Utricular auditory sensitivity of the vocal plainfin midshipman, *Porichthys notatus*

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Abstract

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The detection of behaviorally relevant auditory signals is critical to the reproductive success of many vocal non-mammalian vertebrate species, including songbirds, amphibians, and fishes. Among vocal fishes, the plainfin midshipman (*Porichthys notatus*) has served as a robust model organism for investigating the behavioral, physiological, and neuroanatomical mechanisms underlying the detection, integration, and localization of behaviorally relevant social and reproductive-related vocal acoustic signals. Many of the previous studies investigating the auditory functions of the midshipman inner ear have focused on the saccule and lagena; however, little is known regarding the auditory sensitivity of the utricle. Here, using a well-characterized electrophysiological approach, I investigate the auditory sensitivity of the midshipman utricle. In chapter 2, I characterize the auditory sensitivity of utricular hair cells in nonreproductive male midshipman and show that the utricle exhibits auditory sensitivity similar to the saccule in response to a broad range of behaviorally relevant auditory stimuli. Next, in chapter 3, I show, using surgical manipulations, that the swim bladder of female plainfin midshipman functions to transduce acoustic sound pressure to the utricle, thus effectively enhancing auditory and frequency sensitivity. Finally, in chapter 4, I ask if seasonal variation in reproductive state modulates the auditory sensitivity of the utricle in female plainfin midshipman. We show that the utricle serves an auditory function that is seasonally plastic and highly adapted in reproductive females to detect the dominant frequencies of conspecific vocalizations.

Taken together, the results of these experiments show that the utricle of the plainfin midshipman exhibits robust auditory capabilities, which likely aid in the detection of social and reproductive-related communication. A brief summary, larger implications of the data presented, and future directions are discussed in chapter 5.

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AEP: Auditory evoked potential
ABR: Auditory brainstem response
BF: Best frequency
BM: Body mass
CF: Character frequency
GSI: Gonadosomatic Index
SL: Standard length
SPL: Sound pressure level
T: Telencephalon
M: Midbrain
C: Cerebellum
S: Saccule
U: Utricle
L: Lagena
D: Dorsal
V: Ventral
R: Rostral
C: Caudal

PREFACE

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CHAPTER 1: INTRODUCTION

Vocal-acoustic communication necessitates the production and reception of acoustic signals, and maintenance of animal vocal-acoustic communication systems requires that acoustic signal and receiver systems co-evolve to facilitate successful information transfer between sender and receiver. Among terrestrial and semi-aquatic vertebrates, vocal-producing (i.e., larynx or syrinx) and auditory-receiving (i.e., cochlea or basilar papillae) organs are independent of one another yet have evolved to optimize the detection of biologically relevant signals. Perhaps the most notable are reproductive-related social acoustic signals, whereby, in general, males produce acoustic advertisement vocalizations to attract reproductively ready females for reproduction [e.g., rodents (Barfield and Geyer 1972; Barfield and Thomas 1986), birds (Nowicki and Searcy 2004; Woolley and Moore 2011), and frogs (Ryan 1991; Arch and Narins 2009)]. For these vertebrates, social acoustic signals are detected by the sound pressure sensitive auditory system.

Similar to the vertebrates previously described, teleost fishes have evolved the ability to utilize vocal-acoustic communication to facilitate a range of biologically relevant behaviors. However, unlike the auditory system of terrestrial vertebrates, that of fishes, which is composed of three paired otolithic end organs (saccule, utricle, and lagena), is inherently sensitive to acoustic particle motion. Moreover, the three paired auditory end organs are posited to exhibit differential auditory sensitivity. However, this notion is based on data from a subset of fishes. Thus, a long-term goal for auditory physiologists studying fishes has been to characterize the relative auditory capabilities and functional adaptations that aid in facilitating the detection of acoustic stimuli and social behaviors.

1.1 UNDERWATER SOUND

Just as in air, underwater sound travels as a longitudinal wave through the water medium. However, underwater sound propagates faster and attenuates more slowly than sound in air due to the high molecular density of water (Urick 1967). Underwater sound can be characterized in terms of both sound pressure and particle motion. Sound pressure is the fluctuation of pressure deviations localized within regions of the longitudinal wave and is a scalar measurement of sound that travels omnidirectionally. Particle motion is the vibration of particles within the medium parallel to that of sound propagation and is a vector measurement of sound that carries directional information about the sound source (Larsen and Radford 2018). The underwater acoustic environment is complex and is composed of biotic (i.e., vocalizing invertebrates, fishes, and marine mammals), abiotic (i.e., environmental factors such as wind, rain, and water currents), and anthropogenic (i.e., man-made sounds such as motor vessels, sonar, drilling) sound sources. which have the potential to influence organismal fitness (Slabbekoorn et al. 2010).

1.2 FISH HEARING

The teleost inner ear is a multimodal sensory system that functions to facilitate both vestibular and auditory processes. The inner ear is composed of three semicircular canals that facilitate angular acceleration and three differentially oriented otolithic organs (saccule, utricle, and lagena) that are multimodal and integrate both linear acceleration and particle motion (sound) (Platt and Popper 1981; Schellart and Popper 1992; Popper and Fay 1993; Popper et al. 2003). The functional sensory cells of the inner ear are mechanoreceptive ciliary hair cells, which are contained within the sensory epithelium of each inner ear otolithic end organ (Popper and Fay 1973). These hair cells are composed of numerous small stereocilia and a single elongated kinocilium, which, when displaced via mechanical deflections towards the longest hair cell depolarizes the cellular membrane via an influx of K+ ions resulting in the release of neurotransmitters and the generation of electrical signals (i.e., action potentials) that propagate along the length of the auditory nerve (VIIIth cranial nerve) and are encoded in higher-order auditory centers of the brain (Flock 1965; Katz 1969; Hudspeth 1985; Purves et al. 2001).

