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ORIGINAL PAPER

Ontogeny of auditory saccular sensitivity in the plainfin midshipman fish, *Porichthys notatus*

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Abstract The auditory system of the plainfin midshipman fish, *Porichthys notatus*, is an important sensory receiver system used to encode intraspecific social communication signals in adults, but the response properties and function of this receiver system in pre-adult stages are less known. In this study we examined the response properties of auditory-evoked potentials from the midshipman saccule, the main organ of hearing in this species, to determine whether the frequency response and auditory threshold of saccular hair cells to behaviorally relevant single tone stimuli change during ontogeny. Saccular potentials were recorded from three relative sizes of midshipman fish: small juveniles [1.9–3.1 cm standard length (SL), large juveniles (6.8–8.0 cm SL) and non-reproductive adults (9.0–22.6 cm SL)]. The auditory evoked potentials were recorded from the rostral, middle and caudal regions of the saccule while single tone stimuli (75–1,025 Hz) were presented via an underwater speaker. We show that the frequency response and auditory threshold of the midshipman saccule is established early in development and retained throughout ontogeny. We also show that saccular sensitivity to frequencies greater than 385 Hz increases with age/size and that the midshipman saccule of small and large juveniles, like that of non-reproductive adults, is best suited to detect low frequency sounds (<105 Hz) in their natural acoustic environment.

Keywords Auditory-evoked potentials · Hair cells · Hearing · Saccule · Fish hearing

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Introduction

Sensory systems are important to animals for the detection of biologically relevant stimuli throughout their life history and allow animals to respond to potential threats, detect prey, develop cognitive sensory maps of their surrounding environment, and communicate with conspecifics. Morphological and physiological changes that occur during sensory development can presumably influence behaviors that are adaptive for survival and reproduction (Noakes and Godin 1988; Dangles et al. 2006; Macintosh and Duston 2007; Gannon 2007). Studies that examine ontogenetic changes in the morphology and physiology of sensory systems may ultimately provide valuable insight into how such changes shape the expression of age-dependent adaptive behaviors.

The auditory system is a good example of an important sensory system that is used to extract biologically important information from the natural environment. The acquired acoustic information can then be used to guide and coordinate behavior necessary for survival and reproduction. One auditory system that has become a good model for investigating neural mechanisms of auditory perception that may be shared by all vertebrates is that of the plainfin midshipman fish (*Porichthys notatus*), in part

because acoustic communication plays an important role in the social and reproductive behavior of this species (Bass and McKibben 2003; Sisneros 2009a). Female midshipman use their auditory system to detect and locate nocturnally active, "singing" males that produce multiharmonic advertisement calls to court and spawn with during the breeding season. While it is well established that the adult midshipman auditory system is adapted to encode the acoustic signals of conspecifics and functions in acoustic communication (Bass and McKibben 2003; Bass 2006; Sisneros 2009b), less is known about the response properties and function of this sensory receiver system in the pre-adult life history stages. It remains to be unclear whether juveniles are vocally active during early life history stages and whether the auditory system of juveniles functions in acoustic communication similar to that of adults. Numerous studies have examined ontogenetic changes in the structure and function of the auditory sensory system in various other vertebrates including mammals, birds and amphibians (e.g., see Gray and Rubel 1985; Walsh et al. 1986; Mills et al. 1990; Dmitrieva and Gottlieb 1992; Geal-Dor et al. 1993; Brittan-Powell and Dooling 2000), but relatively few studies have examined ontogenetic changes in the neurophysiological response properties of the auditory system, especially in ancestral vertebrates such as fishes (Popper 1971; Corwin 1983; Kenyon 1996; Iwashita et al. 1999; Wysocki and Ladich 2001; Higgs et al. 2002, 2003; Egner and Mann 2005; Parmentier et al. 2009; Lechner et al. 2010).

Sisneros and Bass (2005) investigated age-related changes in the response properties of the midshipman peripheral auditory system and showed that the resting discharge rate and sensitivity at best frequency (BF) of saccular afferent neurons increased with age/size during ontogeny. Remarkably this study represents the only investigation to date to report ontogenetic changes in the encoding properties of individual auditory neurons for any fish species, which in part is likely due to the difficult nature of these experiments and the rather robust stress tolerance of *P. notatus* for single unit recording methods. Although Sisneros and Bass (2005) provided important data on the changes in the response properties of saccular afferents during ontogeny, their study was to some extent limited because it only reported saccular afferent responses at one sound pressure level (130 dB re 1uPa) due to the limited survival time of individual fish, especially for the smallest juvenile size class. More recently, Sisneros (2007) developed an evoked potential recording technique to determine the frequency response of hair cells within the saccule that can readily be used with smaller fish and is more amenable for investigating ontogenetic changes in auditory saccular sensitivity.

The purpose of this study was to characterize the auditory-evoked potentials from the saccule of *P. notatus* to determine the auditory threshold and frequency response of saccular hair cells to behaviorally relevant single tone stimuli during ontogeny. Here, we compare the saccular hair-cell frequency response properties of three different age/size classes of fish and interpret our findings as they relate to possible age-related adaptations of the midshipman auditory system for survival and communication.

Materials and method

Animal collection and care

We collected both male and female plainfin midshipman fish and grouped them into three classes based on sizes used in previous research (Sisneros and Bass 2005): adults, large juveniles, and small juveniles. Adults were defined as those fish greater than 9 cm standard length (SL), large juveniles were between 6.8 and 8 cm SL, and small juveniles were less than 3.2 cm. Adults and large juveniles were collected in mid February 2006 and in early March 2007 via otter trawl in Puget Sound near Edmonds, WA ($n = 8$ adults; R/V Kittiwake, Bio-Marine Enterprises) and in Monterey Bay near Moss Landing, CA ($n = 24$ adults, 12 large juveniles; R/V John H. Martin, Moss Landing Marine Laboratories), respectively. Small juveniles (<3 cm SL) were collected from intertidal breeding areas during July and August as embryos attached to the underside of rocky nests in Tomales Bay near Marshall, CA ($n = 42$). After being collected, the small juveniles were temporarily maintained in coolers with aerated seawater until they could be transported to temporary holding tanks at the Bodega Marine Laboratory in Bodega Bay, CA and then finally transported to the University of Washington, Seattle, USA.

