The Journal of Experimental Biology 208, 3121-3131
Published by The Company of Biologists 2005
doi:10.1242/jeb.01742

Ontogenetic changes in the response properties of individual, primary auditory afferents in the vocal plainfin midshipman fish *Porichthys notatus* Girard

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Accepted 8 June 2005

Summary

The auditory system of adult midshipman fish *Porichthys notatus* Girard is an important sensory receiver system used during intraspecific social communication to encode conspecific vocalizations, but the response properties and function of this system in the pre-adult stages are unknown. Midshipman fish, like other teleosts, use the saccule as the main acoustic end organ of the inner ear. In this study, we examined the discharge properties and the frequency response dynamics of auditory saccular afferent neurons in pre-adult midshipman (~4–12 months of age) to determine whether encoding of auditory information, inclusive of conspecific vocalizations, changes across life history stages. Extracellular single unit recordings were made from saccular afferents while sound was presented *via* an underwater speaker. Comparisons with adult data show that the resting discharge rate and auditory threshold sensitivity increased with age/size, while temporal encoding of frequency did not show any significant shifts. The results indicate that the saccular afferents of juveniles, like those of non-reproductive adults, are best adapted to temporally encode the low frequency components (≤100 Hz) of midshipman vocalizations. This report represents the first *in vivo* investigation of age-related changes in the encoding properties of individual auditory neurons for any fish species.

Key words: midshipman fish, *Porichthys notatus*, hearing, saccule, auditory neuron, vocalization, ontogeny.

Introduction

While numerous studies have examined ontogenetic changes in the structure and function of the auditory system in mammals and birds (e.g. Ehret and Romand, 1981; Gray and Rubel, 1985; Walsh et al., 1986; Mills et al., 1990; Dmitrieva and Gottlieb, 1992; Geal-Dor et al., 1993; Werner and Gray, 1998; Brittan-Powell and Dooling, 2000), few have examined similar changes in other vertebrates including anuran amphibians (Schofer and Feng, 1981; Boatwright-Horowitz and Megala Simmons, 1995, 1997) and fishes (Popper, 1971; Corwin, 1983; Kenyon, 1996; Higgs et al., 2002, 2003, 2004). Teleost fishes have been functionally divided into two nontaxomic groups based on their utilization of either the swim bladder or other gas-filled structures as accessory hearing organs (Popper and Fay, 1993, 1999). The 'hearing specialists' have either skeletal adaptations (e.g. Weberian ossicles) that connect the anterior part of the swim bladder to the inner ear or possess gas-filled vesicles that are in close or direct contact with the inner ear to enhance the detection of the pressure component of sound waves. In contrast, 'hearing generalists' lack such specialized structural adaptations. The focus of this study was to determine how auditory threshold sensitivity and temporal encoding of pure tone stimuli change during the ontogeny of the plainfin midshipman fish *Porichthys notatus* Girard, a sound-producing hearing generalist.

Previous developmental studies of auditory function in fishes have reported either increases, decreases or no change in auditory sensitivity with age and growth. Based on multiunit recordings from an *in vitro* preparation of the macula neglecta, a nonotolithic end organ of the inner ear in the skate *Raja clavata,* Corwin (1983) was the first to establish an ontogenetic increase in threshold sensitivity in response to vibrational stimuli. Among teleosts, age/size-related increases in behavioral auditory threshold sensitivity have been reported using behavioral conditioning techniques for two distantly related species of hearing generalists, a damselfish (*Pomacentrus* spp.) and the Red Sea bream *Pagrus major* (Kenyon, 1996; Iwashita et al., 1999). Wysocki and Ladich (2001) used the evoked auditory brainstem recording (ABR) technique to show that auditory sensitivity increases prior to the ability to vocalize and communicate acoustically in a hearing specialist, the croaking gourami *Trichopsis vittata*. In contrast to these studies, a recent ABR study shows that auditory threshold sensitivity decreases with an increase in fish size for another species of damselfish, the sergeant major *Abudefduf saxatilis (*Egner and Mann, 2005). A final

ontogenetic pattern has been identified among ostariophysines that are hearing specialists; Popper (1971) reports no shifts in behavioral auditory threshold sensitivity between two subadult groups of goldfish *Carassius auratus* that differed in size (5 cm *vs* 10 cm total length); while Higgs et al. (2002, 2003) used the ABR technique to show that the bandwidth of detectable frequencies, but not auditory threshold sensitivity, increased with age and size in zebrafish *Danio rerio*.

The major goal of the present study was to determine age/size related shifts in the discharge properties and the frequency response dynamics of afferent neurons from the saccule of the plainfin midshipman fish *Porichthys notatus* Girard. We focused on saccular afferents of midshipman fish for several reasons. First, lesion experiments provide strong support for the hypothesis that the saccule is their main organ of hearing (Cohen and Winn, 1967). Second, we have extensively studied the response properties of primary saccular afferents in adults (McKibben and Bass, 1999, 2001; Sisneros and Bass, 2003; Sisneros et al., 2004a). Third, no study has yet demonstrated how, or if, auditory mechanisms are transformed during ontogeny in a hearing generalist. Fourth, it is unknown how auditory sensitivity in a hearing generalist might change concurrently with the ability to vocalize. Studies of vocal teleosts provide the advantage of identifying the significance of changing auditory mechanisms in the context of a well-defined, behaviourally relevant stimulus, namely vocalizations (see Bass and McKibben, 2003; Lu, 2004; Bass and Lu, in press). Fifth, it remains essential to characterize the response dynamics of single-unit auditory neurons if we are to integrate the results of previous ontogenetic studies using ABR and behavioral conditioning techniques with the relative abundance of single-unit recording studies of adults for both hearing specialists and generalists, and other vertebrates in general. Lastly, we were particularly interested in possible shifts in peripheral encoding mechanisms during non-adult stages, given our recent demonstration of steroid-dependent seasonal plasticity in peripheral frequency encoding among adults (Sisneros et al., 2004a).

