Comparison of Electrophysiological Auditory Measures in Fishes

Karen P. Maruska and Joseph A. Sisneros

Abstract Sounds provide fishes with important information used to mediate behaviors such as predator avoidance, prey detection, and social communication. How we measure auditory capabilities in fishes, therefore, has crucial implications for interpreting how individual species use acoustic information in their natural habitat. Recent analyses have highlighted differences between behavioral and electrophysiologically determined hearing thresholds, but less is known about how physiological measures at different auditory processing levels compare within a single species. Here we provide one of the first comparisons of auditory threshold curves determined by different recording methods in a single fish species, the soniferous Hawaiian sergeant fish *Abudefduf abdominalis*, and review past studies on representative fish species with tuning curves determined by different methods. The Hawaiian sergeant is a colonial benthic-spawning damselfish (Pomacentridae) that produces low-frequency, low-intensity sounds associated with reproductive and agonistic behaviors. We compared saccular potentials, auditory evoked potentials (AEP), and single neuron recordings from acoustic nuclei of the hindbrain and midbrain torus semicircularis. We found that hearing thresholds were lowest at low frequencies (~75–300 Hz) for all methods, which matches the spectral components of sounds produced by this species. However, thresholds at best frequency determined via single cell recordings were ~15–25 dB lower than those measured by AEP and saccular potential techniques. While none of these physiological techniques gives us a true measure of the auditory "perceptual" abilities of a naturally behaving fish, this study highlights that different methodologies can reveal similar detectable range of frequencies for a given species, but absolute hearing sensitivity may vary considerably.

Keywords *Abudefduf* • Acoustic • AEP • Hearing • Saccule • Torus semicircularis

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1 Introduction

The ability to detect underwater sounds is of vital importance for fishes that use their auditory and mechanosensory lateral line systems to mediate behaviors such as prey detection, predator avoidance, and social communication, which are crucial for survival and species perseverance. How do fish hear? How well do fish hear, and how do we measure their hearing capabilities? These seemingly simple questions have spawned decades-worth of research on the mechanisms, morphologies, and behavioral functions of fish auditory systems, which have uncovered remarkable diversity in structure and function even though only a limited number of the >30,000 species of fishes have been examined thus far.

The methodologies researchers utilize to measure both spectral hearing range and auditory thresholds in fishes have undergone a historical progression from behavioral techniques, which are laborious and slow to generate entire audiograms, towards quicker electrophysiological techniques that allow audiograms to be completed within a few hours. How well do these different electrophysiological methods reflect the true auditory capabilities of a particular species? What pertinent information can we obtain from each method? Is one method better than another and are the various methods comparable? These questions are difficult to answer without substantial recording examples of different types performed under similar experimental paradigms in diverse representative species. Towards this goal, we present here a comparison of multiple electrophysiological recording methods in a single damselfish species and use it as a framework for discussing the relative utility of different physiological techniques for determining auditory capabilities in fishes.

1.1 Methodologies Used to Measure Auditory Capabilities in Fishes

Techniques used to determine various aspects of fish auditory abilities can be separated broadly into two main categories, behavioral and electrophysiological. Behavioral and psychophysical methods include assays such as avoidance (Tavolga and Wodinsky 1963), operant (Yan and Popper 1991) and classical (Fay and MacKinnon 1969) conditioning, startle response (Bang et al. 2000), and prepulse inhibition (Bhandiwad et al. 2013). These behavioral techniques are advantageous because they measure evoked responses resulting from the integration and perception of the entire auditory scene that is relayed to neural output circuits causing whole animal behaviors. Some disadvantages of these behavioral methods, however, include long training periods and testing trials, unknown relative contributions of lateral line and inner ear components to the response, and the fact that not all behavioral methods work for a particular fish species. In the early days, these behavioral techniques dominated the world of fish bioacoustic research and were perceived as the best way to measure hearing in all animals (Fay 1988). Electrophysiological methods, on the other hand, include both minimally invasive

techniques such as auditory evoked potentials (AEP; formerly called auditory brainstem response, or ABR) (Kenyon et al. 1998; Ladich and Fay 2013), and more invasive approaches such as saccular potentials (Furukawa et al. 1972; Enger et al. 1973; Fay 1974; Sisneros 2007; Vasconcelos et al. 2011), single neuron recordings from auditory primary afferents (Fay 1978a, b; Fay and Ream 1986; Lu et al. 2003; Sisneros and Bass 2003) and single or multi-unit recordings from central auditory nuclei in the brain (Lu and Fay 1993, 1995; Bodnar and Bass 1997, 1999; Edds-Walton and Fay 1998, 2003, 2008; Kozloski and Crawford 2000; Maruska and Tricas 2009b). These electrophysiological methods typically require animal anesthetization and restraint, and depending on the method, are often focused on only a specific subset of the auditory processing pathway, which will subsequently be integrated by the animal to display context-appropriate behaviors. Due to their quick and relatively easy setup, however, electrophysiological methods are particularly useful for testing auditory effects during ontogeny, before and after physiologically relevant (e.g., steroids), acoustical (e.g. noise), or accessory auditory structure (e.g., swim bladder) manipulations (Yan et al. 2000; Scholik and Yan 2001; Egner and Mann 2005; Smith et al. 2006), and for comparing among species, sexes, social status, and reproductive conditions (Kenyon et al. 1998; Maruska et al. 2007, 2012; Ladich and Fay 2013). Thus, while both behavioral and electrophysiological approaches have advantages and disadvantages, their utility for examining auditory abilities in fishes is valuable but will vary based on the research question, species used, and other experiment-dependent limitations. Recent advances in neural telemetry that permit simultaneous neural recordings in freely behaving fishes will also likely make important contributions towards fully understanding the relationships between behavioral and electrophysiological measures of fish auditory and mechanosensory capabilities (Palmer and Mensinger 2002; Maruska and Mensinger 2015; Radford and Mensinger 2014).

1.2 Comparisons of Auditory Capabilities Using Different Methods within a Single Species

To understand the efficacy of determining auditory capabilities in fishes via these diverse techniques, it is imperative to compare measures obtained via several methods within a single species under similar testing conditions. Unfortunately, the existing comparative data on this topic are scant. Auditory abilities using both behavioral and physiological AEP methods have been achieved for only a small representative number of the >30,000 species of fishes, and include the goldfish (*Carassius auratus*), oyster toadfish (*Opsanus tau*), Oscar cichlid (*Astronotus ocellatus*), little skate (*Raja* (*Leucoraja*) *erinacea*), perch (*Perca fluviatilis*), red sea bream (*Pagrus major*), and common carp (*Cyprinus carpio*) [reviewed in Ladich and Fay 2013]. From these comparisons it is clear that there is no universal conversion between behavioral auditory thresholds and AEP-determined thresholds. However, Ladich and Fay (2013) note the generalization that AEPs tend to produce

higher thresholds at low frequencies (<1000 Hz), but lower thresholds at high frequencies (>1000 Hz) compared to behavioral thresholds. This suggests there may be a frequency-dependent effect between different assessment methods.