There is considerable diversity in hair cell patterning, which is dependent upon the fish's environment and can be correlated with its acoustical sensitivity (Popper and Schilt 2008). Regardless, one of the main functions of the inner ear, especially for vocal fishes, is to facilitate the detection of their underwater auditory environment. However, not all fish are capable of detecting the same components of underwater sound. It is well established that all fishes are inherently sensitive to acoustic particle motion; however, only a subset of fishes are sensitive to acoustic sound pressure [for review, see (Putland et al. 2018b;

Popper and Hawkins 2019)]. For this subset, a number of sound pressure detection mechanisms, which function to enhance spectral and auditory sensitivity, have been described [e.g., (Chapman and Hawkins 1973; Coombs and Popper 1979; Popper and Coombs 1980; Schulz-Mirbach et al. 2012a)]. In general, sound pressure detection requires that a reverberant air bubble is closely positioned or coupled to the inner ear. One of the most common mechanisms involves the swim bladder, whereby a received sound pressure wave results in volumetric changes (i.e., resonates) that introduce local particle motion vectors detectable by peripheral auditory structures (Popper and Fay 1999; Ladich and Popper 2004; Braun and Grande 2008). However, given the diversity in the positioning of these reverberant air bubble, fishes have been categorized on a continuum of pressure sensitivity, with fishes that are only sensitive to particle motion (i.e., flatfishes) to fishes that extensively utilize pressure detection (i.e., ostariophysan fishes), with several intermediary groups that lie between these two extremes (Popper and Fay 2011; Putland et al. 2018b).

Regardless, because the otoliths are denser than water and the fish's body is relatively the same density as water, the otoliths lag in phase with the particle motion component of the sound wave, which results in the deflection of sensory hair cells. Similar to mammals, the mechanical deflection of hair cells is converted to electrical signals that propagate along the length of afferent axons in the VIIIth cranial nerve to octaval nuclei located in the hindbrain (i.e., anterior octaval nucleus, descending octaval nucleus and secondary octaval population). Axons from hindbrain project via the lateral lemniscus to the nucleus centralis of the midbrain torus semicircularis [for review, see (Fay and Edds-Walton 2008)].

1.3 METHODS OF MEASURING FISH HEARING

1.3.1 Behavioral measures

Behavioral measures are likely some of the most informative methods for measuring hearing because they require the test fish to elicit a behavioral change in response to a biologically relevant stimulus. For example, auditory reflexive behaviors, which are involuntary (i.e., innate) stereotyped movements in response to an auditory stimulus, are repeatable and do not require conditional behavioral training. Therefore, behavioral experiments, which are robust and easily elicited, take into account the perception of a given acoustic stimuli, which result in measuring the animal's absolute sensitivity when conducted in sufficiently quite environments. Furthermore, because behavioral measures are non-invasive, they allow for measuring the auditory capabilities of fishes that are sensitive and less stress tolerant to the surgery required for invasive auditory physiology experiments, which allows for long-duration and/or longitudinal studies aimed at measuring auditory capabilities consecutively (e.g., following sound exposure or throughout ontogeny).

1.3.2 Electrophysiological measures

Electrophysiological approaches have been some of the most common methods of measuring fish auditory capabilities in the modern era because they are rapid and precise when compared to behavioral methods, such as those outlined above [For review, see (Ladich and Fay 2013)]. Techniques such as single or multi-unit recordings, auditory evoked potential and microphonic potential recordings have been instrumental in understanding various auditory capabilities of fishes, including temporal encoding [e.g., (Fay 1978; Fay and Coombs 1983; Bodnar and Bass 1997)], frequency sensitivity [e.g., (Fay and Edds-Walton 1997a; Weeg et al. 2002)], directional sensitivity [e.g., (Fay 1984; Fay and Edds-Walton 1997b; Lu et al. 2003, 2004)], and auditory plasticity [e.g., (Sisneros and Bass 2003; Sisneros 2009b; Bhandiwad et al. 2017; Rogers et al. 2022a)].

While electrophysiological methods are critical in understanding the sensitivity of the teleost auditory system, there are several limitations that must be addressed. First, some methods, like single/multi-unit and microphonic potential recordings, are technically difficult to perform, can involve invasive surgeries, and require physical restraint of the animal. Even non-invasive methods, such as the auditory evoked potential technique, which has been extensively utilized [for review, see (Ladich and Fay 2013)] requires the animal to be physically restrained. Second, electrophysiological methods are often difficult to perform on small animals, especially those that are early in development; therefore, physiological studies across ontogeny can be more difficult to perform. Additionally, electrophysiological methods measuring auditory thresholds are difficult to compare to behavioral measures as considerable variation in auditory thresholds can be obtained during electrophysiological measures due to factors such as electrode placement, the morphology of the inner ear and skull, and the threshold criteria, which make it interpreting in the context of natural auditory-driven behaviors challenging (Ladich and Fay 2013; Sisneros et al. 2016). For example, auditory evoked hair cell potentials can inform us of hair cell activity but not whether this activity results in the perception of an auditory stimulus. Thus, there is a gap in our understanding of the relationship between behavioral and electrophysiological thresholds.

1.4 BATRACHOID FISHES AS A MODEL FOR FISH HEARING

Some of the most extensively studied species of vocal fishes are found in the Family Batrachoididae (toadfishes and midshipman fish). Batrachoids are primarily a nocturnally active family of fishes that produce a relatively simple repertoire of acoustic signals during social and reproductive behaviors. These fishes are a well-suited model system to investigate mechanisms of acoustic communication because they have evolved a number of adaptations related to their physiology, endocrinology, morphology, and behavior, which aid in mediating intraspecific acoustic communication during social behaviors. As our understanding of these fishes has expanded, so have our technical approaches, thus allowing researchers to develop more complex questions and expand our understanding of how fishes detect, integrate, and respond to the complex acoustic stimuli in their environment.