To our knowledge, this study represents the first *in vivo* analysis of age- and size-related changes in the encoding properties of individual auditory neurons for any fish species. In contrast to our previous work showing changes in frequency encoding that are dependent on adult reproductive state, we now show that the resting discharge properties and auditory threshold sensitivity, but not the frequency response properties, of peripheral auditory neurons change with size (age) among pre-adult midshipman fish.

Materials and methods

Animals

Plainfin midshipman fish *Porichthys notatus* Girard were collected and classified into three groups based on their stage of ontogenetic development. Small juveniles (standard length *SL*=3.1–4.8 cm) were collected while still attached by their yolk sacs to rocks at nest sites during July and August and then reared in the laboratory (Lindholm and Bass, 1993; also see Bass, 1996). Large juveniles (females: *SL*=6.2–9.5 cm; males: *SL*=6.2–11.5 cm) were collected during late September to late February by otter trawl (R/V *John Martin*, Moss Landing Marine Laboratories) in Monterey Bay, off-shore from Moss Landing, California, USA. The classification of juveniles as either small or large rests upon an earlier study of the ontogeny of the vocal motor system in this species (Bass et al., 1996). For that study, animal age was based on analysis of the saccular otolith and showed that animals considered small juveniles are between 130 and 160 days post-fertilization age (d.p.f.); the 130 day time point is likely an overestimate for the present study since the smallest juvenile in the Bass et al. (1996) study was 4.5 cm, while 12 of the 15 animals in the present study were 3.1–4.4 cm in size**.** The age of the large juvenile males in the present study is also based on the report of Bass et al. (1996) and we estimate them to be between 160 d.p.f. and 230 and 370 d.p.f. for juvenile females and males, respectively.

Although previous reports describe the response properties of eighth nerve afferents in adult midshipman (see Introduction), we include some of that data in this report to provide a more complete portrayal of age/size related changes in response properties. Midshipman have two classes of adult males, known as types I and II, which follow divergent reproductive and vocal tactics (for a review, see Bass, 1996). All of the new male data in this study are type I. Only type I males produce advertisement calls to attract gravid females laden with ripe eggs to their nest. We recently showed that elevation of plasma steroid levels of testosterone and 17β-estradiol to levels comparable to those found in gravid females affect auditory saccular afferents in females *via* an induced upward shift in best frequency and in the phase-locking precision at higher frequencies >140 Hz (Sisneros et al., 2004a). Thus, in order to avoid any influences of naturally cycling gonadal steroids on the response properties of the peripheral auditory system we used only adult animals collected by otter trawl during the non-reproductive season (see above). More specifically, we compare the juvenile data to resting discharge data from 15 non-reproductive females and frequency response data from 24 non-reproductive females that were part of a previous study of seasonal changes in the adult peripheral auditory system (Sisneros and Bass, 2003). Additional, unreported resting discharge data are included from six non-reproductive females, and both resting discharge and frequency response data from four non-reproductive males. An earlier report used a higher stimulus intensity to characterize most auditory afferents in type I males that were not classified as either reproductive or non-reproductive (McKibben and Bass, 1999), and so we collected new data from a representative set of non-reproductive males using the same stimulus intensity employed in this study and the previous one of females (Sisneros and Bass, 2003). Adult status (female *SL* >9.5 cm; male *SL* >11.5 cm) was based on the previously reported dimorphic size ranges for males and females (e.g. Bass et al., 1996; Brantley et al., 1993a; Foran and Bass, 1998; Grober et al., 1994).

All animals were maintained in saltwater aquaria at 12–15°C. Juveniles were fed brine shrimp every 2–3 days, whereas adults were fed goldfish and brine shrimp every 3–4 days.

Neurophysiology experiments

Standard electrophysiology techniques and recording methods were used to characterize eighth nerve auditory afferents in the midshipman fish (McKibben and Bass, 1999; Sisneros and Bass, 2003). Animals were anesthetized by immersion in a seawater bath of 0.025% p-amino benzoate (benzocaine; Sigma, St Louis, MO, USA) and then given an intramuscular injection of pancuronium bromide (~0.5 mg kg^{-1}) and fentanyl (~1 mg kg^{-1}) for immobilization and analgesia, respectively. Eighth nerve auditory afferents of the saccule were then exposed by dorsal craniotomy (Fig. 1). The cranial cavity was filled with an inert fluid (Fluorinert FC-75, 3M, St Paul, MN, USA) to enhance clarity and prevent drying. A 2 cm dam of denture cream was built up around the cranial cavity, which then allowed the animal to be lowered just below the water surface. Fish were then positioned such that the saccule was ~10 cm above the surface of the underwater loudspeaker that was embedded in sand on the bottom of a 30 cm diameter, 24 cm high Nalgene tank (similar in design to that used by Fay, 1990). The tank was housed inside an acoustic isolation chamber (Industrial Acoustics, New York, NY, USA) on a vibration isolation table, and all recording and stimulus generation equipment were located outside the chamber. Fish were perfused continuously with fresh seawater at 14–15°C through the mouth and over the gills during the experiments and the condition of the animal was monitored by watching the blood flow in the dorsal vasculature of the brain. All experimental procedures in this study were conducted under the guidelines of the National Institutes of Health for the care and use of animals and were approved by the Cornell University Institutional Animal Care and Use Committee.

Extracellular single unit discharges of the saccular afferent neurons were recorded using glass microelectrodes filled with 4 mol l^{-1} NaCl (~10–40 MΩ). Electrodes were visually guided to the surface of the saccular afferent nerve and advanced using a hydraulic microdrive (Kopf Instruments, Tujunga, CA, USA). Auditory neurons were randomly sampled from the saccular afferent nerve and amplified using standard electrophysiology techniques. Single units were identified with a search stimulus (a single tone from 70–100 Hz) as the electrode was advanced through the nerve. Analog saccular afferent discharges were amplified (Getting 5A, Iowa City, IA, USA), filtered at 150–5000 Hz (Stanford Research Systems SR650, Stanford, CA, USA) and then digitized onto a Macintosh Centris computer running a customized signal-generation/data-acquisition software program (CASSIE, designed by Julian Vrieslander, Cornell University). Visually identified single auditory units isolated during the extracellular recordings were discriminated using a pattern matching algorithm in CASSIE.