There are even fewer examples in which different electrophysiological-based recording methods have been determined in a single species. The goldfish (*C. auratus*), and batrachoidid oyster toadfish (*O. tau*) and midshipman fish (*Porichthys notatus*), are some of the most extensively studied species in terms of auditory capabilities. In addition to several behaviorally generated audiograms (Popper 1971; Enger 1966; Jacobs and Tavolga 1968; Offutt 1968), the goldfish has been examined physiologically by AEP (Kenyon et al. 1998; Smith et al. 2006; Cordova and Braun 2007; Ladich and Wysocki 2009), saccular potentials (Fay 1974; Fay and Popper 1975), single neuron recordings from saccular and lagenar primary afferents (Fay 1978a, b; Fay and Ream 1986) and recordings from various central auditory nuclei (Lu and Fay 1993, 1995; Kirsch et al. 2002; Ma and Fay 2002). The oyster toadfish has an AEP-generated audiogram (Yan et al. 2000), single neuron recordings from saccular primary afferents (Fine 1981; Edds-Walton and Fay 1995; Fay and Edds-Walton 1997), and recordings from central auditory nuclei (Edds-Walton and Fay 1998, 2003, 2005; Fay and Edds-Walton 1999; Edds-Walton et al. 2013) using both speaker and shaker table stimulus delivery methods. The Lusitanian toadfish (*Halobatrachus didactylus*) also has AEP (Vasconcelos et al. 2007; Vasconcelos and Ladich 2008) and saccular potential recordings (Vasconcelos et al. 2011). In addition to behavioral measures (Alderks and Sisneros 2013), the midshipman fish has saccular potential recordings (Sisneros 2007, 2009; Alderks and Sisneros 2011) single neuron recordings from saccular primary afferents (McKibben and Bass 1999; Sisneros and Bass 2003, 2005; Sisneros et al. 2004), and central auditory recordings (Bodnar and Bass 2001a; Bodnar et al. 2001). Primary afferent and central auditory recordings have also been done in the sound-producing mormyrid fish *Pollimyrus adspersus* (Crawford 1993, 1997; Kozloski and Crawford 2000; Suzuki et al. 2002). These limited examples become even further reduced for comparative purposes, however, because (1) many of these studies were not focused on generating audiograms or determining thresholds, but rather, were testing for other specific temporal or spectral processing mechanisms (i.e., used iso-intensity stimuli), and (2) recording methods performed in different laboratories with different experimental setups, including stimulus delivery (e.g., underwater speaker vs. shaker table) and experimental analyses with different threshold criteria, can be variable and difficult to compare. Thus, our current understanding of the relative usefulness of different electrophysiological-based techniques for determining spectral range and auditory thresholds for a given species is still in its infancy. Further, the only species examined thus far with multiple methods are those with either specialized accessory hearing structures like the Weberian ossicles in goldfish, or those endowed with sonic muscles on their swim bladder that use acoustic signaling as a primary mode of communication like toadfish and midshipman. In contrast, nothing is known about the majority of fish species that do not possess these hearing or sonic adaptations. What is needed, therefore, is a comparison of different electrophysiological methods to generate audiograms under similar experimental conditions

within the same species that will allow the assessment of these physiological measures at different auditory processing levels. These types of comparisons should provide insights into what information we can and cannot glean about auditory capabilities from singular recording methods within an individual species.

1.3 Study Species: Hawaiian Sergeant Damselfish, Abudefduf abdominalis

Damselfishes (family Pomacentridae) are a large group of reef fishes with approximately 360 species. Several damselfish genera are known to produce primarily broadband pulsed sounds during territorial and reproductive behavior, which conveys information about species, sex, body size, reproductive readiness, and aggression level (reviewed in Amorim 2006). Previous studies also demonstrate that both the frequency and temporal patterning of the pulsed sounds are critically important for acoustic communication in behaving pomacentrid fishes (Myrberg et al. 1993; Lobel and Mann 1995; Myrberg and Lugli 2006). The Hawaiian sergeant fish, *Abudefduf abdominalis*, is a colonial benthic-spawning damselfish that produces low-frequency, low-intensity pulsed sounds associated with reproductive and agonistic behaviors (Fig. 1). Further, the frequency hearing range matches the spectral content of sounds produced by naturally behaving wild fish (Maruska et al. 2007). During the protracted breeding season, males clean and prepare benthic substrates to attract females for courtship and spawning. After spawning, males remain to guard the nest, care for the developing young until they hatch, and continue to court and spawn with additional females over the course of the breeding season. Similar to other damselfishes examined thus far [see Zelick et al. 1999; Bass and McKibben 2003; Amorim 2006 for reviews], *A. abdominalis* does not appear to possess any special adaptations to enhance the detection of sound pressure, and the anterior edge of the swim bladder is typically several millimeters caudal to the otic capsule (~1.5–3.0 % of SL; Fig. 2a). This species is well suited for comparing different electrophysiological techniques that assess fish hearing because the behaviors associated with sound production including the temporal and spectral sound characteristics, central auditory nerve projections, and response properties of auditory neurons in the brain are already described (Maruska et al. 2007; Maruska and Tricas 2009a, b, 2011). This information facilitates interpretation of the auditory recording data in a biologically relevant context.

The goal of this study was to first characterize the AEP thresholds from the saccule (saccular potentials) in the Hawaiian sergeant fish, and then to compare them to the thresholds measured by the AEP and extracellular single unit recording techniques from the brain in this same species. These data are significant because no other study has directly compared auditory threshold measurements obtained by several different electrophysiological-based techniques from different auditory processing levels in a single soniferous fish under similar testing conditions.

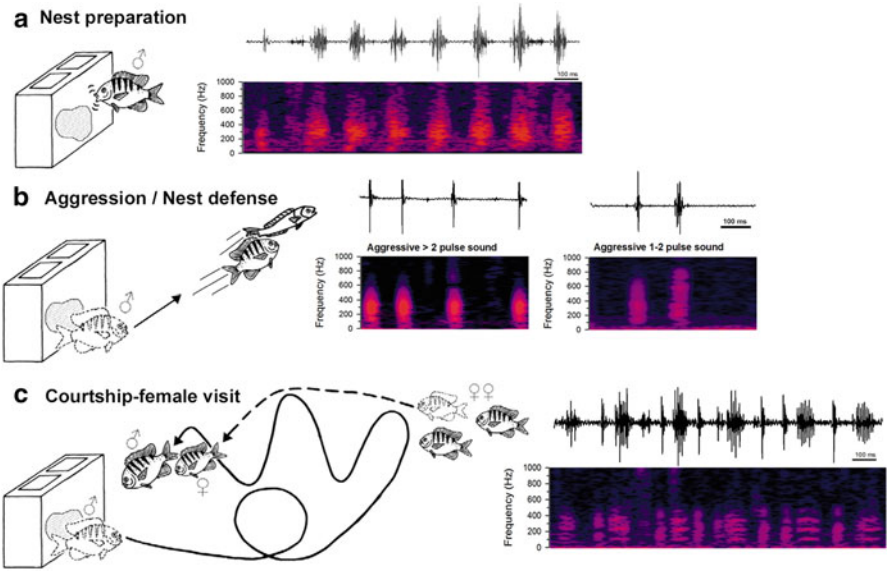

Fig. 1 Behaviors associated with sound production in the Hawaiian sergeant fish *Abudefduf abdominalis*. (**a**) Behavior and sound associated with nest preparation; males clean and prepare substrate adjacent to an existing nest (*dotted circular area*) and produce sounds when they scrape the substrate with their mouths, jaws and teeth. (**b**) Behavior and sound associated with aggression; males chase (*arrow*) both con and heterospecific (e.g., egg-predator wrasse) intruders away from the nest area while producing short-pulse aggressive sounds. (**c**) Behavior and sound associated with courtship–female-visit; males in blue nuptial coloration perform looping and zig-zag swims (*solid arrow line*) in the water column towards passing conspecific females. When a female follows the male back to the nest (*broken arrow line*), the courtship–female-visit sound is produced. Fish with a *dotted outline* in (**b**) and (**c**) represent the initial position, while fish with a *solid outline* represent the final position in the behavior sequence. Scale bars, 100 ms. Sounds are depicted as waveforms (*top*) and sonograms (*bottom*). Modified in part from Maruska et al. (2007)

2 Materials and Methods

2.1 Animals

Adult Hawaiian sergeant fish, *Abudefduf abdominalis*, were caught with hook and line from Kane'ohe Bay, Oahu and used immediately in recording experiments, with the exception of individuals used for saccular potential recordings (see below). At the end of each experiment, fish were measured for standard length (SL) and total length to the nearest 0.5 mm, body mass (BM) to the nearest 0.1 g, and sex was determined by examination of sexually dimorphic genital papillae and gonads under a dissection microscope. Collection, maintenance, surgical, and recording procedures for all fish used in this study were approved by the University of Hawaii Institutional Animal Care and Use Committee.