The plainfin midshipman (*Porichthys notatus*, Girard 1854) is a well-suited species to investigate mechanisms of acoustic communication because they have evolved a number of adaptations related to their physiology, endocrinology, morphology, and behavior that help mediate intraspecific acoustic communication during social behaviors (Bass and McKibben 2003; Sisneros et al. 2004b; Coffin et al. 2012; Forlano et al. 2016; Feng and Bass 2017; Mohr et al. 2017). Plainfin midshipman are a nocturnally active marine fish that produce a relatively simple repertoire of acoustic signals during social and reproductive behaviors that include “grunts”, “growls”, and “hums” (Bass et al. 1999; Sisneros 2009a). Grunts are short-duration, broadband signals produced during aggressive and defensive interactions by all midshipman sexual phenotypes (females and males: type I and II) (Ibara et al. 1983; Brantley and Bass 1994). Growls are long-duration, broadband agonistic signals produced only by type I nesting males during the breeding season in the context of territory and nest defense (Bass et al. 1999; Sisneros 2009a), while hums are long-duration, multiharmonic advertisement signals produced only by breeding type I males to attract gravid females to nest sites for spawning (Brantley and Bass 1994; Bass and McKibben 2003; Forlano et al. 2016). Nocturnally active females rely on their auditory sense to detect and locate “mate calling” males during the late-spring and summer reproductive season. Thus, the bioacoustic ecology and reproductive success of the plainfin midshipman depends on the production and reception of social acoustic signals.

Midshipman have served as an excellent behavioral model for investigating fish hearing because they exhibit innate sound source localizations that are reproductible in a laboratory setting (Zeddies et al. 2010, 2012; Coffin et al. 2014). Thus, midshipman are uniquely suited to investigate both the behavioral and physiological mechanisms of hearing. Previous physiological studies in the plainfin midshipman have shown that the auditory system is remarkably plastic and that a suite of hormonally driven changes along the auditory pathway enhance the detection of auditory signals during the summer reproductive season. For example, it has been shown that the seasonal increases in sex steroids functions to increase the number of sensory hair cells in the saccule, but not the utricle and lagena (Figure 1.1), which results in an approximately 3-fold increase in saccular hair cell auditory sensitivity and increases the bandwidth of detectable frequencies (Coffin et al. 2012). Additionally, seasonally fluctuations in gonadal steroids have been shown to modulate the molecular mechanisms of hair cell transduction at the periphery and central inhibitory pathways, both of which result in enhanced auditory sensitivity during the reproductive season (Rohmann et al. 2013; Perelmuter et al. 2019, 2021). While a number of physiological studies have revealed numerous auditory adaptations, they have primarily focused on the saccule, which leaves gaps in understanding how the other auditory end organs contribute to auditory-driven behaviors.

1.5 QUESTIONS ADDRESSED IN THIS WORK

Investigations into the auditory functions of fish otolithic end organs have primarily focused on the saccule, which is considered to be the main organ of hearing in most fishes (Popper and Fay 1993). Less is known about the functions of the lagena and utricle, but, in general, limited studies suggest that the lagena serves primarily an auditory function (Sand 1974; Fay and Olsho 1979; Lu et al. 2003; Meyer et al. 2004; Vetter et al. 2019) while the utricle may serve both an auditory and vestibular function (Riley and Moorman 2000; Boyle et al. 2001, 2018; Lu et al. 2004; Maruska and Mensinger 2015). In this work, I investigate the auditory sensitivity of the plainfin midshipman (*Porichthys notatus*) utricle and adaptive mechanisms that contribute to enhanced utricular auditory sensitivity by utilizing the auditory evoked hair cell potential recording technique. Using this electrophysiological measure, I have explored the following questions:

What is the auditory role of the midshipman utricle, and is it sensitive to behaviorally relevant acoustic stimuli?

I address this question in chapter 2 by measuring evoked utricular hair cell potentials from type I non-reproductive males to better understand the response characteristics of the utricle to auditory stimuli and to determine whether the utricle serves an auditory function.

Can the utricle encode sound pressure?