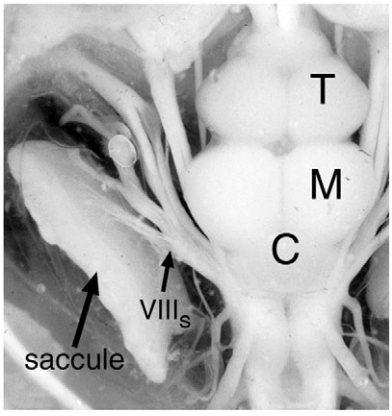

Fig. 1. Dorsal view of midshipman brain and inner ear. Small arrow marks the area of the VIII$_s$ nerve innervating the saccule where recordings were made. C, cerebrum; M, midbrain; T, telencephalon.

Stimulus generation

Acoustic stimuli were synthesized and generated using CASSIE software package on a Macintosh Centris with a 12-bit DA board, attenuated (PA4, Tucker-Davis Technology, Gainesville, FL, USA) and amplified (NAD 3020A, NAD Electronics, Boston, MA, USA) before being played through an underwater loudspeaker (UW-30, Telex Communications, Burnsville, MN, USA). The frequency response of the underwater loudspeaker was measured using a minihydrophone (Bruel and Kjaer 8103) in the position normally occupied by the head of the fish. Relative sound pressure measurements were then made using a spectrum analyzer (Stanford Research Systems SR780), calibrated by peak-to-peak voltage measurements on an oscilloscope, and then adjusted with CASSIE software so that the sound pressures at all frequencies (60–400 Hz) used were of equal amplitude within ±2 dB. Although the midshipman ear may be primarily sensitive to the particle motion component of a sound wave, the determination of sound level in terms of pressure provides a valid characterization of the sound stimuli used here (for an extended discussion, see McKibben and Bass, 1999; also for recent review of underwater sound fields, see Bass and Clark, 2003).

Basic auditory stimuli consisted of eight repetitions of single tones 500 ms in duration, with fall and rise times of 50 ms. Each repetition was presented at a rate of one every 1.5 s. Iso-intensity responses were measured using pure tones presented at 10 or 20 Hz increments from 60 to 400 Hz at a sound pressure of 130 dB re 1 μPa. This sound intensity is consistent with known sound intensity levels for midshipman sounds recorded near the nests (Bass and Clark, 2003).

Data analysis

Resting discharge activity was measured for eight repetitions of the stimulus interval in the absence of an auditory stimulus and then used to generate interspike interval histograms with 1 ms bins.

On isolation of single units showing an auditory response to the search stimulus, the iso-intensity responses were measured for the vector strength of synchronization (VS), which is a metric of the synchronization (i.e. degree of phase locking) of the saccular afferent discharge to the auditory stimulus. Spike train responses of auditory afferents to individual tones were quantified for VS and were calculated from spike train data acquired over the entire stimulus duration. VS is equivalent to the mean vector length for the circular distribution of spikes over the period of the stimulus and was calculated according to Goldberg and Brown (1969) using 2 ms bins. VS varies from 0 for either a random or uniform distribution to 1 for perfect synchronization if all spikes fall in the same bin. A Rayleigh *Z* test was performed to determine whether synchronization to pure tones was significantly different from random ($P<0.05$) (Batschelet, 1981). Only significant VS values were used to generate iso-intensity response curves. We used the VS metric rather than averaged evoked spike rate because VS is known to be less variable than the evoked spike rate (McKibben and Bass, 1999, 2001) and is a more consistent measure for frequency encoding among teleost fishes (Fay, 1978, 1982, 1994), including the midshipman fish (McKibben and Bass, 1999; Sisneros and Bass, 2003).

The best frequency (BF) of a unit was determined as the frequency that evoked the highest VS to the individual tones.

Statistical analysis

The effect of life history stage (small juvenile, large juvenile, and adult) on resting discharge, BF and auditory threshold at BF were determined by one-way ANOVA followed by the Newman–Kuels method for pairwise multiple comparisons. In cases where data sets failed tests of equal variance and a parametric ANOVA or a *t*-test could not be used, data were analyzed using the non-parametric Kruskal–Wallis one-way ANOVA followed by the Dunn's test for pairwise multiple comparisons or the Mann–Whitney *U*-test, respectively. The non-parametric Wilcoxon paired-sampled test was used to test for differences in the iso-intensity profiles of VS median values from nonreproductive adult type I males and adult females. For all tests, α was set at 0.05. An analysis of the slopes for the relationship of VS at BF and resting discharge rate was determined by an analysis of covariance (ANCOVA). Associations between resting discharge rate and *SL*, auditory threshold at BF and *SL*, and VS at BF and resting discharge rate were determined using Pearson's correlation and linear regression. Differences in phase-locking precision at BF between silent and spontaneously active units were determined by a Student's test. Values are reported as means ± S.D. unless otherwise stated.

Results

Resting discharge activity

Of the 138 auditory saccular afferents isolated to determine resting discharge activity, spontaneous resting discharge rates were recorded in the absence of auditory stimulation from 122 units in 51 plainfin midshipman fish: 15 small juveniles (mean *SL*=4.2±0.5 cm), 13 large juveniles (7.9±1.7 cm) and 23 adults (13.9±2.0 cm). Since there is no difference in the resting discharge rates between non-reproductive adult females (median=21.4 spikes s^{-1}; Sisneros and Bass, 2003) and non-reproductive adult type I males (median=26.5 spikes s^{-1}; McKibben and Bass, 1999) (Mann–Whitney *U*-test, *Z*=–1.34, *P*=0.18), data from the 15 nonreproductive adult females in our previous study (Sisneros and Bass, 2003) were pooled with the resting discharge data collected for this study from two non-reproductive adult type I males (*N*=7 units) and six non-reproductive females (*N*=9 units), and then compared to those of small and large juveniles.