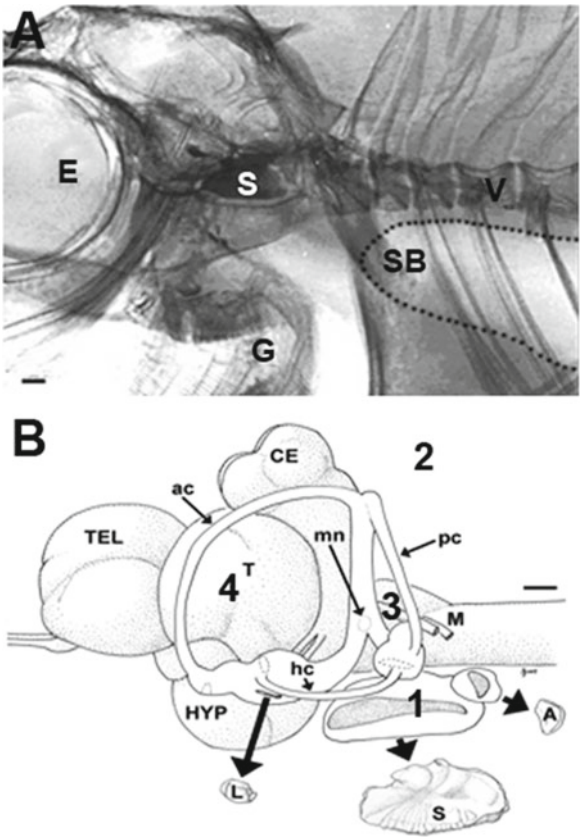

Fig. 2 Relative position of the inner ear to the swim bladder and location of auditory recording sites in the Hawaiian sergeant fish *Abudefduf abdominalis*. (**a**) Representative inverted X-ray to show the relative position of the swim bladder (*dotted outline*, SB) and saccule (S). Anterior edge of swim bladder is ~2–4 mm (~1.5–3.0 % of SL) from caudal edge of the otic capsule. E, eye; G, gills; V, vertebral column. Scale bar, 1 mm. (**b**) Lateral view of *A. abdominalis* brain is shown with otoliths removed (*large arrows*) to illustrate the four recording locations. 1, saccular potentials; 2, auditory evoked potentials (AEP) above the brain; 3, single neuron hindbrain; 4, single neuron midbrain torus semicircularis. A, asteriscus otolith of lagena; ac, anterior semicircular canal; CE, cerebellum; hc, horizontal semicircular canal; HYP, hypothalamus; L, lapillus otolith of utricle; M, medulla; mn, macula neglecta; pc, posterior semicircular canal; S, sagittal otolith of saccule; T, tectum; TEL, telencephalon. Scale bar, 1 mm

2.2 Saccular Potential Recordings

Evoked saccular potentials from the Hawaiian sergeant fish were recorded at the University of Washington. Adult *A. abdominalis* were caught as described above, packaged individually in large bags filled with seawater and oxygen, and transported via overnight air-service to the Department of Psychology at the University

of Washington. Fish were then transferred to holding tanks containing seawater at 20–22 °C and allowed to acclimate for at least 24 h prior to use in experiments. Fish were maintained on a 12 h light:dark cycle and fed daily with fish flakes or frozen squid/fish. Auditory threshold tuning curves were determined from 8 saccular potential recordings in 7 Hawaiian sergeant fish (3 males, 4 females; SL = 130.0 ±0.5 SD mm; BM = 93.2 ± 12.4 SD g).

Methods for recording saccular potentials from the Hawaiian sergeant fish were adapted from those used on the plainfin midshipman fish (Sisneros 2007). Briefly, fish were anesthetized with benzocaine and immobilized by an intramuscular injection of pancuronium bromide. The saccule of the inner ear was exposed by dorsal craniotomy, and the cranial cavity was filled with teleost Ringer's solution to prevent drying and enhance clarity. Fish were positioned so that the saccule was 10 cm above the surface of an underwater loudspeaker (UW-30) that was embedded in sand on the bottom of a 30 cm diameter, 24 cm high Nalgene experimental tank. The tank was positioned on a vibration isolation table and housed within an acoustic isolation chamber (Industrial Acoustics Co.), while all recording and stimulus generation equipment was located outside the chamber. Fish were ventilated continuously with seawater (22–24 °C) pumped through the mouth and over the gills during the experiments.

Acoustic stimuli were generated by the reference output signal of a lock-in amplifier (Stanford Research Systems SR830) that was input to an audio amplifier and underwater speaker (UW-30). The frequency response of the underwater speaker was measured with a mini-hydrophone (Bruel and Kjaer 8103) in the position normally occupied by the fish head. Relative sound pressure measurements were then made with a spectrum analyzer (Stanford Research Systems SR780), calibrated by peak-to-peak voltage measurements on an oscilloscope, and then adjusted with Matlab software so that the sound pressures at all tested frequencies (75–385 Hz) were of equal amplitude (within ±2 dB). Auditory stimuli consisted of 8–10 repetitions of single 500 ms duration tones with rise and fall times of 50 ms. Each repetition was presented at a rate of 1 every 1.5 s. Pure tone stimuli were presented at 10 Hz increments from 75 to 145 Hz and 20 Hz increments from 165 to 385 Hz. To determine threshold tuning responses, pure tone stimuli were presented at sound pressures from 100 to 145 dB re: 1 μPa in incremental steps of 3 dB.

Saccular potentials were recorded with glass microelectrodes (tip diameter, 1–2 μm) filled with 3 M KCl (1–10 MΩ). Electrodes were visually guided and placed into the endolymph of the saccule close to the sensory macula. Analog saccular potentials were preamplified (100×), input to a digital signal processing lock-in amplifier, and then stored on a PC computer running a custom data acquisition Matlab software control program. The lock-in amplifier yields a DC RMS voltage output signal that is proportional to the component of the signal whose frequency is exactly locked to the reference frequency. The reference frequency was set to the second harmonic of the stimulation frequency signal (i.e., twice the fundamental frequency) since the maximum evoked potential from the saccule of teleost fishes occurs at twice the stimulus sound frequency due to the presence of nonlinear and oppositely oriented hair cell populations within the saccule (Cohen and Winn 1967;

Furukawa and Ishii 1967; Hama 1969; Fay 1974; Zotterman 1943). Noise signals at frequencies other than the reference frequency are rejected by the lock-in amplifier and do not affect the measurements.

Threshold tuning curves were constructed by characterizing the input–output measurements of the RMS amplitudes of the evoked saccular potentials over the range of stimulus intensities at the tested frequencies. Background noise measurements were also recorded for 8–10 repetitions of the stimulus interval at each of the test frequencies with no auditory stimulus present prior to the recording of each threshold tuning curve, and were then used to establish subthreshold saccular potential response levels. Auditory threshold at each stimulus frequency was designated as the lowest stimulus intensity that evoked a saccular potential that was at least 2 SD above the background noise measurement. The frequency that evoked the lowest saccular potential threshold was defined as the best frequency.

2.3 Auditory Evoked Potential (AEP) Recordings

AEP tuning curves were determined from 7 Hawaiian sergeant fish collected in late July (6 males, 1 female; SL = 132.4 ± 7.0 SD mm; BM = 100.1 ± 18.5 SD g). To ensure fish were in similar reproductive condition to those used for saccular potential recordings, these fish were collected and tested immediately prior to the fish that were collected and shipped to the University of Washington in early August. AEPs were performed identical to that described in Maruska et al. (2007), except that additional stimulus frequencies in 25 Hz increments were tested between 100 and 400 Hz. This finer frequency resolution was performed to more closely match the frequencies used in saccular potential recordings, and because natural *A. abdominalis* sounds and best hearing sensitivity is within this low frequency spectral range. Briefly, immobilized fish were positioned in an experimental tank (30 cm diameter, 36.5 cm high, water level 29.5 cm high; fish positioned 16.5 cm above speaker) above an underwater speaker (UW-30, Lubell Labs) and stainless steel sub-dermal electrodes (Rochester Electro-Medical, Inc.; 6–12 kΩ) were placed beneath the skin in the head musculature above the hindbrain (recording electrode) and between the eyes (reference electrode). Fish were continuously ventilated with fresh seawater during all experiments. Acoustic stimuli were generated with a Cambridge Electronics Design (CED, Cambridge, UK) Micro 1401 controlled by Spike 2 software and delivered to the speaker via CED 3505 attenuator and amplifier (UMA 352, Peavey Electronics). Stimuli consisted of 2000 repetitions of 20 ms pulses (for ≥200 Hz: 10 ms plateau with rise and fall times of 5 ms; for 100 Hz: 10 ms plateau, rise, and fall; for 80 Hz: 13 ms plateau, rise, and fall). Sequential alternation of stimulus phase during the 2000 repetitions was used to eliminate stimulus artifacts in the AEP recordings. Trials began at suprathreshold intensities and were decreased in 5 dB steps to a sound level below the presumed threshold before moving to the next test frequency. Sound levels produced by the speaker were calibrated with a B&K hydrophone (model 8103; sensitivity −211 dB re: 1 V/μPa)

placed in the experimental tank at the position the fish head normally occupies, amplified (Nexus amplifier) and signal averaged by the Spike 2 script to determine sound pressure levels in dB_{rms} re: 1 μPa.