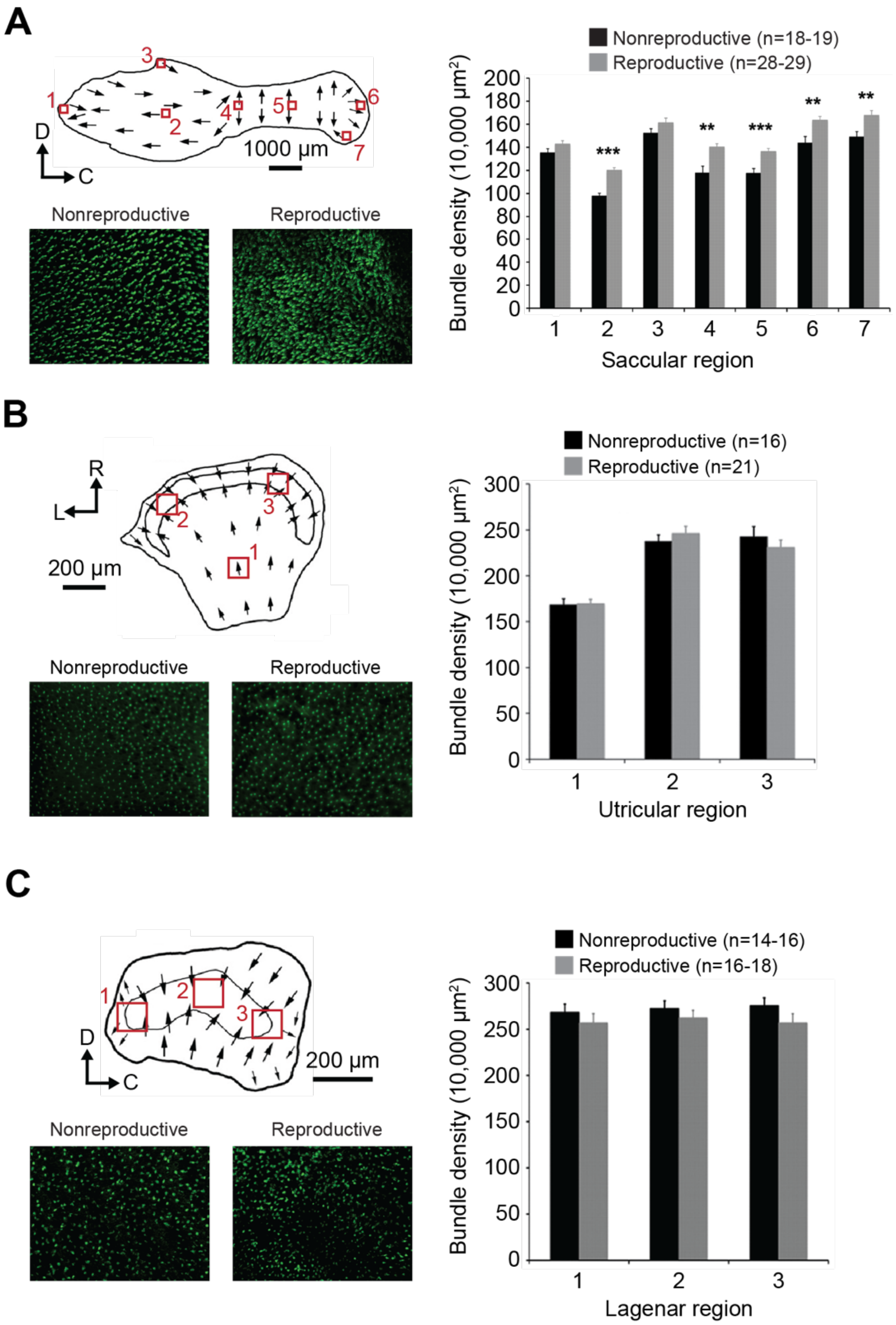
I address this question in chapter 3 using surgical manipulations of the swim bladder to determine if the swim bladder serves as an accessory auditory structure that indirectly excites the utricle in female plainfin midshipman.

Does utricular auditory sensitivity change seasonally with reproductive state?

I address this question in chapter 4 by recording the utricular hair cell auditory sensitivity from non-reproductive and reproductive female plainfin midshipman.

Taken together, these chapters explore how the utricle, an end organ that has primarily been thought to serve a vestibular function, serves a primary auditory function in the vocal plainfin midshipman. I show that similar to the saccule, which is considered the main auditory end organ for most fishes, the utricle is sensitive to behaviorally relevant acoustic stimuli, encodes acoustic sound pressure, and is seasonally modulated.

Figures


A
3
1
6
2
4
5
7
D
C
1000 µm
Nonreproductive
Reproductive
Nonreproductive (n=18-19)
Reproductive (n=28-29)
Bundle density (10,000 µm²)
200
180
160
140
120
100
80
60
40
20
0
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1
2
3
4
5
6
7
Saccular region
B
R
L
2
3
1
200 µm
Nonreproductive
Reproductive
Nonreproductive (n=16)
Reproductive (n=21)
Bundle density (10,000 µm²)
300
250
200
150
100
50
0
1
2
3
Utricular region
C
1
2
3
D
C
200 µm
Nonreproductive
Reproductive
Nonreproductive (n=14-16)
Reproductive (n=16-18)
Bundle density (10,000 µm²)
300
250
200
150
100
50
0
1
2
3
Lagenar region

Figure 1.1: Midshipman auditory inner ear. A, Saccule, **B**, utricle, and **C**, lagena hair cell orientation maps (top left), representative micrographs (bottom left), and bundle densities (right) for nonreproductive (black) and reproductive (gray) female midshipman. Red squares represent regions where hair cell bundle density was quantified. Data are plotted as mean ± SE. Asterisks indicate significant reproductive state differences:**p < 0.01, ***p < 0.001. This figure was modified from Coffin et al. (2012).

CHAPTER 2

Auditory evoked potentials of utricular hair cells in the plainfin midshipman, *Porichthys notatus*

Running title: Utricular potentials of plainfin midshipman

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Abstract

The plainfin midshipman, *Porichthys notatus*, is a soniferous marine teleost fish that generates acoustic signals for intraspecific social communication. Nocturnally active males and females rely on their auditory sense to detect and locate vocally active conspecifics during social behaviors. Previous work showed that the midshipman inner ear saccule and lagena are highly adapted to detect and encode socially relevant acoustic stimuli, but the auditory sensitivity and function of the midshipman utricle remain largely unknown. Here, we characterize the auditory evoked potentials from hair cells in the utricle of non-reproductive type I males and test the hypothesis that the midshipman utricle is sensitive to behaviorally relevant acoustic stimuli. Hair cell potentials were recorded from the rostral, medial, and caudal regions of the utricle in response to pure tone stimuli presented by an underwater speaker. We show that the utricle is highly sensitive to particle motion stimuli produced by an underwater speaker positioned in the horizontal plane. Utricular potentials were recorded across a broad range of frequencies with lowest particle acceleration (dB re: 1 ms^{-2}) thresholds occurring at 105 Hz (lowest frequency tested; mean threshold = -32 dB re: 1 ms^{-2}) and highest thresholds at 605 to 1005 Hz (mean threshold range = -5 to -4 dB re: 1 ms^{-2}). The high gain and broadband frequency sensitivity of the utricle suggests that it likely serves a primary auditory function and is well suited to detect conspecific vocalizations, including broadband agonistic signals and the multiharmonic advertisement calls produce by reproductive type I males.