Auditory neurons that did not display resting discharges

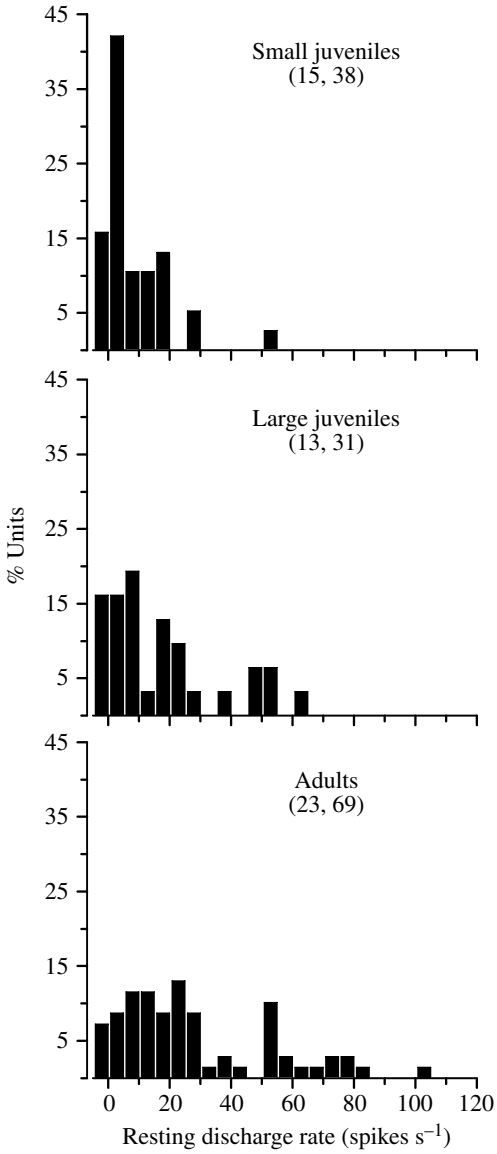

Fig. 2. Resting discharge rate histogram of auditory saccular afferent neurons recorded from juvenile and adult midshipman fish (*P. notatus*). The numbers of animals and auditory saccular afferent neurons tested are indicated, respectively, in parentheses.

were classified as silent units and comprised 15.8%, 16.1% and 7.2% of the total saccular afferent population in the small juvenile, large juvenile and adult size classes, respectively. Resting discharge rates ranged from 0 to 51.6 spikes s^{-1} for small juveniles (N=38 units), 0 to 62.8 spikes s^{-1} for large juveniles (N=31 units) and 0 to 100.4 spikes s^{-1} for adults (N=69 units) (Fig. 2). Mean resting discharge rates increased with size from small juvenile to adults and were positively correlated with standard fish length (Fig. 3; r=0.59, H_0: β=0, t=3.33, P<0.005). Although there was no difference in resting discharge rates between large juveniles (mean=17.4±18.1 spikes s^{-1}, median=9.6 spikes s^{-1}, N=31) and adults (mean=27.2±24.1 spikes s^{-1}, median=20.9 spikes s^{-1}, N=69), the median resting discharge rate for adults was 5.6 times greater than that for small juveniles (mean=8.1±10.4 spikes s^{-1}, median=3.7 spikes s^{-1}, N=38) (Kruskal-Wallis one-way ANOVA, Dunn's test, H=23.6, d.f.=2, P<0.01). Thus, there is an approximate sixfold increase in resting discharge rate during development from the small juvenile to the adult size class.

Frequency sensitivity of saccular afferents to auditory stimuli

Responses to single tone stimuli at 130 dB re 1 μPa were recorded for 163 auditory saccular afferent neurons in 54 midshipman fish: 27 units in 12 small juveniles (mean SL=3.9±0.6 cm), 35 units in 14 large juveniles (mean SL=8.0±1.5 cm), and 101 units in 29 adults (mean SL=13.7±2.2 cm). Because there was no significant difference in the iso-intensity profiles of median VS values from the five nonreproductive adult type I males (N=13 units) recorded for this study and those collected from the non-reproductive adult females (SL >9.5 cm; N=88 units) in our previous study (Sisneros and Bass, 2003) (Wilcoxon paired-sampled test, P=0.16), data were pooled and then used for comparison to juveniles. Iso-intensity responses measured for spike synchronization revealed similar shapes of the iso-intensity response curves and BFs for the three size classes. Median VS declined gradually from 60 Hz to 400 Hz in all three size class (Fig. 4). The BFs ranged from 60 Hz to 200 Hz for all three size classes (Fig. 5) and there was no difference in mean BF among the three study groups (one-way ANOVA, F=1.41, d.f.=2, 160, P=0.25).

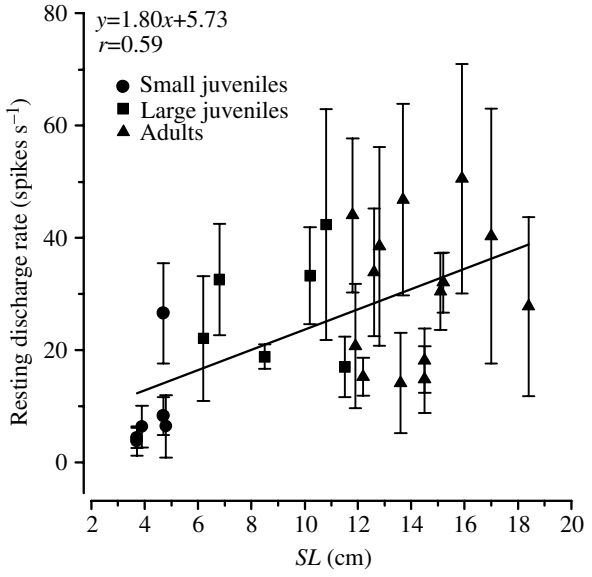

Fig. 3. Relationship of resting discharge rate of auditory saccular afferent neurons with standard length (SL) recorded from juvenile and adult midshipman fish. Note that resting discharge rate increases with SL. Values are means ± S.E.M. for each animal.