Embryos attached to small rocks were kept on their natal rock in aquaria until they became large enough to detach naturally and become free-swimming juveniles. Small juveniles were maintained to a size of 2–3 cm SL at which point they were used in experiments. All housed fish were maintained in chilled saltwater aquaria at 15°C and fed a diet of live fish and brine shrimp two to three times a week. Fish were kept on a reversed light cycle so that experiments could be performed during the day (dark phase) when midshipman would be most active, since *P. notatus* is nocturnal.

Because the reproductive status of the adult midshipman is known to affect hearing sensitivity (Sisneros and Bass 2003), the ratio of gonad mass to body mass (gonad somatic index or GSI; defined here as 100 * (gonad mass/body mass—gonad mass), according to Tomkins and Simmons 2002) was measured to determine the reproductive status of the experimental fish. All fish used in this

study were in non-reproductive condition (Brantley et al. 1993; Grober et al. 1994; Bass et al. 1996).

Experimental procedures

Surgical procedures for exposing the saccule were similar to those used in previous studies (Sisneros 2007, 2009b). Fish were anesthetized by immersion in a 0.025% ethyl p-aminobenzoate saltwater bath followed by an intramuscular injection of pancuronium bromide (0.5 mg/kg) for immobilization. We then injected 0.25% bupivacaine (1 mg/kg) at the incision site for local analgesia. The saccule was exposed via a dorsal craniotomy and then teleost ringer solution was added to the cranial cavity as needed to prevent drying. A denture cream dam approximately 2–3 cm high was built around the cranial opening that allowed the entire animal to be lowered just below the surface of the water. During the experiment fresh chilled seawater (15 ± 1°C) was pumped into the mouth and over the gills. We monitored blood flow in the dorsal vasculature of the brain to ensure the animal was alive and had adequate oxygen since the brain and saccule is sensitive to low oxygen levels. The experimental fish were placed in a Nalgene tank (30 cm diameter, 24 cm high) similar to Fay (1990) and positioned 10 cm above the surface of an underwater speaker that was embedded in gravel. The tank was located on a vibration isolation table housed inside an acoustic isolation chamber (Industrial Acoustics, New York, NY). All of the recording and stimulus generation equipment was located outside the isolation chamber.

Stimulus generation

Acoustic stimuli were generated using the sinusoidal output signal from a lock-in amplifier (Stanford Research Systems SR830) that passed the stimulus signal through an audio amplifier to an underwater loud speaker (UW-30, Telex Communications, Burnsville, MN). Prior to each experiment we tested the speaker's frequency response characteristics by placing a mini-hydrophone (Bruel and Kjaer model 8103) 10 cm above the underwater speaker, in the position normally occupied by the fish's head during an experiment, and then measured the peak-to-peak voltage on an oscilloscope. This peak-to-peak voltage was then used by custom Matlab software to control an automated compensation script to calibrate the speaker so that pressure level at all test frequencies (75–1,025 Hz) was of equal amplitude within ±2 dB re 1μPa. We then made sound pressure measurements of the stimulus frequencies relative to each other using a spectrum analyzer (Stanford Research Systems SR780) to verify the speaker calibration. Test frequencies were 500 ms pure tones presented at 10 Hz increments from 75 to 85 Hz, 40 Hz increments from 105 to 785, and 80 Hz increments from 865 to 1,025 Hz. We presented 10 repetitions of each tone at a rate of 1 tone every 1.5 s. In order to measure threshold tuning responses, pure tone stimuli were presented in 3 dB increments at sound pressures from 97 to 154 dB re 1μPa.

To measure and compare the evoked iso-level responses of the saccule, we recorded the saccular potentials for each test frequency at a sound pressure level of 130 dB re 1μPa. In order to control for differences in the absolute magnitude of the evoked saccular potentials and compare the shape of the iso-level response profiles, we normalized the iso-level response data by expressing the saccular potential data relative to a value of 0 dB that was assigned to the maximum evoked potential at the corresponding stimulus frequency (i.e., best frequency). The normalized data were then used to construct the iso-level response profiles. The sound pressure level of 130 dB re1μPa was used in this study because it is consistent with biological relevant sound pressure levels of type I midshipman calls (e.g., the male advertisement call or "hum") that have been recorded near nest sites (Bass and Clark 2003) and can be used for comparison with previous studies (Sisneros 2007, 2009b).

Although batrachoidid fish such as toad fish and midshipman, which lack specialized structures for hearing, are thought to primarily detect acoustic particle motion, we report in this study hearing thresholds in terms of sound pressure for technical reasons and for the comparison with our previous findings (Sisneros 2007, 2009b). We recognize that the use of sound pressure to describe hearing thresholds should not be considered in terms of absolute values but it should provide an interpretable measure of sound stimuli as proposed in other studies (Vasconcelos and Ladich 2008; Vasconcelos et al. 2007; Wright et al. 2010; Casper and Mann 2009). The determination of sound level in terms of particle motion or displacement is difficult due to the confounding nature of the directionality of particle motion within small tanks (Parvulescu 1967; Fay and Popper 1980). Although sound pressure is scalar and does not have a vector component, previous studies have confirmed that the primary axis of acoustic particle motion in this type of tank is primarily vertical and orthogonal to the surface plane of the underwater speaker (McKibben and Bass 1999). Other studies have also confirmed that the reflection of the acoustic stimuli from the walls and water surface in tank of this type does not alter the sound pressure waveform of the acoustic signal (Bodnar and Bass 1997, 1999). For a more extended discussion of this issue see McKibben and Bass (1999); Weeg et al. (2002); Sisneros (2007).