Introduction

Soniferous teleost fishes rely on the auditory inner ear and lateral line to detect and encode behaviorally-relevant, social acoustic signals (Ladich 2004; Bass and Ladich 2008; Kelley and Bass 2010; Tricas and Webb 2016). For these fishes, the otolithic end organs of the inner ear (saccule, utricle and lagena) function as biological accelerometers that detect linear acceleration and respond to the fish's direct displacement by local particle motion (Platt and Popper 1981; Fay 1984; Popper and Fay 1993; Schulz-Mirbach et al. 2018). Investigations into the auditory functions of the otolithic end organs have primarily focused on the saccule, which is considered to be the main organ of hearing in most fishes (Popper and Fay 1993). Less is known about the functions of the lagena and utricle, but, in general, limited studies suggest that the lagena serves primarily an auditory function (Sand 1974; Fay and Olsho 1979; Lu et al. 2003; Meyer et al. 2004; Vetter et al. 2019) while the utricle may serve both an auditory and vestibular function (Riley and Moorman 2000; Boyle et al. 2001, 2018; Lu et al. 2004; Maruska and Mensinger 2015). Recently, Maruska and Mensinger (2015) showed in the soniferous oyster toadfish (*Opsanus tau*) that the utricle and its afferents are capable of detecting and encoding social acoustic signals, and that the toadfish inner ear utricle can serve both an auditory and vestibular function.

Some of the most extensively studied species of soniferous fishes are found in the Family Batrachoididae (toadfishes and midshipman fish). The plainfin midshipman (*Porichthys notatus*, Girard 1854) is a well-suited species to investigate mechanisms of acoustic communication because they have evolved a number of adaptations related to their physiology, endocrinology, morphology, and behavior that help mediate intraspecific acoustic communication during social behaviors (Bass and McKibben 2003; Sisneros et al. 2004b; Coffin et al. 2012; Forlano et al. 2016; Feng and Bass 2017; Mohr et al. 2017). Plainfin midshipman are a nocturnally active marine fish that produce a relatively simple repertoire of acoustic signals during social and reproductive behaviors that include "grunts", "growls", and "hums" (Bass et al. 1999; Sisneros 2009a). Grunts are short-duration, broadband signals produced during aggressive and defensive interactions by all midshipman sexual phenotypes (females and males: type I and II) (Ibara et al. 1983; Brantley and Bass 1994). Growls are long-duration, broadband agonistic signals produced only by type I nesting males during the breeding season in the context of territory and nest defense (Bass et al. 1999; Sisneros 2009a), while hums are long-duration, multiharmonic advertisement signals produced only by breeding type I males to attract gravid females to nest sites for spawning (Brantley and Bass 1994; Bass and McKibben 2003; Forlano et al. 2016). Nocturnally active females rely on their auditory sense to detect and locate "mate calling" males during the late-spring and summer reproductive season. Thus, the bioacoustic ecology and reproductive success of the plainfin midshipman depends on the production and reception of social acoustic signals.

The auditory sensitivity of the midshipman saccule and lagena is known to be well-suited to detect conspecific vocalizations during the breeding season in all three sexual phenotypes (females and males: type I and II) (Sisneros 2009b; Rohmann and Bass 2011; Bhandiwad et al. 2017; Vetter and Sisneros In Review). Previous works showed that reproductive state-dependent changes occur in saccular sensitivity of females and males (type I and II) such that reproductive animals are highly tuned to detect and encode conspecific vocalizations (Sisneros and Bass 2003; Sisneros 2009b; Rohmann and Bass 2011; Bhandiwad et al. 2017). In addition, recent work by Vetter et al. (2019) showed that the auditory sensitivity of the lagena is also well suited to detect and encode conspecific vocalizations, especially when close to the sound source. Although the auditory sensitivity of the saccule and lagena are both well-established in the midshipman, the sensitivity and function of the utricle remains largely unknown.

The objective of this study was to characterize auditory evoked potentials from hair cells in the utricle of the plainfin midshipman and to test the hypothesis that the utricle is sensitive to behaviorally-relevant acoustic stimuli. We focus on the utricular potentials of type I non-reproductive males to better understand the response characteristics of the utricle to auditory stimuli and to determine whether the utricle serves an auditory function. In addition, we compare the auditory evoked response characteristics of the utricle to that of the saccule and lagena from our previous studies and interpret our findings as they relate to the detection and reception of conspecific acoustic communication signals.

Methods

Animal collection and husbandry

Non-reproductive adult plainfin midshipman fish, *Porichthys notatus* Girard 1854, were collected via otter trawls (*R/V John H. Martin*, Moss Landing Marine Laboratories) in Monterey Bay near Moss Landing, CA at depths ranging from 85 to 100 m during the non-reproductive midshipman season. Fish were then transported to the University of Washington where they were housed in 35 L recirculating saltwater tanks that were maintained at 13 ± 2 °C and kept on a 9/15-h light/dark photoperiod. Before each physiology experiment, standard length (SL; cm) and body mass (BM; g) were recorded. Following each physiology experiment, sex via visual inspection of the gonads and gonadosomatic index (GSI, defined here as 100 x gonad mass/[BM – gonad mass]) according to Tomkins and Simmons (2002) were determined. The GSI and standard length of the fish used in the present study were consistent with those reported for type I male midshipman in previous physiology studies (Sisneros 2007; Rohmann and Bass 2011; Vetter et al. 2019). All utricular potential recordings were performed within 23 days following trawl collection to minimize any effects of captivity on auditory sensitivity.