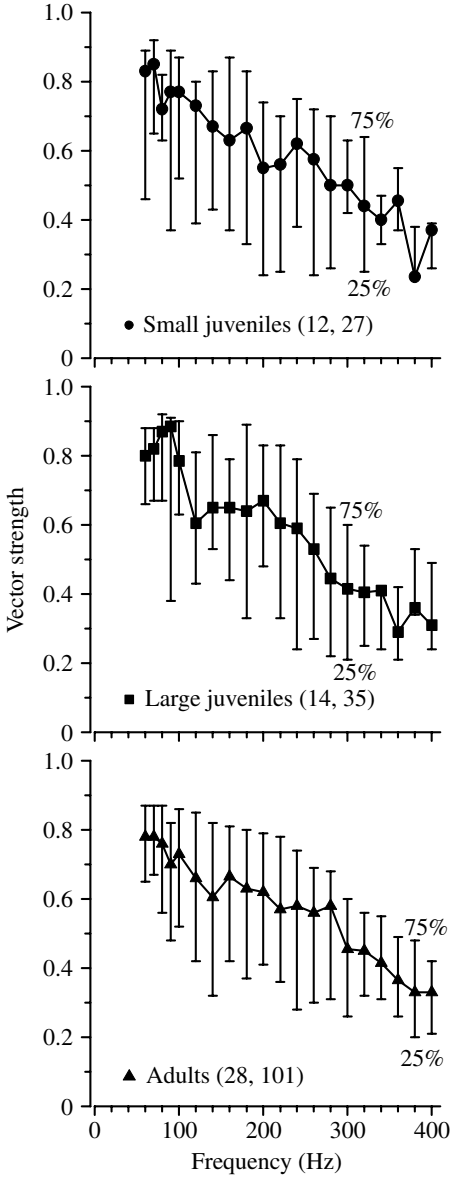

Fig. 4. Iso-intensity response curves for the entire population of juvenile and adult midshipman auditory saccular afferent neurons to 130 dB (re 1 μPa) iso-intensity tones. The numbers of animals and auditory saccular afferent neurons tested are indicated, respectively, in parentheses. Iso-intensity curves based on vector strength (VS) of synchronization show VS values for each frequency tested in terms of the median (black filled symbols), 25th percentile (bottom bar) and 75th percentile (top bar).

Auditory threshold at BF was determined for 16 saccular primary afferents from 12 fish (four units in three small juveniles, two units in two large juveniles, and ten units in seven adults). The auditory threshold at BF increased with size from small juvenile to adults and was negatively correlated with standard fish length (Fig. 6; r=–0.65, H_0: β=0, t=–3.19, P<0.01). Although there was no difference in threshold at BF between large juveniles (104±5 dB re 1 μPa, N=2) and adults (102±5 dB re 1 μPa, N=10), the mean sound pressure at the auditory threshold at BF for small juveniles (118±11 dB re 1 μPa, N=4) was approximately 5–6 times higher than that for large juveniles and adults, respectively (one-way ANOVA, Newman–Kuels method, F=7.84, d.f.=2, 13, P<0.01). Thus, auditory sensitivity at BF increases during ontogeny from the small juvenile to the adult size class.

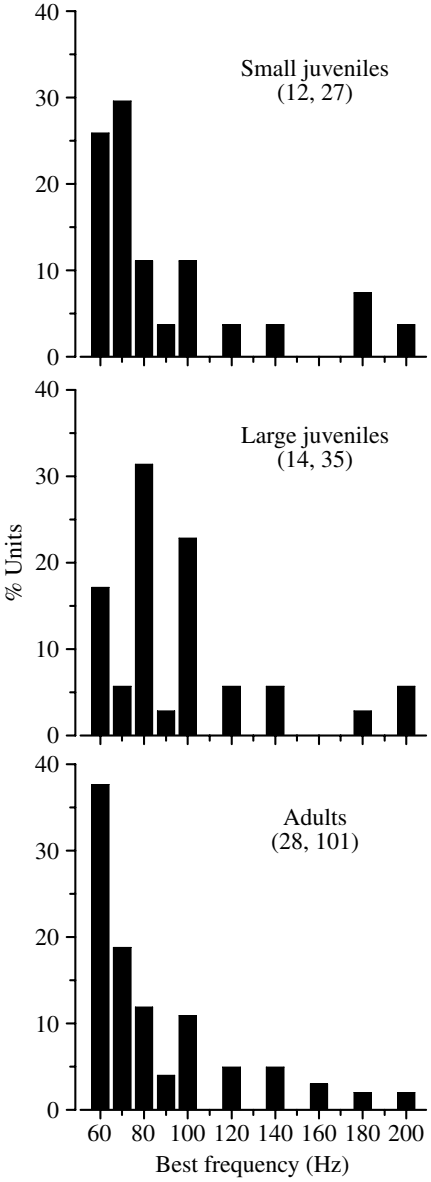

Fig. 5 Best frequency histogram of auditory saccular afferent neurons recorded from juvenile and adult midshipman fish (*P. notatus*). The distribution of best frequencies (BFs) for auditory saccular afferents is based on the vector strength of synchronization to iso-intensity tones of 130 dB (re 1 μPa). The numbers of animals and auditory saccular afferent neurons tested are indicated, respectively, in parentheses.

Relationship between VS at BF and resting discharge rate

A weak but significant linear relationship was discovered between phase-locking precision at BF and resting discharge rate for a subsample of auditory saccular afferents that were analyzed for both resting discharge rate and VS at BF. The VS of synchronization at BF was negatively correlated with the resting discharge rate for small juveniles (r=–0.55, H_0: β=0, t=–2.58, P<0.05), large juveniles (r=–0.50, H_0: β=0, t=–2.74, P<0.05) and adults (r=–0.42, H_0: β=0, t=–3.48, P<0.01). Because the slopes of these regressions did not differ among the three size classes (ANCOVA, F=0.14, d.f.=2, 100, P=0.86), the data were pooled, and the linear relationship for VS at BF and resting discharge rate was plotted (Fig. 7) (r=–0.48, H_0: β=0, t=–5.51, P<0.001). In addition, an analysis of phase-locking precision at BF on the basis of discharge type (silent *vs* spontaneous units) revealed that the mean VS at BF was higher for silent units (0.94±0.08, N=12) than for spontaneously active units (0.84+0.12, N=92) (t-test, t=2.68, d.f.=102, P<0.01). Thus, these results show that VS at BF decreases with increasing resting discharge rate and that silent units have higher phase-locking precision at BF than spontaneously active units.