Saccular potential recordings

Methods for recording saccular potentials from the midshipman were adapted from previous studies in goldfish (Furukawa and Ishii 1967; Furukawa et al. 1972; Fay and

Popper 1974) and were the same as those in previous midshipman studies (Sisneros 2007, 2009b). Saccular potential recordings were made using glass microelectrodes (tip diameter: 1–2 μm) filled with 3 M KCl (1–10 MΩ). Electrodes were visually guided into the endolymph of the saccule roughly 2–5 mm from the closest hair cell bed (saccular macula) in either the left or right saccule. The smallest juveniles tested were unable to survive a complete dorsal craniotomy, so the tissue above the skull dorsal to the saccule was removed and the electrode was inserted through the skull and into the saccule. In these small juveniles, the otoliths were readily visible through the thin translucent skull and were used as a reference to guide the placement of the electrode into the saccule. The electrode was placed in one of three positions within the saccule: rostral, middle, or caudal (Sisneros 2007). Analog saccular potentials were preamplified (10×, Getting 5A), input into a lock-in amplifier (10×, SR830, Stanford Research Systems) and then stored on a computer running a custom data acquisition Matlab script. The lock-in amplifier yields a DC voltage output that is proportional to the component of the signal whose frequency is locked to the reference frequency. The reference frequency was set to the second harmonic of the stimulation frequency (i.e., twice the stimulation frequency) while the sensitivity of the lock-in amplifier was set to 50 mV with a time constant of 100 ms. The lock-in amplifier filters out noise signals at frequencies other than the reference. We used the second harmonic of the stimulus frequency as the reference frequency because the greatest evoked potential from the saccule of teleost fishes occurs at twice the stimulus frequency due to the nonlinear response and opposite orientation of hair cell populations within the saccule (Zotterman 1943; Cohen and Winn 1967; Furukawa and Ishii 1967).

Background noise measurements were performed prior to recording each threshold tuning curve and used for determining the threshold. Noise measurements were similar to that of the saccular potentials recordings with sound but instead were performed by recording ten repetitions at each stimulus frequency with the loud speaker turned off so that no auditory stimulus was present. Auditory threshold was designated as the lowest stimulus level at each stimulus frequency that evoked a response that was at least two standard deviations above the background noise measurement. We considered any response greater than this threshold criterion an evoked saccular potential. Threshold tuning curves were constructed by recording the lowest stimulus level that evoked a saccular potential for each stimulus frequency.

Statistical analysis

Iso-level data was analyzed using a standard one-way ANOVA to determine the effects of size class and recording position on BF, the magnitude of the evoked potential, relative gain (sensitivity) and relative range of the saccular response. When a significant omnibus test resulted, the data were further analyzed using a Bonferoni posthoc test for multiple planned comparisons (Howell 2007). We were unable to use repeated measures ANOVA to analyze the average threshold tuning curve data, due to the amount and uneven distribution of missing values (missing data was concentrated at higher test frequencies). Missing values resulted when we were unable to record an evoked potential at a particular test frequency within the experimental amplitudes used (97–154 dB re 1μPa). We instead used growth curve modeling (Llabre et al. 2004) to analyze the threshold tuning data. The effects of size class, recorded saccular region (rostral, middle, caudal), and sex on auditory threshold were determined using an ANOVA on the regression coefficients of the growth curve modeled data followed by Bonferoni posthoc test for multiple planned comparisons. Analyses were carried out on computer using SPSS and systat statistical software with alpha set to 0.05 for all tests.

Results

Iso-level response of the saccular potentials

We recorded auditory evoked saccular potentials from three relative sizes of midshipman fish: small juveniles, ranging in size from 1.9 cm to 3.1 cm SL (mean SL = 2.5 ± 0.2 SD cm, $n = 42$); large juveniles, ranging in size from 6.8 cm to 8.0 cm SL (mean SL = 7.6 ± 0.4 SD cm, $n = 12$); and non-reproductive adults, ranging in size from 9.0 cm to 22.6 cm (mean SL = 13.8 ± 3.5 SD cm, $n = 32$) with GSIs that ranged from 0.036 to 1.486 (mean GSI = 0.238 ± 0.414 SD) for male midshipman, and from 0.234 to 12.809 (mean GSI = 1.413 ± 3.182 SD) for female midshipman. Iso-level response profiles of the evoked saccular potentials were generated from the presentation of pure tone stimuli that ranged from 75 to 1,025 Hz at 130 dB (re 1μPa). Figure 1 shows representative iso-level response curves of the evoked saccular potentials from the three size classes. In general, the iso-level profiles from the three size groups consisted of response curves that had BFs ≤85 Hz (BFs, defined as the frequency that evoked the greatest saccular potential) with evoked potentials rapidly declining above BF to that of the baseline levels below threshold (noise levels) above 145–185 Hz. Because there were no differences in the BFs of non-reproductive adults collected in California (mean BF = 80 ± 2 SD Hz) and Washington (mean BF = 82 ± 3 SD Hz) (t test, $t = 0.29$, $p = 0.77$), the adult data were pooled and then used to compare with that of small and

large juveniles collected from the California midshipman population. BFs ranged from 75 to 145 Hz for all three size groups, with the majority of BFs occurring at 75–85 Hz (small juvenile = 88%, large juvenile = 95%, adults = 91%). The mode of BFs based on the iso-level response curves did not differ among the three size classes (one-way ANOVA, $F = 0.876$, df = 2, 102, $p = 0.42$). Nor did the mode of BFs differ among the three recording positions, rostral, middle, and caudal (one-way ANOVA, $F = 0.069$, df = 2, 102, $p = 0.934$).

Although there were no differences in BFs among the three size classes, there were significant differences in the magnitudes of the evoked potentials recorded from the saccule in juveniles and adults. The mean response magnitude of the saccular potentials was greater in small juveniles (35.18 ± 35.78 SD μVs, $n = 42$ records) as compared to that of adults (18.93 ± 20.51 SD μVs, $n = 43$ records) and large juveniles (9.46 ± 11.89 SD μVs, $n = 20$ records) (one-way ANOVA, $F = 7.397$, df = 2, 102, $p < 0.001$, Bonferroni post hoc tests: between small juvenile and adults, $p < 0.05$; between small juveniles and large juveniles, $p < 0.005$). There were no differences in the evoked potentials recorded from the saccule of large juveniles and adults (Bonferroni post hoc, A*LJ, $p = 0.58$).