Utricular potential measurements

The methodology for recording utricular hair cell potentials was similar to the technique used in previous studies that measured auditory evoked potentials from hair cells in the midshipman saccule and lagena (Sisneros 2007, 2009a; Alderks and Sisneros 2011; Bhandiwad et al. 2017; Vetter et al. 2019; Colleye et al. 2019; Vetter and Sisneros In Review). Briefly, midshipman were first anesthetized by immersion in a 0.025% ethyl *p*-aminobenzoate (benzocaine) buffered saltwater bath and then given an intramuscular injection of cisatracurium besylate (~3 mg/kg of BM) and bupivacaine HCL (~1 mg/kg of BM) for immobilization and analgesia, respectively. Next, a craniotomy was performed on the dorsal surface of the skull to expose the right and left utricles, and then the cranial cavity and inner ear were filled with chilled teleost Ringer solution. Note that the position of the utricle is lateral to the caudal part of the telencephalon (forebrain), and the otolith (lapillus) lies in the horizontal plane in relation to the midshipman brain and head. In addition, the utricle is oriented in a plane that is approximately orthogonal to the plane of saccule orientation (approximately vertical) [see Figure 2.1, but also see the following references for other visual descriptions of the utricle and its orientation (Cohen and Winn

1967, McKibben and Bass 1999, Sisneros 2009)]. To prevent saltwater contamination of the inner ear during experimental testing, a hydrophobic barrier of approximately 4-5 cm (height) by 1 cm (thick) made of denture adhesive cream (Fixodent, Proctor and Gamble Company, Cincinnati, OH, USA) was constructed around the craniotomy (Figure 2.2C). The fish was then suspended using acoustically transparent film (Figure 2.2D), which allowed the fish to be lowered below the water line in the center of the experimental tank (40 cm diameter, 20 cm water depth). The fish's head was then positioned using a custom-built acrylic head holder (Figure 2.2B) such that the utricle and inner ear cavity were 4 cm below the water's surface. Once the fish was secured, a small silicone tube was then inserted in the buccal cavity of the fish so that chilled saltwater (13 – 15°C) could be perfused over the fish's gills throughout the experiment (Figure 2.2A). For all experiments, the experimental testing tank was maintained on a vibration-isolation table (TMC Vibration Control, Peabody, MA, USA), which was situated inside a sound attenuation chamber (Industrial Acoustics, New York, NY, USA). All additional experimental equipment was maintained outside of the sound attenuation chamber.

The auditory evoked potentials of hair cells in the utricle were recorded using glass microelectrodes filled with 3 M KCl (impedance: 2.0 – 8.0 MΩ) that were visually guided into the otic capsule and positioned along rostral, medial, or caudal regions of the utricle. Auditory evoked hair cell potentials were recorded from both left and right utricles. The analog evoked potential signals were pre-amplified (10×; Model 5A, Getting Instruments, San Diego, CA, USA), bandpass filtered (0.07 to 3 kHz), and then amplified (10×) via a digital filter (model SR650, Stanford Research Systems, Sunnyvale, CA, USA). Signals were then sent to a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA), which yielded an output signal that was proportional to the relative amplitude of the utricular hair cells' response to the pure tone, stimulus frequency that was locked to the reference frequency. The reference frequency of the lock-in amplifier was set to the second harmonic of the stimulus frequency, which corresponds to the greatest evoked potentials due to populations of oppositely oriented hair cells in the inner ear of teleost fishes (Cohen and Winn 1967; Furukawa and Ishii 1967; Sisneros 2007; Lozier and Sisneros 2019). All data was stored on a computer that used a custom MATLAB script, which acquired data and controlled stimulus timing. Each experimental recording session began with control trials that measured electrical background noise conditions (no auditory stimulus present), which was then followed by stimulus trials at the various tested frequencies and amplitudes.

Acoustic stimulus and calibration

The methodology used for acoustic stimulus presentation and calibration was similar to that used in previously published work (Sisneros 2007, 2009b; Alderks and Sisneros 2011; Bhandiwad et al. 2017; Vetter et al. 2019; Colleye et al. 2019). Acoustic stimuli were generated by a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA), which sent pure tone signals to an audio amplifier (BG-1120, TOA Corporation, Hyogo, Japan) and then to an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA). Since the midshipman utricle end organs reside primarily within the xy-plane (Cohen and Winn 1967; Coffin et al. 2012; Mohr et al. 2017) and the hair cells of the utricle are oriented within the horizontal plane (Coffin et al., 2012), the underwater speaker was positioned upright within a custom fabricated speaker mount on the bottom of the experimental tank (40 cm diameter, 20 cm water depth) with the speaker submerged 2 cm below the water's surface (Figure 2.2). Another reason why we chose to position the underwater speaker in the horizontal plane is because we were unable to record auditory evoked utricular potentials when we positioned the speaker beneath the animal (vertical axis of sound projection). The vertically oriented speaker produced particle motion stimuli primary along the vertical (z) axis of the water column, which is orthogonal to the horizontal orientation of the utricle and resulted in no measurable auditory evoked hair cell potentials (i.e., the utricular potential measurements were no different from that of recorded electrical background levels with no sound stimuli). Acoustic stimuli consisted of single 500 ms pure tones repeated 8 times at a rate of one every 1.5 s. Acoustic stimuli were randomly presented at the following frequencies: 105, 125, 185, 205, 285, 305, 405, 605, 705, 805, 905, and 1005 Hz. The tested frequencies were chosen because they encompass the dominant bandwidth frequencies contained within type I male midshipman advertisement vocalizations and avoid any potential interference associated with acoustic tank resonance frequencies and electrical noise (60 Hz and its harmonics).

