily rhythms in intrinsic saccular physiology, we cannot rule out the alternative possibility that photoperiod-related changes in peripheral physiology depend on acute shifts in central efferent activity that would have

been masked by the paralytic, pancuronium bromide used in our preparation that acts by blocking nicotinic acetylcholine receptors also present at the efferent-hair cell synapse (Glowatzki and Fuchs, 2000). Efferent action on hair cell and afferent physiology in fish lasts in the order of milliseconds (Furukawa, 1981) to more than 1 s (Boyle et al., 2009) in the saccule and semicircular canals, respectively. Thus, any daily rhythm in saccular efferent activity would have to occur over the time course of pancuronium bromide blockade of efferent-hair cell synapses during the *in vivo* preparation used here.

### Timing of annual reproductive rhythms

The observation that type I males collected by trawl in December 2009 were further along in the transition from non-reproductive to reproductive states than the males collected in February 2009 demonstrates the year-to-year variability in the midshipman reproductive cycle. Previous studies on circulating steroid levels as well as gonadal condition conducted over a 2 year period (2001–2002) identified December through February as a non-reproductive period and March through April as the pre-nesting transitional state (Sisneros et al., 2004b). Plasma steroid levels, testis morphology, and auditory physiology all indicated that animals were already in a pre-nesting condition by December 2009. The variability in the annual onset of transition to a reproductive state and subsequent changes to the vocal-acoustic system, including the auditory inner ear and vocal motor system, suggests an equally variable external circannual trigger. The occurrence of El Niño conditions during the winter season of 2010 (Lee and McPhaden, 2010) suggests warming ocean water temperatures may have affected the midshipman breeding population, as has been demonstrated in other fish stocks (Stenseth et al., 2002). Animals may begin the transition from non-reproductive to reproductive state and invest in gonadal development based on internal somatic cues such as nutritional state (Carrillo et al., 2009), which could vary year to year based on food availability due to environmental changes such as water temperature. Regardless of what cues the animal to transition from non-reproductive to reproductive states, this study points out the importance of using a variety of metrics including gonad morphology and circulating steroid levels in determining the reproductive state of the animal, and that collection date alone is not a foolproof indicator of reproductive state for this species.

### Seasonal plasticity in male saccular physiology, gonadal condition and plasma testosterone levels

To our knowledge, this is the first study to demonstrate seasonal plasticity in auditory hair cell physiology in male fish. A previous study of the Hawaiian sergeant damselfish (*Abudefduf abdominalis*) reported no seasonal differences in auditory evoked potentials in either sex (Maruska et al., 2007). Similarly, the Lusitanian toadfish (*Halobatrachus didactylus*), a member of the same family as midshipman (Batrachoididae), does not exhibit seasonal plasticity in saccular thresholds (Vasconcelos et al., 2010). This dichotomy may reflect differences between species that remain on or near their shallow water breeding grounds [Lusitanian toadfish (Amorim et al., 2006); damselfish (Maruska et al., 2007)] and those that migrate from shallow breeding grounds to deeper offshore waters (midshipman) (Sisneros et al., 2004b) where the acoustic cutoff frequency is lower (Bass and Clark, 2003).

Although previous studies have shown reproductive-state-related changes in saccular physiology, it was assumed this plasticity was influenced by steroids (Sisneros, 2009) as is the case with saccular afferents (Sisneros et al., 2004a). The current study is the first to provide evidence that the seasonal state of saccular hair cells correlates with seasonal changes in circulating steroid (testosterone) levels. Although onset of reproductive auditory physiology correlates with increasing plasma testosterone levels, elevated testosterone levels do not appear to be necessary to maintain this phenotype for up to a month (Fig. 3B, Fig. 4). However, circulating plasma testosterone levels may not reflect local levels within the peripheral auditory system. It remains to be determined whether steroids, including testosterone and its metabolites (estradiol, 11-ketotestosterone) (see Bentley, 1998) are necessary and sufficient to induce seasonal changes in saccular hair cell function. The presence of both androgen (Forlano et al., 2010) and estrogen (Forlano et al., 2005) receptors in the saccular epithelium of midshipman as well as the enzyme aromatase, which converts testosterone to estradiol, in the saccular branch of the eighth nerve (Forlano et al., 2005) suggests testosterone may act both directly *via* androgen receptors and indirectly *via* estrogen receptors on saccular hair cells, as proposed for saccular afferents (Sisneros et al., 2004a). This pathway would work similarly in both males and females despite sex differences in circulating steroids (estradiol in females, 11-ketotestosterone in type I males) (Brantley et al., 1993b; Knapp et al., 1999; Sisneros et al., 2004b).