AEPs recorded via the sub-dermal electrodes were differentially amplified and band-pass filtered (DP-301, Warner Instruments), and then digitized on a CED Micro 1401 analog to digital interface run by Spike 2 software. A total of 2000 repetitions were averaged for each sound intensity and frequency, and power spectra (FFT, 512 or 1024 points) of these averaged waveforms were calculated to examine peaks at twice the stimulus frequency that result from the opposed orientation of hair cells and non-linearities in the auditory system. Thresholds were defined as the lowest sound level to show a repeatable AEP waveform above background noise and an FFT peak at twice the stimulus frequency. AEP recordings obtained here were similar to those reported previously for this species using identical experimental setups (Maruska et al. 2007).

2.4 Single Neuron Recordings in the Auditory Hindbrain and Midbrain

Single cell extracellular auditory neuron recordings from the hindbrain and midbrain previously measured in *A. abdominalis* for a separate study (Maruska and Tricas 2009b) were used here for comparison with the newly generated saccular potential and AEP recording data. These recordings were performed in the auditory medulla and midbrain torus semicircularis, and full methodological details can be found in Maruska and Tricas (2009b). Briefly, immobilized fish were positioned in an acrylic head holder above an underwater speaker (UW-30) in an experimental tank (30 cm diameter; fish positioned 10 cm above speaker) on a vibration isolation table inside a sound isolation chamber (Industrial Acoustics). Fish were ventilated continuously with seawater (23–25 °C) pumped through the mouth and over the gills during the experiments. The brain was exposed by dorsal craniotomy and the cranial cavity filled with Fluorinert fluid (FC-75, 3M) to enhance clarity, prevent drying, and reduce bleeding.

Extracellular single neuron recordings were made with carbon fiber (Carbostar-1, Kation Scientific, Inc., 400–800 kΩ) or glass (15–35 MΩ, filled with 4 M sodium chloride) microelectrodes advanced through the midbrain torus semicircularis (TS) or octaval nuclei of the hindbrain (primarily descending octaval nucleus) as an auditory search stimulus was presented (100–200 Hz at 124–126 dB_{rms} re: 1 μPa). Neural action potentials were amplified (500×–10,000×) and band-pass filtered (100–5000 Hz) with a Neurolog system (Digitimer, Inc.) and then converted to digital files with a CED power 1401 system run by Spike 2 software. Acoustic stimuli were generated by the CED digital to analog interface controlled by Spike 2 software, attenuated, and amplified before being sent to the underwater speaker. Stimulus characteristics were similar to those described above for AEP experiments except that 100 repetitions of 40 ms (10 ms rise and fall, 20 ms plateau) were used

for each test intensity and frequency to facilitate quicker generation of the entire audiogram data while the single neural recording was stable. Sound pressure levels were calibrated with a B&K hydrophone as described above for AEPs.

Thresholds were determined for each test frequency by beginning with a suprathreshold intensity followed by decreasing intensities in 5 dB increments until the neuron no longer responded to the stimulus. Threshold was defined as the lowest intensity to produce a Rayleigh statistic, or Z value, of ≥ 4.5 (Lu and Fay 1993; Batschelet 1981). The Z value measures the significance of phase-locking and is defined as $R^2 \times N$, where N is the total number of action potentials sampled, and R is the synchronization coefficient, or vector strength calculated according to (Goldberg and Brown 1969). The degree of phase-locking is generally a good predictor of auditory frequency encoding among vertebrates for low frequency systems (≤ 1 kHz) (Fay 1978b; Javel and Mott 1988; Sisneros and Bass 2003).

The four different recording locations compared in this study are depicted in Fig. 2b (saccular potential, AEP, hindbrain and midbrain single neurons), and all experiments used the same underwater speaker positioned beneath the fish as a stimulus. While the Hawaiian sergeant fish is likely most sensitive to particle motion rather than sound pressure, due to technical limitations and for comparisons to other studies, we only characterized the stimulus for all recordings in terms of sound pressure levels (dB re: 1 μPa) measured and calibrated in the experimental tanks with a hydrophone. We agree, however, that future studies on fish hearing should attempt to measure both sound pressure and particle motion in their experimental setups whenever possible as recently suggested by Popper and Fay (2011). This information would allow for better interpretation of auditory capabilities in biologically relevant contexts, as recent work shows differences in threshold curves expressed in terms of pressure versus particle motion primarily for species with special adaptations to transfer pressure fluctuations from the swim bladder to the inner ear (Horodysky et al. 2008; Wysocki et al. 2009; Radford et al. 2012).

3 Results

3.1 Saccular Potential Recordings

Similar to previous studies (Fay 1974; Fay and Popper 1974; Sisneros 2007), saccular potentials from the Hawaiian sergeant fish were evoked maximally at twice the stimulus frequency rather than at the same stimulus frequency (Fig. 3). This double frequency effect is due to hair cell populations with opposite orientations and is also dependent on the nonlinearity of the saccular potential such that the cancellation of two sinusoidal waveforms 180° out of phase with each other is avoided (Fay 1974). Best frequency was defined as the frequency that evoked the saccular potential with the lowest threshold and ranged from 109 to 124 dB re: 1 μPa at 75 Hz (the lowest frequency tested) for all individuals tested. The majority of saccular potential tuning curves showed lowest thresholds at this best frequency of

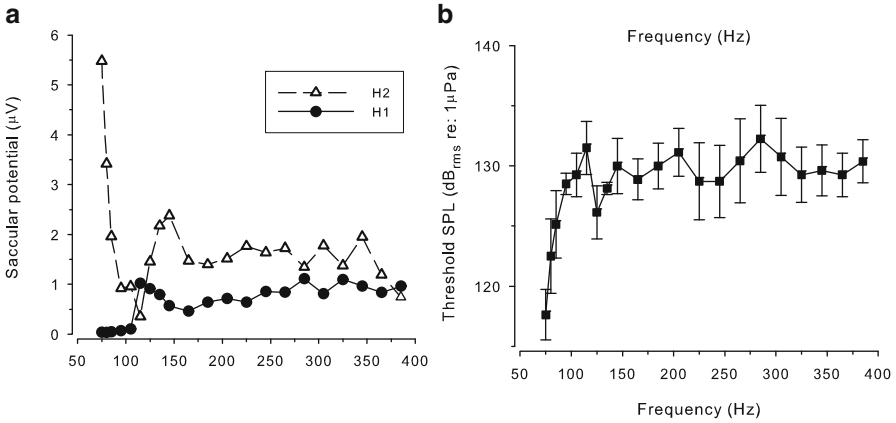

Fig. 3 Saccular potential recordings from the Hawaiian sergeant fish *Abudefduf abdominalis* show best sensitivity to low frequencies. (**a**) Representative example of iso-intensity curves of saccular potentials evoked at the same stimulus frequency (H1, the first harmonic or fundamental frequency) and at twice the stimulus frequency (H2, second harmonic) from the saccule in response to single tones at 130 dB re: 1 μPa. Both recordings were taken from the same position within the saccule. (**b**) Threshold tuning curve based on evoked potentials from the saccule. Threshold at each stimulus frequency was determined as the lowest stimulus intensity in dB re: 1 μPa that evoked a saccular potential that was at least 2 SD above the background noise measurement. Data are plotted as mean ± SD. $N = 7$ fish, 8 recordings

75 Hz, with an increase in threshold from 80 to 115 Hz and then a plateau in response from 115 to 385 Hz (Fig. 3). When thresholds were compared with a repeated measures one-way ANOVA, 75 Hz differed from all other test frequencies except 80 and 85 Hz (RM ANOVA; $F_{(7,136)} = 4.32$; $p < 0.001$; Holm–Sidak posthoc comparisons, $p < 0.05$). There were no other differences in threshold among test frequencies.