Discussion

To our knowledge, this study is the first to investigate the encoding properties of single-unit auditory neurons during the

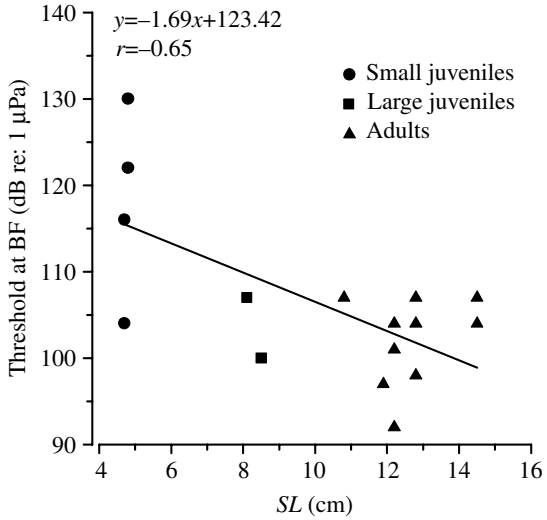

Fig. 6. Relationship of auditory threshold (decibels, re 1 μPa) at best frequency (BF) of auditory saccular afferent neurons with standard length (*SL*) recorded from juvenile and adult midshipman fish. Note that threshold at BF decreases with *SL*.

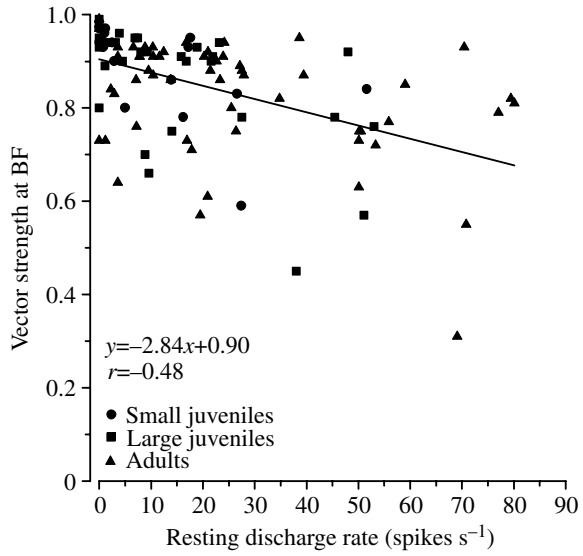

Fig. 7. Relationship of vector strength of synchronization at best frequency (BF) with resting discharge rate of auditory saccular afferent neurons recorded from juvenile and adult midshipman fish.

ontogeny of any fish species. The results demonstrate that the resting discharge properties and auditory threshold sensitivity, but not the frequency response properties, of peripheral auditory neurons change with age (size). The following discussion will first compare the results to those of previous ontogenetic studies and then their relation to the encoding of vocal communication signals for this species.

Resting discharge activity

The auditory primary afferent neurons in the saccular nerve of midshipman and in many other teleost fishes form a relatively heterogeneous population of spontaneously active units. The resting discharge rates were highly variable and skewed in their distribution (the majority were <25 spikes s^{-1}) for all three size classes of the midshipman fish. The range (Fig. 2) and rate (Fig. 3) of the resting discharge activity increased with fish size. The high variability of resting discharge rates for saccular afferents is common in other teleost fishes and can vary from 0–200 spikes s^{-1} for sculpin *Cottus scorpius* (Enger, 1963), 0–250 spikes s^{-1} for the Atlantic cod *Gadus morhua* (Horner et al., 1981), 0–184 spikes s^{-1} for catfish *Ictalurus punctatus* (Moeng and Popper, 1984), 0–310 spikes s^{-1} for goldfish *Carassius auratus* (Fay, 1981), 0–180 spikes s^{-1} for toadfish *Opsanus tau* (Fay and Edds-Walton, 1997), and 0–162 spikes s^{-1} for sleeper goby *Dormitator latifrons* (Lu et al., 1998). Such differences in the resting discharge rates among the various teleost species may be due to a wide range of factors that include species differences in the discharge properties of the peripheral auditory system and/or the number of hair cells that are innervated by individual primary afferent neurons. However, some of this variability is most likely due to the influence of temperature on neuronal response properties. Temperature is known to have a significant influence on the thresholds for membrane depolarization and spike initiation of neurons (Carpenter, 1981). The previous studies were conducted using both tropical and temperate fish species at their respective ambient temperatures.

The mean resting discharge rate for adult midshipman fish (29.4 spikes s^{-1}) was higher than that reported for most species of adult teleost fishes, except for the Atlantic cod *G. morhua*, which had a rate as high as 94 spikes s^{-1} (Horner et al., 1981). Mean rates for other adult teleosts range from 16 spikes s^{-1} in the sleeper goby (Lu et al., 1998) to 13–22 spikes s^{-1} in the goldfish (Fay and Ream, 1986). In contrast to mean spike rates, the percent occurrence of silent units recorded for juvenile (about 16%), and adult (7.2%) midshipman was much lower than that previously reported for other teleosts, again with the exception of the Atlantic cod (6%; Horner et al., 1981). The percent occurrence of silent units reported for other teleosts range from about 30–45% (Enger, 1963; Fay, 1978; Moeng and Popper, 1984).

Resting discharge rates of adult midshipman were approximately six times those of small juveniles. This ontogenetic increase in resting discharge rate likely facilitates an increase in the sampling and encoding of frequency information as well as auditory threshold sensitivity. Previous work on mammals indicates that auditory sensitivity (i.e. auditory intensity thresholds) of primary afferent neurons is inversely correlated with resting discharge rate (Liberman, 1978; Geisler et al., 1985). The intensity thresholds of primary auditory afferents in adult cats with low resting discharge rates (<0.5 spikes s^{-1}) are, on average, at least 20 dB higher than those with high resting discharge rates (>18 spikes s^{-1}) at any particular characteristic frequency (Liberman, 1978). A similar relationship between auditory threshold sensitivity and resting discharge rate was found for midshipman saccular afferents across the different ontogenetic stages studied here.