Prior to each physiology experiment, calibration of the acoustic stimuli was performed by positioning a mini-hydrophone (model 8103, Bruel and Kjaer, Naerum, Denmark), which was connected to a conditioning amplifier (gain = 100 mV/Pa, Nexis 2692-0S1, Bruel and Kjaer, Naerum, Denmark), 10 cm perpendicular from the face of the underwater speaker and 4 cm below the water's surface to coincide with the position of the midshipman inner ear during auditory evoked hair cell potential measurements. Acoustic stimuli were calibrated by measuring the peak-to-peak (pk-pk) voltage (V_{pk-pk}) amplitude on an oscilloscope (Tektronix, Beaverton, OR, USA), and then equalized in sound pressure level (SPL; dB re: 1 μPa) using a custom MATLAB (MathWorks Inc., Natick, MA, USA) script, which measured the power spectral density for all tested frequencies. The signal (V_{pk-pk}) sent to the speaker was scaled until the measured peak-to-peak SPL (SPL_{pk-pk}) output from the speaker was 130 ± 0.5 dB re: 1 μPa.

Particle acceleration (dB re: 1 ms^{-2}) measurements were conducted using a calibrated neutrally buoyant waterproofed triaxial accelerometer (Model VW3567A12; Sensitivity at 100 Hz: 10.42 mV/ms^{-2} (*x*-axis), 10.03 mV/ms^{-2} (*y*-axis), 10.37 mV/ms^{-2} (*z*-axis); PCB Piezotronics, Depew, NY, USA) that connected to a signal conditioner (Model: 482A16; PCB Piezotronics, Depew, NY, USA), which was used to amplify the signal (gain = ×100/axis). Measurements were then sent to a data acquisition system (Model: NI USB-6009, National Instruments, Austin, TX, USA) and visualized using LabVIEW software (National Instruments, Austin, TX, USA). Particle acceleration (dB re: 1 ms^{-2}) measurements were made by placing the triaxial accelerometer 4 cm below the water's surface and 10 cm perpendicular from the cone of the speaker, which corresponded with the position of the midshipman inner ear during testing, and was calibrated in response to each tested frequency across the entire intensity range. Using a custom LabVIEW (National Instruments, Austin, TX, USA) script, particle motion amplitude measurements (V_{pk-pk}) for each axis (*x*-, *y*-, and *z*-axis) were corrected for the gain (sensitivity) of the accelerometer. Figure 2.3A illustrates the variation of stimulus particle acceleration (dB re: 1 ms^{-2}) along the *x*-, *y*-, and *z*-axes at three sound pressure levels (dB re: 1 μPa) tested within our experimental tank.

Acoustic impedance measurements

The small dimensions (40 cm diameter, 20 cm water depth) and material (Nalgene plastic) of the experimental testing tank directly influence the acoustic environment in which auditory evoked potential recordings were performed. Therefore, as suggested by Popper and Fay (2011) and more recently by Popper et al. (2019), the acoustical impedance (*Z*) of the experimental tank environment should be measured and compared to the acoustic impedance of seawater in a free-field environment, thus allowing for more meaningful comparisons of different experimental tank acoustic environments in other physiology and behavior studies. The *Z* is the complex ratio of sound pressure to particle velocity and is expressed in Rayls [1 Rayl = 1 (Pa s)/m] and was determined in the experimental test tank across all tested frequencies at three sound pressure levels (151, 142 and 133 dB re: 1 μPa). The experimental tank's *Z* was measured and then compared to the *Z* of theoretical "seawater" ($Z_{\text{theoretical seawater}}$ = 1.559 MRayls) in a free-field environment with a salinity of 35 ppt at 15 °C (Bradley and Wilson 1966; Erbe 2011). Additionally, the phase (Φ) of the complex *Z* was also determined across all test frequencies at three sound pressure levels (151, 142 and 133 dB re: 1 μPa) by comparing the phase difference between the particle velocity and sound pressure waves. All measurements and analyses for *Z* and phase (Φ) of the complex acoustic impedance were similar to that in previously published studies (Vetter et al. 2019; Colleye et al. 2019).

The *Z* of our experimental tank was determined by simultaneously measuring the sound pressure (dB re: 1 μPa) and particle acceleration (dB re: 1 ms^{-2}) for each tested frequency. Simultaneous measurements were conducted at the position that would be normally occupied by the midshipman inner ear during the physiology experiment using a mini-hydrophone (model 8103, Bruel and Kjaer, Naerum, Denmark) connected to a conditioning amplifier (gain = 100 mV/Pa, Nexis 2692-0S1, Bruel and Kjaer, Naerum, Denmark) to record sound pressure (dB re: 1 μPa), whereas particle acceleration (dB re: 1 ms^{-2}) was measured using a calibrated neutrally buoyant waterproofed triaxial accelerometer (Model VW3567A12; Sensitivity at 100 Hz: 10.42 mV/ms^{-2} (*x*-axis), 10.03 mV/ms^{-2} (*y*-axis), 10.37 mV/ms^{-2} (*z*-axis); PCB Piezotronics, Depew, NY, USA) that was connected to a signal conditioner (Model: 482A16; PCB Piezotronics, Depew, NY, USA) that amplified the particle acceleration signal (gain = ×100/axis). Particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa) measurements were recorded using a data acquisition system (NI myDAQ 16-bit analog to digital conversion at 200 kS s^{-1}, National Instruments, Austin, TX, USA) that was controlled by a custom-written program in LabVIEW software (NI LabVIEW 2016, National Instruments, Austin, TX, USA). Analysis of the complex acoustical impedance followed Colleye et al. (2019) and Vetter et al. (2019).