In order to compare the range of the response magnitudes or relative gain (sensitivity) of the evoked saccular potentials for each size class, the iso-level data were normalized and expressed relative to a value of 0 dB at the BF in each recording and then averaged to construct a relative gain plot (Fig. 2). Although there was no difference in the range of relative gain from 75 to 945 Hz between small juveniles (range 43 dB) and adults (range 37 dB), the range of relative gain across test frequencies for small juveniles was 12 dB greater than that of large juveniles (range 31 dB) (ANOVA, $F = 5.784$, df = 2, 102, $p < 0.005$, Bonferoni post hoc test: between small juvenile and adults, $p = 0.27$; between small juveniles and large juveniles, $p < 0.005$). Thus, there were no differences in the shape or range of the relative gain of the iso-level response profiles between the three size classes when expressed by recording position.

Auditory saccular sensitivity

Auditory threshold tuning curves were constructed for whole populations of hair cells in the rostral, middle, and caudal regions of the saccule in all three size classes of fish. Representative tuning curves from the three recording regions and size classes are shown in Fig. 3. In general, the threshold tuning curves consisted of profiles with lowest thresholds at frequencies ≤145 Hz that increased steadily to highest thresholds at frequencies above 545 Hz. Best frequencies (BF, defined as the frequency that evoked the lowest saccular potential threshold) ranged from 75 to 145 Hz for all three size classes, and the threshold at BF ranged from 97 to 139 dB (re 1μPa) for the three size classes. The distribution of BFs based on the threshold tuning profiles did not differ by saccular recording region (rostral, middle, or caudal) (two-way ANOVA, effect of recording position, $F = 0.14$, df = 2, 96, $p = 0.87$) or by size class (two-way ANOVA, effect of size class, $F = 0.897$, df = 2, 96, $p = 0.41$; interaction of size class and recording position, $F = 0.791$, df = 4, 96, $p = 0.53$). Also, the threshold at BF also did not differ by recording position (two-way ANOVA, effect of recording position, $F = 3.053$, df = 2, 96, $p = 0.052$) or by size class (two-way ANOVA, effect of size class, $F = 2.079$, df = 2, 96, $p = 0.131$; interaction of size class and recording position $F = 1.101$, df = 4, 96, $p = 0.361$).

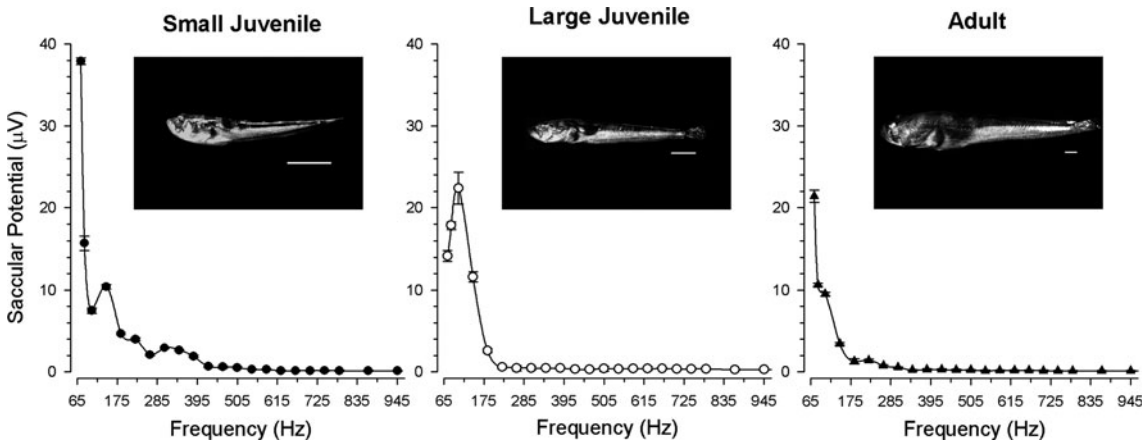

Fig. 1 Representative iso-level response profiles of evoked saccular potentials to pure tones at 130 dB (re 1 μPa) recorded from small juvenile (*solid circles*), large juvenile (*open circles*), and adult (*solid triangles*) midshipman fish (Scale bar = 1 cm). The data plotted represent the mean evoked potential for 10 stimulus presentations and are plotted as means ± 1 SD (some SD bars are obscured by the *symbols*). Note the similar shape of the response profiles for the three size classes and that the greatest evoked saccular potentials occur at the lower frequencies

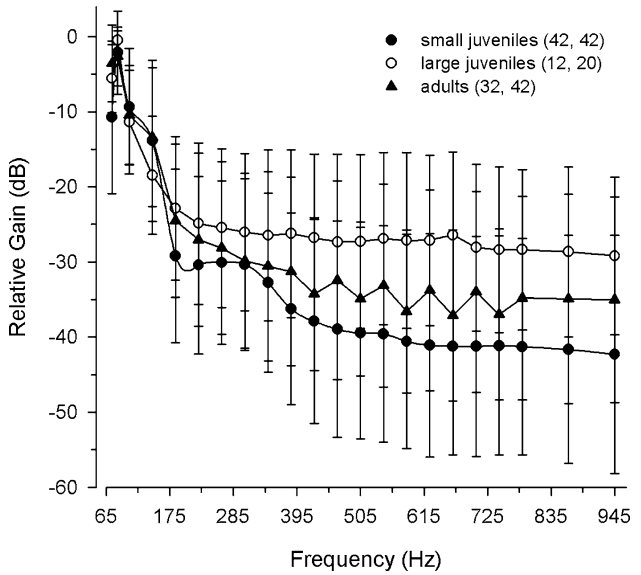

Fig. 2 Relative gain plots of the evoked potentials recorded from the saccule of small juvenile (*solid circles*), large juvenile (*open circles*), and adult (*solid triangles*) midshipman based on the responses to iso-level pure tones at 130 dB (re 1 μPa). The iso-level response data were normalized to a relative value of 0 dB to control for the absolute sensitivity of the saccule from different recording positions and to compare across different animals. A relative value of 0 dB was assigned to the peak response for each recording and the remaining data for other frequencies were expressed in relative dB (re Best Frequency Sensitivity). Data are plotted as means ± 1 SD. The number of animals and records for each size class are indicated in *parenthesis*