An age-related change in the morphology of the saccular afferents is one possible means that could alter the response properties of the saccule. For example, changes in the saccular afferent diameter could alter the saccular afferent discharge properties and auditory threshold sensitivity. Variations in afferent diameter could affect the space constants for excitatory potentials generated by the hair-cell neurotransmitter and influence the thresholds for membrane depolarization and afferent discharge behavior (Liberman, 1982; Geisler et al., 1985). Alternatively, age-related increases in the number of saccular hair cells and an increase in the convergence ratio of hair cells to saccular afferent neurons could also contribute to changes in the response properties and threshold sensitivity of the saccule. Future studies that examine the relationships of resting discharge rate, auditory threshold sensitivity, saccular hair cell numbers and afferent morphology, and the functional role of silent units will provide valuable insight into the mechanisms responsible for the ontogenetic changes in the encoding properties of the midshipman peripheral auditory system.

Auditory response properties of midshipman saccular afferents and functional significance of the ontogenetic changes

Our results indicate that the auditory saccular afferents of midshipman are broadly tuned to relatively low-frequency auditory stimuli throughout ontogeny. We show that the peripheral frequency sensitivity of adults is similar to that of both small and large juveniles. Adult females show a seasonal, reproductive-state dependent plasticity of peripheral frequency encoding (Sisneros and Bass, 2003) that is dependent on seasonal shifts in circulating plasma levels of testosterone and estradiol (Sisneros et al., 2004a). Non-reproductive adults (Sisneros et al., 2004b), like juveniles (Brantley et al., 1993b; R. Knapp and A. H. Bass, unpublished observations), have basal levels of gonadal steroids. The similarity in the frequency response properties of saccular afferents between juvenile and non-reproductive adult midshipman (also for non-reproductive type I males; see McKibben and Bass, 1999), is consistent with their shared steroid hormone profiles and the role of elevated levels of circulating steroids in the induction of upward shifts in frequency encoding.

In contrast to the maintenance of a similar pattern of frequency encoding for saccular afferents from small juvenile to non-reproductive adult status, we showed that auditory threshold sensitivity at BF increased with fish size (age). Auditory threshold sensitivity at BF of large juveniles and adults was approximately 5–6 times that of small juveniles. We were unable to demonstrate a significant increase in auditory threshold sensitivity from large juveniles (104 dB re 1 μPa) to adults (102 dB re 1 μPa), but this may be due to the relatively low sample size of the large juvenile size class for which we were only able to obtain two recordings of auditory threshold sensitivity at BF for this size class. An increase in auditory threshold sensitivity with size is known to occur in other hearing generalists, as demonstrated *via* behavioral conditioning experiments (Kenyon, 1996, Iwashita et al., 1999), but increases in physiologically measured, auditory threshold sensitivity have only been demonstrated in one non-teleostean fish, the elasmobranch skate (Corwin, 1983). In contrast, a recent study of a hearing generalist, the sergeant major damselfish *Abudefduf saxatilis*, indicated a decreased ABR-measured auditory threshold sensitivity with an increase in fish size from post-settlement juvenile to adult (Egner and Mann, 2005). Studies of hearing specialists indicate either a lack of increased auditory threshold sensitivity with size (age) using behavioral (Popper, 1971) and ABR (Higgs et al., 2002, 2003) methods, or a small increase in auditory sensitivity over a restricted range using ABR (Wysocki and Ladich, 2001). Differences in physiologically determined levels of auditory sensitivity among the fish species so far tested may yet be due to the differences in either the recording technique, for example, multi-unit eighth nerve recordings (Corwin, 1983) *vs* ABR (Higgs et al., 2002, 2003; Egner and Mann, 2005) *vs* single-unit eighth nerve recordings (this study) and/or the presence of accessory hearing structures among hearing specialists that may influence the development of auditory sensitivity in fishes (also see Higgs et al., 2003). Further study using all of the above methods in both a hearing generalist and a hearing specialist would help to resolve these different hypotheses.

The phase-locking precision at BF was negatively correlated with resting discharge rate for midshipman saccular afferents for all three size classes of midshipman fish, as previously reported for adult females (Sisneros and Bass, 2003) and mammalian auditory afferents (Johnson, 1980; Palmer and Russell, 1986). The maintenance of this relationship throughout different stages of ontogeny may describe the limits to which a synchronized response can be represented in the discharge pattern of auditory saccular afferent neurons.

Plainfin midshipman fish generate three types of vocal communication signals during the adult life history stage (Ibarra et al., 1983, Brantley and Bass, 1994, Bass et al., 1999). Both male reproductive morphs and females produce broadband, short duration (50–200 ms) ‘grunts’, important for agonistic encounters (Fig. 8A). However, only nesting males are known to produce ‘grunt trains’, which are a rapid succession of single grunts used to fend off potential intruders into their nests. The pulse repetition rate of the grunt ranges from 97–110 Hz. A second type of agonistic signal known as a ‘growl’ is also produced by nesting type I males in agonistic encounters. Growls (Fig. 8C) are multiharmonic and relatively long in duration (>1 s). Growls have an initial grunt-like signal followed immediately by a multi-harmonic component with a fundamental frequency (F_0) of 59–116 Hz that gradually changes throughout the call. Nesting males also produce a third type of vocal signal known as the ‘hum’, which is a long duration (>1 min) multiharmonic advertisement call (Fig. 8E). Hums have F_0 values similar to that grunts and growls that range from 90–100 Hz, contain several prominent harmonics

Fig. 8. (A) Representative example of a single grunt recorded at 16°C from a nesting type I male midshipman fish. Bar, 10 ms. (B) Comparison of the power spectrum of the representative grunt shown in A (grey trace) and the frequency sensitivity of auditory saccular afferent neurons recorded from juvenile (small and large) and adult (non-reproductive and reproductive) midshipman fish. (C) Representative example of a growl recorded at 16°C from a nesting type I male midshipman fish. Bar, 500 ms. (D) Comparison of the power spectrum of the representative growl in C (grey trace) and the frequency sensitivity of auditory saccular afferent neurons recorded from juvenile (small and large) and adult (non-reproductive and reproductive) midshipman fish. (E) Representative example of a hum recorded at 16°C from a nesting type I male midshipman fish. Bar, 10 ms. (F) Comparison of the power spectrum of the representative hum in E (grey trace) and the frequency sensitivity of auditory saccular afferent neurons recorded from juvenile (small and large) and adult (non-reproductive and reproductive) midshipman fish. Iso-intensity response curves are based on vector strength (VS) of synchronization to iso-intensity tones of 130 dB (re 1 μPa) and show median VS values for juvenile and adult midshipman (filled circles, squares and triangles from Fig. 4; open triangle, non-reproductive female midshipman fish from Sisneros and Bass, 2003).

ranging up to 400 Hz that typically contain as much or more spectral energy than the F_0, and are produced by nesting males during the breeding season to attract gravid females for spawning.