3.2 *Auditory Evoked Potential Recordings*

AEPs were obtained from all test fish and showed similar averaged response waveforms for a given frequency across all individuals (Fig. 4). FFT analyses of averaged AEP waveforms also showed peaks at twice the stimulus frequency for intensities at and above threshold. Best frequencies ranged from 80 to 125 Hz for all individuals tested (120–121 dB_{rms} re: 1 μPa). Auditory thresholds determined by AEP showed

Fig. 4 (continued) determine threshold (1024 points). Five different stimulus intensities at 100 Hz are shown. Bottom trace (*green*) shows the stimulus waveform. FFT analyses illustrate peaks at approximately twice the stimulus frequency from 130 to 115 dB. Threshold for this individual fish at this test frequency was 115 dB_{rms} re: 1 μPa. (**b**) Threshold tuning curve for AEPs (*left* y-axis) with overlay of spectral content (*right* y-axis) of different natural sounds produced by the Hawaiian sergeant fish. AEP data (*triangles*) are plotted as mean ± SE, $N = 7$ fish

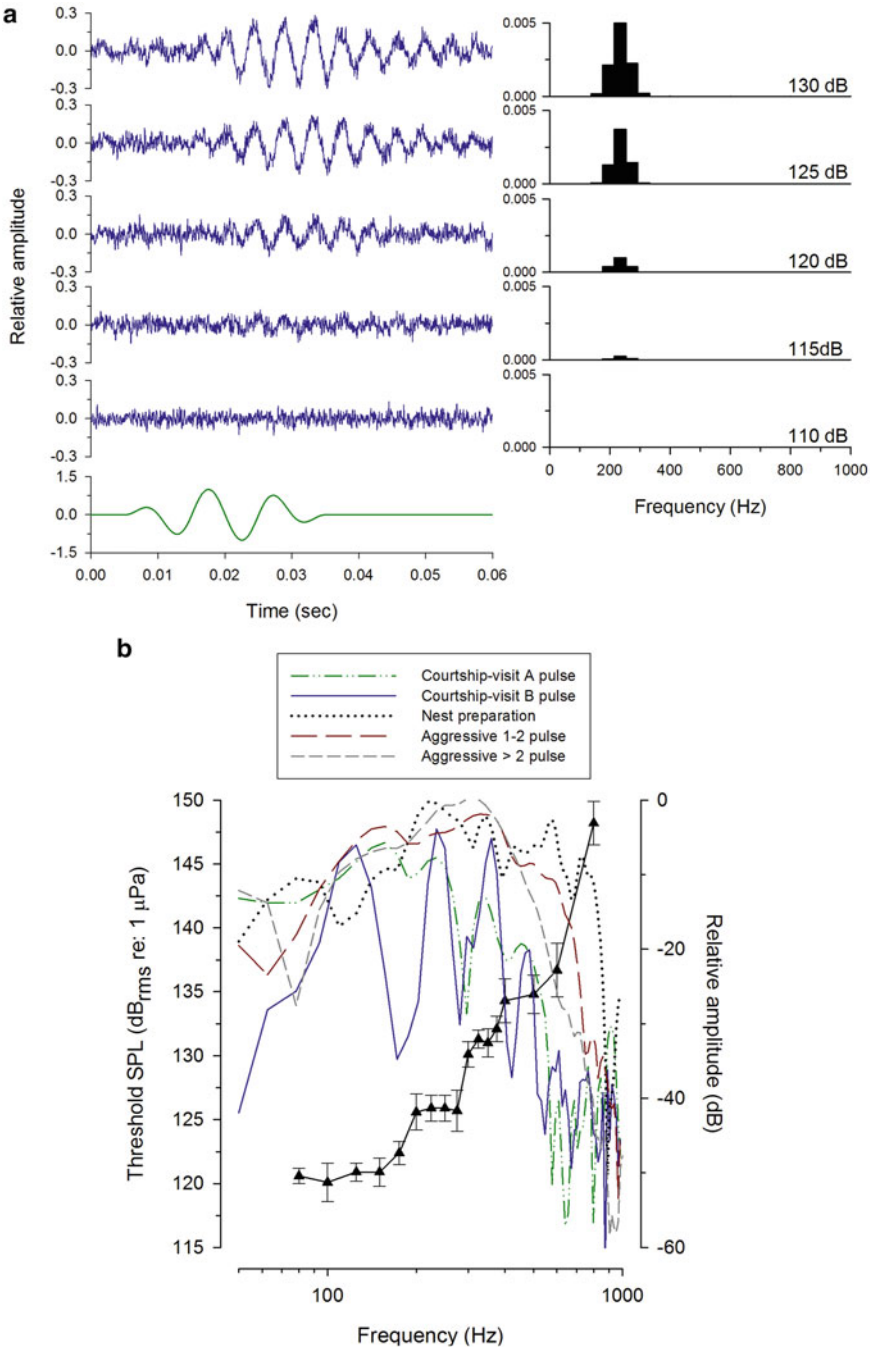

Fig. 4 Auditory evoked potential (AEP) recordings from the Hawaiian sergeant fish *Abudefduf abdominalis* show low-frequency sensitivity that matches the spectral content of sound production. (**a**) Representative example of averaged AEP waveforms (*left*) and FFT analyses (*right*) used to

best sensitivity at low frequencies (≤300 Hz) for all tested fish, and there was a 30 dB difference in threshold values between the frequency of best sensitivity (80–125 Hz) and worst sensitivity (800 Hz). AEP thresholds did not differ between 80 and 275 Hz, but these lower frequencies differed from those at 300–600 Hz, and threshold at the highest test frequency (800 Hz) differed from all other frequencies (RM ANOVA, $p<0.001$; $F_{(6,96)}=56.29$; Holm–Sidak posthoc comparisons, $p<0.05$).

3.3 *Comparison of Saccular Potentials, AEPs, and Single Neuron Recordings in* A. abdominalis

The tuning curves determined by saccular potential, AEP, and single unit auditory hindbrain and midbrain recordings are plotted together in Fig. 5 and represent four different threshold measurements at levels from auditory hair cells to midbrain neurons. There are several important points to note from this figure. First, the lowest thresholds are at the low frequencies for all curves from 75 to 85 Hz for saccular