### Mechanisms for saccular hair cell plasticity

Several studies describe seasonal changes in midshipman auditory physiology (Sisneros, 2009; Sisneros and Bass, 2003) (current study) but little progress has been made in identifying the mechanism(s) of such plasticity except for identifying that it can be induced, at least at the afferent level, by steroid implants for 3–5 weeks (Sisneros et al., 2004a). The occurrence of estrogen receptors in the auditory epithelium of vertebrates including fishes [midshipman (Forlano et al., 2005); cichlid (Maruska and Fernald, 2010)], songbird (Noirot et al., 2009), rodents (Stenberg et al., 1999) and humans (Stenberg et al., 2001) suggests a widespread occurrence of steroid-dependent auditory plasticity among vertebrates. Several mechanisms might account for this plasticity (see also Sisneros et al., 2004a). The first is hair cell addition. Corwin correlated auditory hair cell addition into adulthood in sharks with increased auditory sensitivity (Corwin, 1983). Pre-nesting increases in local estrogen due to either circulating estrogen (females) or local aromatization of circulating testosterone (both sexes) within the saccule may trigger hair cell proliferation prior to the reproductive season as has been shown in midshipman vocal muscle (Brantley et al., 1993a). Although hair cell addition to the saccule of reproductive midshipman might account for a general decrease in thresholds compared with non-reproductive animals, the properties of these new hair cells would have to be such that a higher proportion of them have higher best frequencies in order to account for the frequency dependence of seasonal changes in saccular thresholds.

Seasonal changes in the ionic composition of saccular endolymph could result in changes to the ionic driving forces for currents that play roles in electrical tuning of hair cells. It has been proposed in mammals that estrogen receptors alpha and beta, both of which are present in cells of the stria vascularis and Reissner's membrane involved in ion and fluid homeostatis, may alter ionic gradients within the inner ear (Stenberg et al., 1999). Estrogen receptor mRNA is expressed in supporting cells with similar functions within the saccular epithelium of midshipman (Forlano et al., 2005) [see Forlano et al. (Forlano et al., 2010) for androgen receptor]. Estrogen inhibits ion transport through $K^+$ channels in the stria vascularis of gerbils *via* non-genomic mechanisms, which potentially include

membrane estrogen receptors or direct interactions with ion channel protein complexes (Lee and Marcus, 2001). Although the time course of seasonal changes in midshipman suggests a long-term, transcriptionally dependent response, estrogen may be changing ion channel expression to produce protracted changes in endolymph composition.

A potential mechanism with the appropriate time scale and frequency specificity is the seasonal change in expression of ion channels regulating the electrical resonance, and thus frequency selectivity, of auditory hair cells. As noted previously (Sisneros and Bass, 2003), a similar mechanism for steroid-dependent frequency sensitivity has been proposed for electroreceptors in weakly electric fish (Zakon and Meyer, 1983). One of the primary determinants of electrical resonant frequencies of vertebrate auditory hair cells is the expression of large conductance, calcium-activated potassium (BK) channels (Fettiplace and Fuchs, 1999). The frequency range at which BK channel diversity accounts for electrical tuning in turtles and other non-mammalian vertebrates (Fettiplace and Fuchs, 1999) encompasses the frequency range of midshipman hearing. BK channels play an analogous role in the membrane oscillations of saccular hair cells in goldfish (Sugihara, 1994; Sugihara and Furukawa, 1989), and have been identified as a major outward current in saccular hair cells of toadfish (*Opsanus tau*) (Steinacker and Romero, 1991), a species in the same family (Batrachoididae) as midshipman (Nelson, 2006). The rapid kinetics of both activation and deactivation of BK currents are necessary for electrical resonance in toadfish saccular hair cells (Steinacker and Romero, 1992).

The *slo1* gene that encodes the pore-forming α-subunit of BK channels is duplicated in midshipman and both genes are expressed in the saccular epithelium (Rohmann et al., 2009). Expression of transcripts of one of these two genes (*slo1a*) is upregulated in saccular epithelium of reproductive compared with non-reproductive adults (Rohmann and Bass, 2010). Whether upregulation of *slo1* expression during the reproductive season results in changes in BK currents, and is regulated by testosterone (e.g. Mahmoud and McCobb, 2004) and/or estrogen (e.g. Holdiman et al., 2002; Zhu et al., 2005) to account for steroid-dependent physiological plasticity are questions we are currently investigating.

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