The results of this study indicate that the saccular afferents of juveniles, like those of non-reproductive adults, are best adapted to temporally encode the low frequency components (≤100 Hz) of midshipman vocalizations. The saccule of

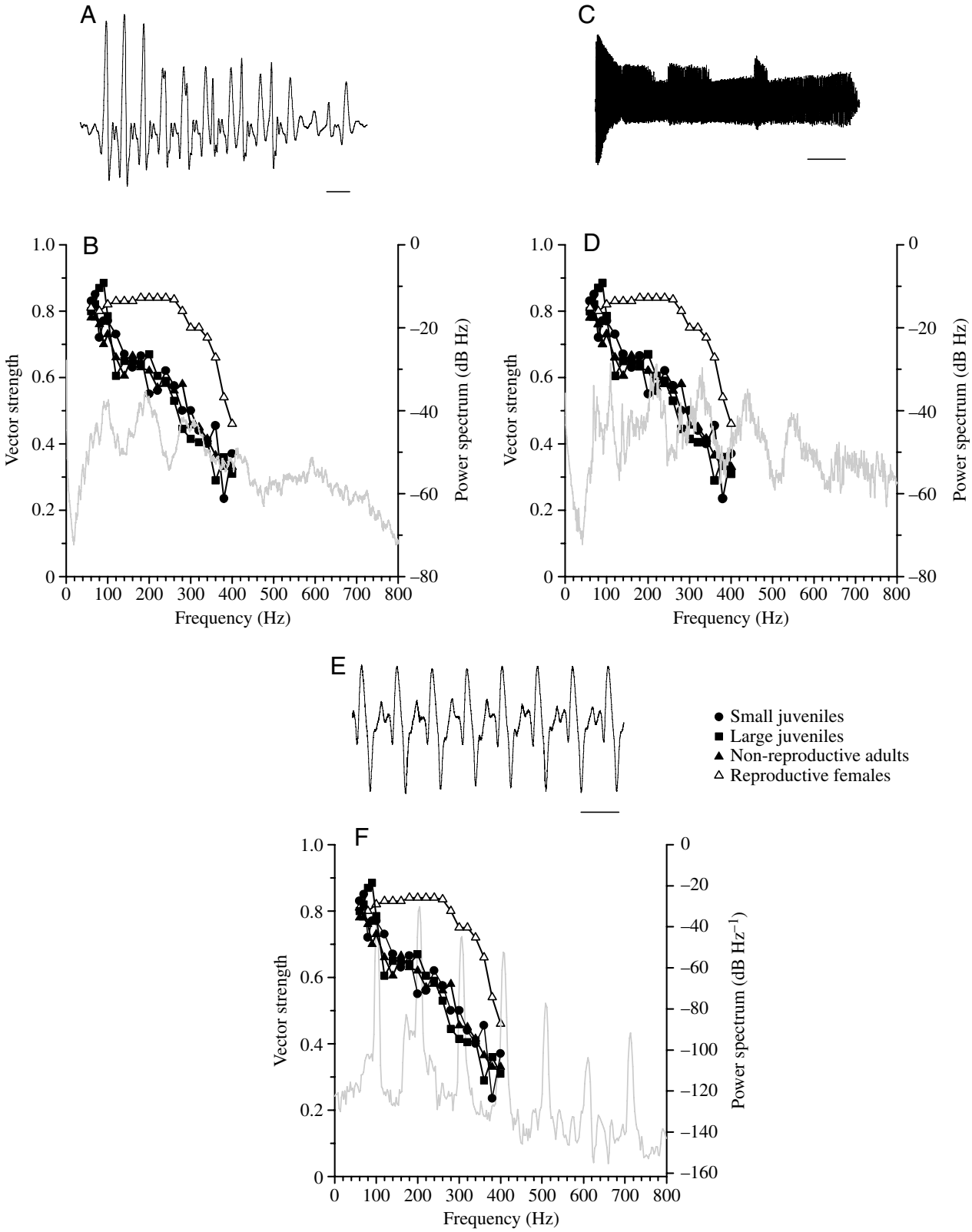

Fig. 8. See previous page for legend.

reproductive females shows enhanced temporal encoding of the higher harmonics of the hum (Sisneros and Bass, 2003; Sisneros et al., 2004a) and the growl (Fig. 8C,D), thereby likely improving the receiver's probability of conspecific detection, identification and localization during the summer breeding season (for further discussion, see Sisneros et al., 2004a; Bass and Clark, 2003; peripheral encoding in reproductive males has yet to be studied). It is currently unknown if juveniles are sonic, but we expect them only to make isolated grunts like females and type II males (see Brantley and Bass, 1994), especially given the similarities in the morpho-physiological properties of their vocal motor systems (Bass, 1995; Bass et al., 1996). Field measurements of transmission distance show that the F_0 of midshipman-like calls falls off rapidly with increasing distance from the sound source (Fine and Lenhardt, 1983; also see Bass and Clark, 2003), which in this case would be a conspecific. Given this, the auditory system of juveniles seems best adapted for the detection of calling conspecifics at close range.

Research support from NIH NIDCD DC00092 (A.H.B.) and 1F32DC00445 (J.A.S.). We thank M. Marchaterre and P. Forlano for field assistance, two anonymous reviewers for their helpful comments on the manuscript, and the University of California's Bodega Marine Laboratory for logistical support.

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