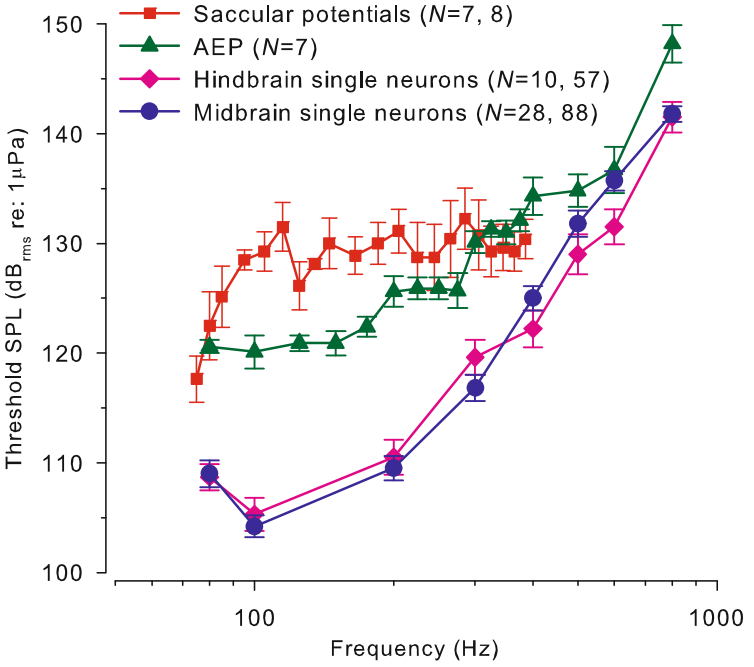

Fig. 5 Comparison of auditory threshold tuning curves in the Hawaiian sergeant fish *Abudefduf abdominalis* determined by different electrophysiological recording methods. Data are plotted as mean ± SE. *N* = number of animals, number of recordings for saccular potentials; number of animals for AEP recordings; and number of animals, number of neurons for hindbrain and midbrain single unit recordings

potentials, and 80 to 300 Hz for AEP and single cell recordings in the brain. Second, the highest thresholds were observed in the saccular potential recordings. This is likely because the potentials are recorded from a small region of the hair-cell based sensory macula from the saccule on one side of the fish head. Thus, there is little neural convergence and no summation of the response from both inner ears, as would be present in the AEP and single unit recordings from the brain. A similar difference in thresholds (~10–20 dB) was seen between saccular potentials and AEP thresholds in the Lusitanian toadfish (see Fig. 6). Third, the hindbrain single unit curve shows similar sensitivity to the midbrain units at the low frequencies (80–200 Hz), but broader tuning at the higher frequencies (300–800 Hz). Thus there is possibly a low pass filtering mechanism between the hindbrain and midbrain in the

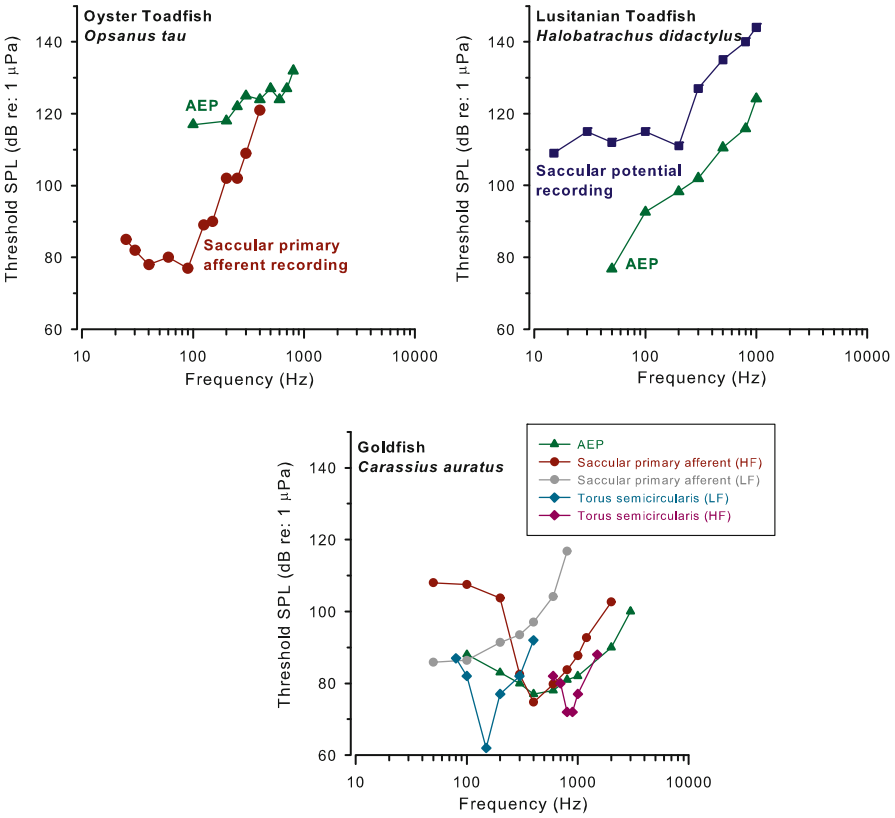

Fig. 6 Representative examples of auditory tuning curves obtained by different electrophysiological recording methods in several fish species. Values were estimated from previously published figures and data from the following papers: Oyster toadfish (Yan et al. 2000; Fine 1981); Lusitanian toadfish (Vasconcelos et al. 2007, 2011); Goldfish (Lu and Fay 1993; Fay 1978a; Ladich and Fay 2013; Fay and Ream 1986). Threshold sound pressure levels (SPL) reported for neural recordings in the goldfish were converted from dB re: 1 dyne/sq.cm to dB re: 1 µPa for comparisons. HF, high frequency neurons; LF, low frequency neurons

Hawaiian sergeant fish, as shown for several other species (Feng and Schellart 1999). Fourth, the dynamic range of threshold values from lowest to highest sensitivity across similar ranges of frequencies is greatest for the midbrain single unit recordings (37.6 dB), followed by hindbrain units (33.7 dB), AEPs (28.1 dB), and saccular potentials (12.8 dB). Fifth, there is an approximately 15–20 dB difference in sensitivity at the best frequency of 100 Hz between the single unit recordings and the AEP recordings, and a 25 dB difference between the single units and the saccular potential recordings at this frequency. The reason for these differences in sensitivity is not known, but may be related to recording locations (e.g., peripheral vs. central auditory system) and methodology, or properties inherent to different portions of the auditory processing pathway.

4 Discussion

The goal of this study was to generate auditory threshold tuning curves in the Hawaiian sergeant fish using saccular potentials and AEP recordings, and then compare them to previously determined single neuron recordings from different auditory brain nuclei to determine how threshold measures at different processing levels compare in a single teleost species. Our results show that the Hawaiian sergeant fish is most sensitive to low frequency tone stimuli (≤300 Hz), regardless of recording technique, which matches the spectral content of their sound production during agonistic and reproductive behaviors. Relative hearing thresholds, however, differed by as much as 5–25 dB between the different recording methods, with largest differences occurring at these same low frequencies (≤300 Hz). Our results are interpreted below with the aim of discussing the utility of different electrophysiological methods in fish hearing and bioacoustics research, as well as their biological implications for the study species.

4.1 Saccular Potentials and AEP Recordings in the Hawaiian Sergeant Damselfish

Saccular potential recordings in *A. abdominalis* revealed best hearing sensitivities at low frequencies (<125 Hz). Several previous studies used evoked potentials to determine the sensitivity and response dynamics of saccular inner ear hair cells in teleost fishes (Adrian et al. 1938; Furukawa et al. 1972; Fay 1974; Sisneros 2007; Alderks and Sisneros 2011), and they are easily identified because they are evoked at twice the stimulus frequency due to the presence of nonlinearities and oppositely oriented hair cell populations in the fish saccule (Furukawa and Ishii 1967; Hama 1969; Fay 1974; Fay and Popper 1974). This frequency doubling effect is also evident in FFT analyses of AEP recordings and is present in the lateral line system (Flock 1965) for similar reasons, but is absent in the cochlea and vestibular system

because the hair cells are oriented in only one direction and the evoked potential occurs at the stimulus frequency (de Vries and Bleeker 1949; Tasaki et al. 1954). The magnitude of the saccular potentials in the Hawaiian sergeant fish were generally lower than and did not have the dynamic range of those observed in the midshipman and Lusitanian toadfish measured with the identical experimental setup (Sisneros 2007; Alderks and Sisneros 2011; Vasconcelos et al. 2011). This difference could be due to several factors including electrode placement in the saccule (either distance between recording electrode and hair cells, or position of electrode in regions with hair cells oriented off the vertical stimulation axis), especially since the saccule in *A. abdominalis* is located deep within the otic capsule beneath the medulla. This location makes it difficult to position electrodes in this area compared to the more easily accessible and laterally positioned saccule in batrachoidid fishes. Alternatively, the Hawaiian sergeant fish saccule may just be less sensitive than the midshipman to stimuli along the dorso-ventral axis. Nevertheless, the tuning curves obtained by saccular potential recordings in the Hawaiian sergeant fish are within the range of thresholds obtained by the AEP technique in this species and in the congener *A. saxatilis* (Egner and Mann 2005). Recordings from individual endorgans like the saccule in fishes provide important information about the response properties of hair cells, which are the first processing level of the auditory system. These types of recordings are also valuable for comparisons to recordings done at subsequent processing levels. For example, saccular (and lagenar and utricular) recordings can be used to evaluate whether changes in auditory sensitivity due to circulating hormones or noise exposure occur at the level of the macula and hair cells, or elsewhere along the auditory pathway.