The saccular threshold tuning curves for the three size classes are summarized in Fig. 4 and show an increase in auditory threshold above 85 Hz that gradual diminishes above 545 Hz. We applied a quadratic regression model to analyze the threshold tuning curve data because it provided the best fit for the majority of the data (mean $R^2 = 0.86 \pm 0.11$ SD, min $R^2 = 0.48$, max $R^2 = 0.98$). There were no differences in saccular tuning profiles between the three size classes of fish based on slope (ANOVA, $F = 1.466$, df = 2, 101, $p = 0.236$), intercept (ANOVA, $F = 0.94$, df = 2, 101, $p = 0.394$), or curvilinear component (ANOVA, $F = 1.424$, df = 2, 101, $p = 0.246$). A separate analysis of auditory threshold tuning for the three size classes based on saccular recording region was performed and revealed no differences in the saccular tuning profiles for each recording position among the three size classes based on slope (two-way ANOVA, effect of recording position, $F = 1.751$, df = 2, 95, $p = 0.179$; interaction of size class and recording position, $F = 0.85$, df = 4, 95, $p = 0.497$), intercept (two-way ANOVA, effect of recording position, $F = 0.22$, df = 2, 95, $p = 0.803$; interaction of size class and recording position, $F = 0.829$, df = 4, 95, $p = 0.51$), or curvilinear component (two-way ANOVA, effect of recording position, $F = 1.036$, df = 2, 95, $p = 0.359$), however, there was a slight interaction effect between the size class and recording position on the curvilinear component of the modeled regression lines (two-way ANOVA, interaction of size class and recording position, $F = 2.632$, df = 4, 95, $p < 0.05$). The overall similarity of the filter shapes of the threshold tuning profiles for the three size classes based on saccular recording position indicates that there is no difference in tuning across the saccule during ontogeny (Fig. 5).

Although there were no differences in the saccular tuning profiles for the three size classes, there was an ontogenetic difference in the maximum detectable frequency by size class. We recorded evoked saccular potentials in all small juveniles (100%) at 265 Hz ($n = 42$ records) while only 40 and 2% of the small juvenile recordings contained evoked potentials at 545 and 745 Hz, respectively (Fig. 6). Similarly, we recorded evoked saccular potentials in all large juveniles (100%) at 265 Hz ($n = 20$) while 50 and 5% of the large juvenile recordings contained evoked potentials at 625 and 865 Hz, respectively. In contrast, we recorded evoked saccular potentials in all adults (100%) up to 225 Hz ($n = 43$) while 49 and 12% of the adult recordings contained evoked potentials at 705 and 945 Hz, respectively. We were unable to record the evoked saccular potentials of fish from any of the three size classes at frequencies higher than 945 Hz using the sound levels reported in this study.

Discussion

The aim of this study was to characterize the frequency response and auditory thresholds of saccular hair cells to behaviorally relevant stimuli throughout development in the plainfin midshipman. Our results indicate that the frequency response and threshold sensitivity of the midshipman saccule is established early in development and retained throughout ontogeny. We also show that the ability of the saccule to detect higher frequency sounds (>385 Hz) increases with age/size. This report adds considerable new quantitative data regarding the ontogeny of the frequency response range of relative gain and auditory threshold of saccular hair cells for this species. In this discussion we interpret our results as they relate to the vocal-acoustic communication and life history of the plainfin midshipman fish and other ontogenetic studies of fish hearing.

Evoked saccular potentials

In general, saccular potentials are thought to result from the summation of evoked receptor potentials produced by populations of hair cell populations within the fish saccule.