Our finer low-frequency resolution tuning curve generated for the Hawaiian sergeant fish by AEP is similar to that previously determined using fewer test frequencies (Maruska et al. 2007). The additional frequencies, however, further highlight that this species is most sensitive to tonal stimuli of ≤200 Hz, with slightly lower sensitivity but with similar thresholds across the range of 200–285 Hz, and then with a steady drop in auditory sensitivity from 300 to 800 Hz. The low thresholds measured across this frequency range overlaps the dominant spectral energy found in all of the natural agonistic and courtship sounds produced by this species (<80–400 Hz) (Maruska et al. 2007), illustrating a match between hearing ability and sound production for communication. Low frequency acoustic information is also likely important for all fishes to survey complex "soundscapes" for mediating other non-communicative behaviors such as prey detection, predator avoidance, and assessment of ambient noise and environmental disturbances (Fay 2009). This low-pass frequency hearing is similar to most other fishes that do not have accessory auditory specializations (e.g., midshipman and toadfish) but instead rely on the otolithic endorgans that detect acoustic particle motion by acting as inertial accelerometers (Fay and Edds-Walton 1997; Sisneros 2007). Fishes that do possess adaptations to detect the pressure component of sound stimuli, on the other hand, typically have enhanced high-frequency hearing abilities (e.g., goldfish, mormyrids, clupeids, labyrinth fishes). However, even these species that detect high frequencies (≥800 Hz) have some saccular primary afferent and central neurons tuned to low

frequencies (≤200 Hz) in addition to those tuned to higher frequencies (Lu and Fay 1993; Fay and Ream 1986; Suzuki et al. 2002). This suggests that the maintenance of low frequency encoding may be a general characteristic found in all fish auditory systems. This low frequency hearing may be driven by environmental constraints of the underwater environment that favor the detection of low frequency sounds that propagate farther distances than high frequency sounds, as well as facilitate the localization of sound sources using directional particle motion cues (Zeddies et al. 2012).

4.2 Comparison of Different Auditory Physiology Recording Techniques

Our comparison of different electrophysiological recording techniques illustrates the limitation of comparing data sets among studies that use different methods, and the value of using multiple techniques to examine auditory encoding in a single species. In the Hawaiian sergeant fish, different recording techniques revealed a similar detectable range of frequencies, but the thresholds or sensitivity measures varied considerably among methods. For example, auditory thresholds varied by as much as 10–25 dB among techniques, with the greatest differences occurring at low frequencies (75–400 Hz). Since the spectral content of the sounds produced by the Hawaiian sergeant fish is also at these same low frequencies to which their auditory system is most sensitive, the threshold differences have important biological implications. This generalization of comparable frequency range but varying thresholds appears to hold true for other species such as batracoidids, but not for goldfish, which shows more overlap in thresholds obtained by different recording techniques (Fig. 6). The oyster toadfish, for example, also shows differences in thresholds between AEP and primary afferent recordings from the saccular nerve, with a 40 dB difference between the techniques at 100 Hz. In the Lusitanian toadfish, differences of 10–25 dB are also evident between AEP and saccular potential recordings across the low frequency range tested. These observed differences in auditory sensitivity among recordings in the same species could be due to methodology differences (e.g., electrode placement, threshold criteria, tank acoustics), or inherent biological characteristics of each recording location (e.g., summation, convergence, relative inputs from inner ear and lateral line) that are important for the animals perception of its auditory world.

While our study attempted to keep as many experimental conditions constant across recording methods as possible, there were several unavoidable variations that cannot be ruled out as contributors to the observed threshold differences. For example, the experimental tank, as well as the position of the entire fish and saccule beneath the water surface in AEP experiments differed from that of the other three techniques in which the saccule was closer to the water surface due to the surgical intervention required for electrode placement. Since the acoustics in small tanks and near the air–water interface can be complex (Parvulescu 1967; Akamatsu et al. 2002),

it is possible that variations in tank dimensions and position of the saccule relative to the water surface has important consequences for threshold determination. However, tank dimensions and fish position were essentially identical between saccular potential recordings and single neuron recordings in the brain, suggesting that the differences in threshold between these techniques are due to biological rather than methodological variations. Nevertheless, future studies should carefully consider and characterize particle motion and sound pressure levels throughout their experimental tank, as well as any other subtle procedural variations.

One important auditory sensitivity measurement missing from our data set in the Hawaiian sergeant fish is a behavioral audiogram determined by classical conditioning or psychophysical methods. Behavioral auditory thresholds are often, but not always, lower than any electrophysiologically determined thresholds and may be the best indicator of true hearing abilities in a species. However, they are extremely time-consuming and difficult to generate in some fish species, especially those that do not respond to the training paradigms. Physiologically determined audiograms are valuable because they provide a good estimate of the frequency hearing abilities of a species (i.e., spectral range), including a measure of best frequency, in a comparatively shorter amount of time, even though they may underestimate hearing sensitivity at certain frequencies in some species. However, this underestimation is not a universal relationship among all fishes. For example, behavioral thresholds are lower than (Fay 1974; Kojima et al. 2005), greater than (Kenyon et al. 1998), or similar to (Fay 1978a, b; Kenyon et al. 1998; Ladich 1999, 2000) physiologically determined thresholds in different species [see also Ladich and Fay 2013 for a review], suggesting that differences may be species-specific and dependent on experimental factors that vary among labs. Based on their extensive comparison of AEP and behavioral tuning curves in many fishes, Ladich and Fay (2013) note that AEPs tend to produce higher thresholds at low frequencies (<1000 Hz) and lower thresholds at high frequencies (>1000 Hz) compared to behaviorally generated audiograms, suggesting there is also a frequency dependent effect between these two methods.

The type of auditory recording method employed in a study will depend largely on the research question addressed, species used, and the available resources. For example, AEPs have become popular in recent years because they are relatively quick to perform, easy to learn, inexpensive to setup, applicable to almost any species, and are minimally invasive allowing repeated measurements in the same individuals. AEPs are therefore valuable for obtaining rapid information on the frequency range and threshold tuning for a particular species, as well as doing before and after comparisons following manipulation or "intervention" to test some aspect of hearing (e.g., exploring temporary hearing changes that result from noise exposure). Single neuron recordings, on the other hand, require more expensive equipment, invasive surgical approaches, complex analysis tools, and expertise to perform and interpret. Neural recordings that examine auditory responses at different points along the ascending pathway, however, are quite valuable for providing important information on specific auditory processing and filtering mechanisms that occur at different levels within the central auditory system. This type of information cannot be obtained

from recordings such as AEPs that likely average the response across multiple levels of the auditory processing pathway. In most AEP studies, what appear to be recorded are the evoked double frequency responses of the hair cells and their afferents along with some auditory brainstem and midbrain activity (Corwin et al. 1982). In contrast, single neuron recordings can reveal specific filtering and response properties of auditory neurons. These properties include the low pass filtering system observed between the hindbrain and midbrain in the Hawaiian sergeant fish (Maruska and Tricas 2009b), the sharpening of directional response properties that occurs along the auditory pathway in the toadfish (Edds-Walton and Fay 2005), and as a generalization, the decrease in spontaneous activity, increased latency, and sharpened tuning in the ascending auditory pathway from primary afferent to hindbrain to midbrain neurons that exists in several fish species (Feng and Schellart 1999). Thus, peripheral and central neural recordings have uncovered many important aspects of fish auditory processing capabilities such as temporal encoding (Fay 1977; Fay and Coombs 1983; Carr 1986; Bodnar and Bass 1997; Kozloski and Crawford 2000; Bodnar et al. 2001), frequency selectivity, role of inhibition in shaping frequency responses, filtering properties, and phase-locking ability (Fay 1978a, b; Lu and Fay 1996; Kawasaki and Guo 1998; Sisneros and Bass 2003; Maruska and Tricas 2009b), directional sensitivity (Fay 1979; Lu et al. 1998; Edds-Walton and Fay 2003, 2005), integration with other senses (Schellart 1983; Prechtl et al. 1998; Fay and Edds-Walton 2001), and effects of hormones and neuromodulators on the auditory system (Sisneros et al. 2004; Maruska and Tricas 2011).