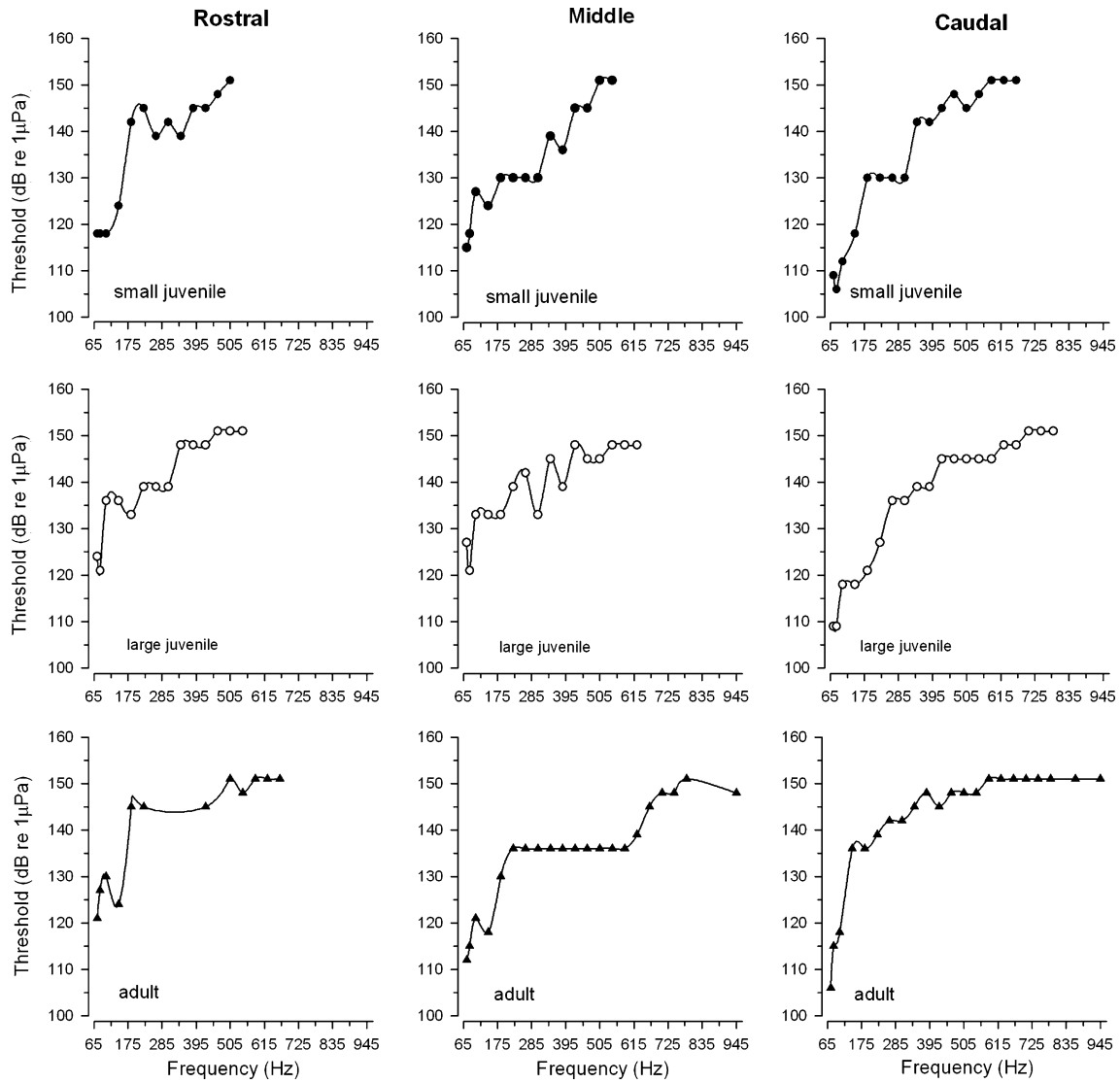

Fig. 3 Representative examples of individual auditory threshold tuning curves for small juveniles (*solid circles*), large juveniles (*open circles*), and adults (*solid triangles*) based on the saccular potentials from three recording positions in the saccule: rostral (*first column*), middle (*middle column*), and caudal (*third column*). The auditory threshold for each frequency was determined as the lowest stimulus intensity in dB (re 1 µPa) that evoked a saccular potential greater than 2 SD above the background noise measurements

The saccular potentials of midshipman and other teleost fishes are evoked greatest at twice the stimulus frequency due to opposite oriented hair cell populations in the saccule that produce two summed evoked potentials for each stimulus cycle of a pure tone (Flock 1965; Wersall and Flock 1965). This double frequency effect of the saccular potentials is thought to be primarily due to the nonlinearity in the generation of the summed hair-cell potential response, which avoids the complete cancelation of the two summed waveforms from the opposing sets of hair cell populations (Fay 1974). This study takes advantage of the double frequency response of saccular potentials by using a lock-in amplifier to yield an output signal that is "locked" or referenced to the second harmonic of the stimulus (i.e., an evoked response that is twice the stimulus frequency). Based on the iso-level response profiles, the saccular potentials of small juveniles evoked at 130 dB re 1 µPa were greater in magnitude than that in large juveniles and adults (Fig. 1) and had a greater range of relative gain across test frequencies than that of large juveniles (Fig. 2). These findings are somewhat surprising considering that small juveniles have a smaller saccule and presumably fewer saccular hair cells compared to that of large juveniles and adults.

One possible explanation for the magnitude differences in the saccular potentials of small juveniles versus that of adults and large juveniles could be due to the size of the saccule and the recording position of the electrode.

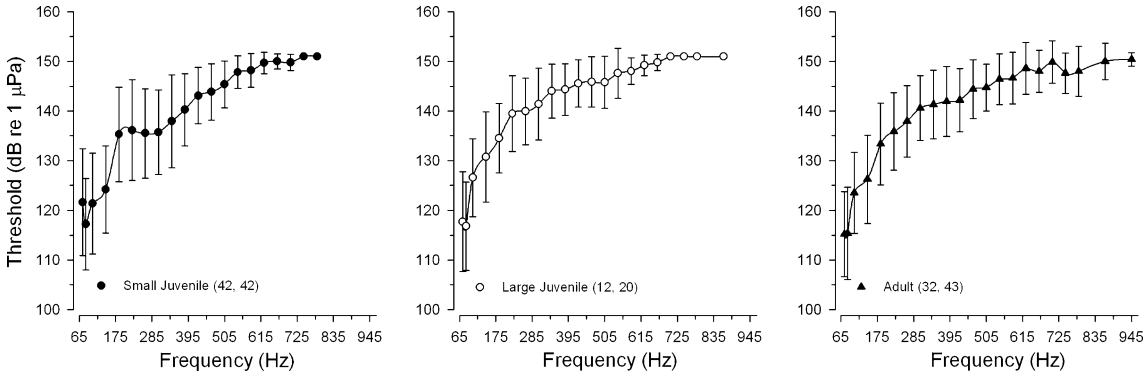

Fig. 4 Auditory threshold tuning curves for small juvenile (*solid circles*), large juvenile (*open circles*), and adult (*solid triangles*) midshipman based on evoked saccular potentials. The auditory threshold for each frequency was determined as the lowest stimulus intensity in dB (re 1 μPa) that evoked a saccular potential greater than 2 SD above the background noise measurements. All data are plotted as mean ± 1 SD. The number of animals and records for each size class are indicated in *parenthesis*. Note the similar tuning filter characteristics for all three size classes

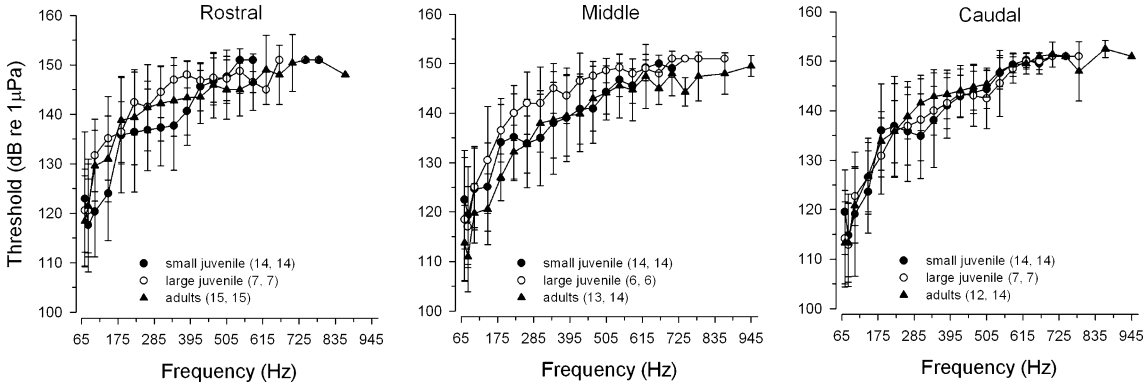

Fig. 5 Auditory threshold tuning curves for small juvenile (*solid circles*), large juvenile (*open circles*), and adult (*solid triangles*) midshipman recorded from the rostral, middle, and caudal regions of the saccule. The auditory threshold for each frequency was determined as the lowest stimulus intensity in dB (re 1 μPa) that evoked a saccular potential greater then 2 SD above the background noise measurements. All data are plotted as mean ± 1 SD. The number of animals and records for each size class are indicated in parenthesis. Note the widely overlapping error bars and similar filter shapes for all size classes and recording positions. This overall similarity indicates that there is no difference in tuning across the saccular regions during ontogeny

The adult saccule and its corresponding macula is approximately 3–4 times larger than that in small juveniles, and the magnitude of the recorded evoked saccular potentials should vary depending on the distance between the recording electrode and the sensory bed of hair cells within the saccule. Thus, for small juveniles the size of the saccule may have allowed the electrode to be positioned closer in proximity to hair cells to produce a greater evoked potential measurement. Congruently, the smaller size of the saccule and proximity of the recording electrode in relation to the auditory eighth nerve in small juveniles possibly may have permitted the measurement of evoked potentials from the saccular afferent terminals innervating opposite oriented hair cell populations within the saccule. Alternatively, the differences in the evoked saccular potential magnitudes may have been due to a greater density of hair cells in the saccule of small juveniles as compared to that in adults and large juveniles. Although this explanation seems unlikely since small juveniles would presumably have fewer numbers of saccular hair cells, but the possibility exists that those hair cells may have been more densely arranged in the saccule. Future studies that examine the distribution, morphology, and orientation patterns of hair cells within the saccule of the midshipman will be required in order to resolve our observed ontogenetic differences in the iso-level response profiles of saccular hair cells.

Ontogenetic retention of saccular sensitivity

Perhaps the most surprising result of this study was that there was no change in auditory saccular sensitivity among the three size classes, which indicates that there is an

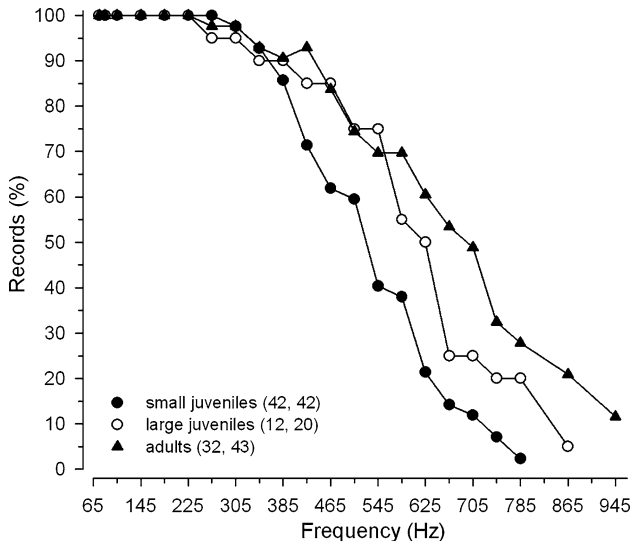

Fig. 6 Distribution of the percentage of saccular potential recordings that were above threshold at given test frequencies for small juvenile (*solid circles*), large juvenile (*open circles*), and adult (*solid triangles*) midshipman. For example at 625 Hz, 20% of the records for small juveniles were above threshold at that frequency while 50% of the recordings for large juveniles were above threshold and 60% of the recordings for adults were above threshold. The number of animals and records for each size class are indicated in parenthesis. Note that the probability of detecting higher frequencies greater than 385 Hz increased with size/age

ontogenetic retention of saccular sensitivity with age/size. We show that the auditory saccular hair cells are broadly tuned to low frequency auditory stimuli throughout development and that the saccular sensitivity of adults is similar to that of both small and large juveniles. These results are in contrast to previous findings which demonstrated an ontogenetic increase in auditory sensitivity at BF and resting discharge rate at the level of the saccular afferent neurons (Sisneros and Bass 2005). Sisneros and Bass posited that their results could be explained by an age related increase in the number of saccular hair cells and/or convergence ratio of hair cells to afferent neurons to increase saccular afferent sensitivity. In general, teleost fish continue to add hair cells postembryonically throughout their lifetime (Platt 1977; 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 (Corwin 1983; Sento and Furukawa 1987). Corwin (1983) and Sento and Furukawa (1987) showed that an increase in auditory sensitivity was correlated with increases in the number of hair cells innervated by individual primary afferent neurons. However, Popper (1971) was unable to demonstrate such changes in behavioral auditory sensitivity with age/size between two subadult groups of goldfish (*Carasius auratus*), which presumably exhibited hair cell addition with size/age. Popper and colleagues later proposed a model of fish hearing that is congruent with their results and predicts that hair cell addition with fish growth will maintain hearing sensitivity as the relative size and positions of different structures associated with fish audition change during ontogeny (Popper et al. 1988; Rogers et al. 1988). Future ontogenetic studies of hair cell addition coupled with developmental anatomical studies of the midshipman auditory periphery will provide valuable insight into the mechanisms that allow for increases in afferent sensitivity while retaining hair cell sensitivity within the saccule during development.