Tuning curves from single neuron recordings, however, are difficult to compare directly to techniques such as AEP and saccular potentials because the auditory system contains neurons of many different types and response dynamics, particularly in the auditory nuclei of the brain. Thus, some individual neurons in the same fish can show differences in threshold of 20–40 dB to the same frequency, be untuned, broadly tuned, or sharply tuned, be tuned to only low, mid, or high frequency stimuli, and vary in their degree of phase-locking (Fay 1978a; Fay and Ream 1986; Lu and Fay 1993; Feng and Schellart 1999; Edds-Walton and Fay 2003; Maruska and Tricas 2009b). This individual variation may also contribute to the often lower thresholds detected with peripheral or central single neuron recordings compared to AEP and saccular potentials in the Hawaiian sergeant, toadfish, and goldfish (Fig. 6). Further, there are also differences in temporal processing features (e.g., overall envelope encoding, waveform structure detection) among individual neurons in the same brain area (Fay and Coombs 1983; Crawford 1997; Bodnar and Bass 1999, 2001b). These neural response characteristics are important for understanding how fishes encode the auditory scene and their perceptual world or "umwelt," which cannot be detected from behavioral, AEP, or saccular potential recordings. In fact, single auditory neurons in the midbrain and hindbrain of the Hawaiian sergeant fish are more sensitive to playbacks of natural courtship and aggressive sounds than to single frequency tonal stimuli (Maruska and Tricas 2009b). This indicates that thresholds to the tonal stimuli typically used in electrophysiology recording studies may be higher than that measured if more natural sounds which contain complex spectral and temporal characteristics were used. Thus, single neuron recordings are

extremely useful for studying how salient information from sounds received at the inner ear is transformed along the auditory pathway and ultimately integrated with other senses and internal physiology to allow context-appropriate behavioral decisions.

Other important factors to consider when comparing different electrophysiological techniques are the relative contributions of the different endorgans (saccule, lagena, and utricle) and the mechanosensory lateral line system to the recorded "auditory" response, which may account for some of the observed differences in thresholds across techniques (Table 1). The majority of fish auditory research has concentrated on the largest endorgan, the saccule, but most species will also have significant inputs from the lagena and utricle that are likely species-specific but not yet completely understood. A recent study conducted in the goldfish also demonstrated that the lateral line system contributes to AEPs at low frequencies (Higgs and Radford 2013), and this is likely true for many species. In contrast, potentials recorded directly from the sensory macula or primary afferents of the saccule, utricle, or lagena would not contain input from the mechanosensory system, and the segregation of auditory and lateral line inputs to the hindbrain nuclei in fishes suggests most recordings from these medullary areas only contain inner ear information (McCormick 1999). Recordings from auditory-responsive regions of the midbrain torus semicircularis, diencephalic, and telencephalic nuclei, however, may contain bimodal or multimodal neurons that receive both lateral line and inner ear information, and in some cases visual and somatosensory cues as well (Schellart 1983; Lu and Fay 1995; Prechtl et al. 1998; Kirsch et al. 2002). Since most electrophysiological recording experiments use small experimental tanks with often

Table 1 Summary of potential sensory system contributions to hearing thresholds determined by different techniques

	Auditory system (inner ear)	Mechanosensory lateral line system
Behavioral or psychophysical methods	Saccule, lagena, utricle (both sides)	Canal and superficial neuromasts (whole body)
Auditory evoked potentials	Saccule, lagena, utricle (both sides)	Canal and superficial neuromasts (whole body)
Otolithic endorgan potentials	Single otolithic endorgan only (saccule, utricle, or lagena)[a]	None
Primary afferent recordings	Single otolithic endorgan only (saccule, utricle, or lagena)[a]	None
Hindbrain auditory nuclei single neuron recordings	Saccule, lagena, utricle[b] (primarily ipsilateral)	Minimal to none
Midbrain auditory torus semicircularis single neuron recordings	Saccule, lagena, utricle (contralateral and ipsilateral)	Canal and superficial neuromasts (whole body)[c]

[a]Endorgan potentials and primary afferent recordings represent only that individual endorgan being recorded from

[b]Endorgan contribution is dependent on which hindbrain nucleus recordings are made from

[c]There is evidence for bimodal neurons that respond to both mechanosensory and auditory stimuli in the torus semicircularis of some fish species

complex and unknown particle displacement fields (Parvulescu 1964, 1967; Akamatsu et al. 2002), it is important to recognize the relative contribution of otolithic endorgan versus lateral line system input to "hearing thresholds" across species. While in most cases it may not matter to the fish whether a biologically relevant stimulus is detected by the inner ear, lateral line, or both, it does become important when characterizing the response dynamics of individual sensory systems [see Braun and Sand 2014 for discussion of overlap between lateral line and auditory systems in fishes, and also Higgs and Radford, in this volume].

5 Conclusions and Future Directions

Our study comparing auditory threshold tuning curves measured by different electrophysiological methods in a single species highlights the great variability in thresholds within an animal's spectral range of best sensitivity among the different techniques, suggesting that single curves generated for a particular species should be interpreted with caution. Despite our current knowledge, there are still many remaining questions and important areas of future work, several of which are briefly mentioned below.

1. More studies should be performed using multiple recording methods within a single species, as well as in representatives of diverse species with different anatomical specializations. These studies should help clarify the methodological and biological reasons for the different thresholds measured across multiple levels of the auditory pathway from peripheral endorgan hair cells to central processing levels in the brain. Ideally these studies should be conducted in the same lab with identical experimental setups using similar stimulus delivery (i.e., speaker or shaker system) and threshold criteria, as well as characterization of the stimulus in terms of both sound pressure and particle motion.
2. To truly understand the auditory capabilities of a particular species, multiple electrophysiological recording techniques should also be combined and compared with behavioral audiograms within a single species. These data could then be used in combination with an assessment of the ambient noise and sound propagation properties of the fish's natural habitat to gain a better understanding of the ecology and evolution of a species' auditory system. Electrophysiology recordings using playbacks of natural sounds in addition to tonal stimuli will also be informative. The enormous diversity of fish auditory sensitivities, inner ear morphologies, and accessory hearing structures should provide fruitful future comparisons for the selective pressures that have shaped the evolution of the auditory system.
3. More electrophysiological recordings are also needed from the other putative auditory endorgans, the utricle and lagena. In comparison with the numerous studies on the saccule, there are few physiological recordings from these other endorgans in fishes (Fay and Olsho 1979; Lu et al. 2003, 2004; Maruska and

Mensinger 2015; Meyer et al. 2010, 2012), and therefore limited understanding of how they contribute to auditory sensitivity and directional hearing abilities that should be further explored. Similarly, the relative contribution of the mechanosensory lateral line system to "hearing" thresholds and its overlap in acoustic sensitivity with the inner ear should be carefully considered when reporting auditory capabilities of different species (see Higgs and Radford, in this volume).

4. Lastly, moving forward, there is a need for studies that examine the relative role of the auditory system as only one sensory component of a fish's entire perceptual world, or umwelt. Fishes must constantly assess simultaneous incoming information from multiple sensory channels (auditory, mechanosensory, visual, chemosensory, somatosensory, vestibular, and in some cases electrosensory) and integrate it to make context-appropriate behavioral decisions about crucial tasks related to their survival and reproduction such as when to eat, when to flee from predators, and when to reproduce. Perception of the complex underwater "soundscape," therefore, represents just one aspect of the multimodal input used for neural computations, and future work is needed to determine the relative importance of auditory information in mediating different behaviors in all fishes, the most diverse and speciose group of vertebrates.

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