Alternatively, Sisneros and Bass (2005) posited that their reported ontogenetic changes in the response properties of saccular afferent neurons could have been due to changes at the level of the CNS via the efferent auditory pathway. Using the saccular potential recording technique (Sisneros 2007), we were able to show that the auditory sensitivity and tuning of saccular hair cells did not change during ontogeny which now leads us to suggests that the previously reported changes occurred either post-synaptic to the hair cell and/or via the saccular efferents. In the closely related oyster toadfish (*Opsanus tau*), efferent synaptic terminals are found both on the dendrites of afferent neurons and on the hair cells (Holstein et al. 2004). Activation of efferent neurons generally increases the resting discharge rate of afferents and reduces the sensitivity (gain) of the hair-cell receptor potentials in the semicircular canals to rotary stimuli (Boyle et al. 2009). In addition, efferent feedback can increase the signal-to-noise ratio of saccular responses in conditions where a signal is masked by noise which could potentially help unmask biologically relevant signals (Tomchik and Lu 2005, 2006). Similar mechanisms of efferent activation and central neural control may play a role in the tuning and sensitivity modulation of saccular hair cells and their afferent responses in the midshipman inner ear.

Functional significance of the ontogenetic retention of saccular sensitivity

The results of this study indicate that the saccule of small and large juvenile midshipman is best adapted to detect low frequency sounds ($\leq$105 Hz) in their natural environment. The early development and retention of this low frequency sensitivity in juveniles is consistent with adaptations to increase survival by enhancing their ability to detect low frequency periodic stimuli often associated with potential predators and prey. Although there is no evidence that juveniles produce conspecific vocalizations for communication, we show that the saccule of juveniles, like that of non-reproductive adults, is well adapted to detect the low frequency components of midshipman vocalizations, similar to the findings of Sisneros and Bass (2005) for saccular afferent sensitivity. The detection and encoding of the low

frequency components of midshipman vocalizations may very well be important for the interception and eavesdropping of conspecific vocalizations during social encounters. In adults, the detection and production of conspecifics vocal signals is very important for acoustic communication during social and reproductive behaviors (Bass et al. 1999; Bass and McKibben 2003). Our results show that soon after detaching from their natal rock small free-swimming juveniles possess the same auditory saccular sensitivity similar to larger non-reproductive adults. It would be interesting to know in future work at what stage of embryonic development does the saccule become functional, especially since the developing embryos are thought to be repeatedly exposed to the rather high sound levels of the male's advertisement over the duration of their embryonic development. Thus, future research will be necessary to determine when the midshipman auditory system becomes functional and how such auditory saccular sensitivity is retained during post-embryonic development.

In contrast to the similar tuning profiles of juveniles and adults, we show that there is an ontogenetic increase in the ability of the midshipman saccule to detect frequencies higher than 385 Hz with age/size (Fig. 6). The large increase in the percentage of saccular potential recordings that were above threshold for adults at frequencies from 425 to 945 Hz indicate that adults have a higher probability of detecting frequencies >385 Hz than small and large juveniles. This increase in the ability to detect higher frequencies is likely to be adaptive for adults in social communication. Previous work by Sisneros and Bass (2003) showed that adult females undergo a seasonal plasticity of peripheral frequency sensitivity that is dependent on seasonal shifts in circulating plasma levels of testosterone and estradiol (Sisneros et al. 2004a, b). Non-reproductive adults and juveniles have similar basal levels of circulating hormones hormone profiles. The similarity of saccular tuning between juveniles and non-reproductive adults is consistent with their shared steroid hormone profiles and the role of seasonal elevated steroid levels to induce an upward shift in peripheral frequency sensitivity. These seasonal changes in peripheral frequency sensitivity occur at the level of the saccular hair cell (Sisneros 2009b) and auditory afferents (Sisneros and Bass 2003) act to enhance the detection and encoding of the higher harmonic frequency components of the male's advertisement call. These higher harmonic components are thought to propagate further than the call's fundamental frequency due to the physical and environmental constraints of the shallow water breeding habitat that limit sound transmission (Bass and Clark 2003; Fine and Lenhardt 1983). This novel form of auditory plasticity in the midshipman is thought to provide an adaptable mechanism that enhances the coupling between sender and receiver in this communication system and acts to increase the probability of mate detection and localization during the breeding season (Sisneros at 2004a).

Ontogenetic studies of hearing in other fishes

Relatively few neurophysiological and behavioral studies have been performed on the ontogeny of hearing in fishes. In cases where such studies have been performed, the results have often been contradictory and sometimes confusing. Behavioral studies in several species of teleosts have shown decreases in auditory threshold with age/size (Kenyon 1996; Iwashita et al. 1999). However, studies in the goldfish *Carasius auratus* using similar conditioning techniques concluded that threshold did not undergo ontogenetic shifts (Popper 1971). Similar cases exist for auditory physiology studies using the auditory-evoked potential (AEP) technique. Such AEP studies have demonstrated decreases in auditory threshold during ontogeny in the croaking gourami, *Trichopsis vittata* (Wysocki and Ladich 2001), and in the Lusitanian toadfish, *Halobatrachus didactylus* (Vasconcelos and Ladich 2008) while another study using the same technique showed an increase in hearing thresholds with age/size in the damselfish, *Abudefduf saxatilis* (Egner and Mann 2005). In contrast, similar AEP studies of the zebrafish, *Danio rerio*, have shown that auditory thresholds do not change during ontogeny but the maximum detectable frequency did increase with size/age (Higgs et al. 2002, 2003). The above reported ontogenetic differences in auditory sensitivity are most likely due to species-specific differences related to their evolutionary history and environmental habitat, and direct comparison of the findings from the previous ontogenetic fish hearing studies is difficult at best due to the wide array of techniques used to determine auditory sensitivity. Future studies that employ more than one method (behavioral and physiological) to determine auditory sensitivity should help resolve differences that maybe attributed to technique rather than to species-specific differences.

In sum, we show using the evoked saccular potential recording technique that there is an ontogenetic retention of auditory saccular sensitivity with size/age in the plainfin midshipman fish. However, we also report an ontogenetic increase in the ability of the midshipman saccule to detect frequencies higher than 385 Hz with age/size, which may be important for the detection of social acoustic signals during the adult life history stage. Future neurophysiological studies of the midshipman saccular hair cells and auditory afferents are needed to reveal the possible mechanisms that enable the ontogenetic retention of auditory saccular sensitivity.

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