The complex phase of Z is equal to the phase difference ($\Delta\Phi p,v$) between the particle velocity (v) and the pressure (p). The phase (Φ) of the complex Z in our experimental test tank was determined by measuring the phase difference ($\Delta\Phi$) between the particle acceleration (a) and pressure (p), where $\Delta\Phi p,a = \Phi p - \Phi a$. All measurements were recorded with a data acquisition system (NI myDAQ 16-bit analog to digital conversion at 200 kS s^{-1}, National Instruments, Austin, TX, USA) that was controlled by a custom-written program in LabVIEW software (NI LabVIEW 2016, National Instruments, Austin, TX, USA). For sinusoid waves, such as the pure tones examined in our study, the phase of particle acceleration (a) will always lead the phase of particle velocity (v) by 90 deg. Therefore, the phase difference ($\Delta\Phi p,v$) between the particle velocity and acoustic pressure waves was determined by (Eq. 1):

$$\Delta\Phi p, v = \Delta\Phi p, a + 90^\circ \quad \text{(Eq. 1)}$$

All measurements were within the near-field approximation; however, we do not expect a simple relationship between velocity and pressure because of the complex nature of our experimental tank conditions. Figure 2.3 displays both the Z (Figure 2.3B) and $\Delta\Phi p,v$ (Figure 2.3C) at all frequencies examined for three sound pressure levels (151, 142, and 133 dB re: 1 μPa) along the x-axis (rostral-caudal). Acoustic impedance and $\Delta\Phi p,v$ along the y- (lateral) and z-axes (dorsal-ventral), respectively, are also provided (Figure S2.1).

Analyses

The auditory threshold tuning curves for utricular potentials based on particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa) were determined via input-output measurements of the evoked utricular hair cell potentials over the range of tested frequencies and amplitudes. The recorded acoustic noise floor measurements were used to establish the subthreshold levels for the utricular potentials (-71 ± 1 dB re: 1 ms^{-2}; 76 ± 1 dB re: 1 µPa). The auditory threshold for utricular potentials was defined as the lowest stimulus level that yielded a mean utricular evoked potential that was greater than two standard deviations above the background electrical noise measurement. The frequency that evoked the lowest utricular threshold was defined as the characteristic frequency (CF), while best frequency (BF) was defined as the frequency that elicited the highest utricular potential voltage in the iso-intensity analyses. To determine if individuals' best frequencies differed across recording regions (rostral, medial, and caudal), a non-parametric Friedman test was conducted, due to normality violations (Shapiro-Wilk normality test; W = 0.58, $p < 0.001$). Particle acceleration level (dB re. 1 ms^{-2}) thresholds were calculated as the combined magnitude vector of particle acceleration in dB scale (Eq. 2) (Wysocki et al. 2009; Vasconcelos et al. 2010; Bhandiwad et al. 2017; Vetter 2019; Colleye et al. 2019; Rogers et al. 2020) as follows:

$$\text{dB re: } 1\ \text{ms}^{-2} = 20\ \text{Log}_{10}(\sqrt{x^2 + y^2 + z^2}) \qquad \text{(Eq. 2)}$$

Results

Auditory evoked utricular potentials were recorded from 15 adult, non-reproductive type I male midshipman fish with standard lengths (SL) that ranged from 15.8 – 24.3 cm (20.4 ± 2.6 cm; mean ± SD), body masses that ranged from 46.0 – 183.2 g (99.7 ± 43.4 g), and gonadosomatic indices (GSI) that ranged from 0.3 to 2.6 g (1.5 ± 0.6 g). All adult type I males tested in this study were within the size range reported in previous physiology studies for type I male midshipman (Sisneros 2007; Rohmann and Bass 2011; Vetter et al. 2019).

Auditory thresholds for both particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa) level were determined for populations of hair cell receptors in rostral (n = 10 records), medial (n = 11 records) and caudal (n = 10 records) regions of the utricle. Evoked utricular hair cell potentials were recorded in response to sound pressure levels that ranged from 106 to 154 dB re: 1 µPa. Figure 2.4 illustrates representative iso-intensity response profiles of utricular hair cell potentials in response to pure tones (105–1005 Hz) at the highest sound level tested (154 dB re: 1 µPa). The iso-intensity response curves of the utricle consisted of BFs that ranged from 105 to 205 Hz, with the majority (52%) occurring at 105 Hz (Figure 2.5). Because individuals' best frequency did not differ across recording regions (Friedman test, $\chi^2 = 4.43$, d.f. = 2, $p = 0.11$) iso-intensity response curves from all three regions were grouped for further analysis. Additionally, a subset of recordings (n = 12, 18) displayed a prominent secondary peak (2° peak) ranging from 185 to 505 Hz, with a majority (44%) occurring at 205 Hz (Figure 2.5).

Auditory threshold curves based on particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa) level were constructed from utricular potentials recorded from rostral, medial and caudal regions of the utricle. Figure 2.6 illustrates representative individual auditory threshold tuning curves based on particle acceleration (dB re: 1 ms^{-2}; top) and sound pressure (dB re: 1 μPa; bottom) levels. Across all recordings, the CFs ranged from 105 Hz to 205 Hz for both the particle acceleration (median CF = 105 Hz) and sound pressure (median CF = 105 Hz) tuning curves (Figure 2.7). Lowest utricular auditory thresholds occurred at 105 Hz (lowest frequency tested; mean particle acceleration threshold = -32 dB re: 1 ms^{-2} , mean sound pressure threshold = 119 dB re: 1 μPa) and gradually rose to highest threshold levels at 605 to 1005 Hz (mean particle acceleration threshold range = -5 to -4 dB re: 1 ms^{-2} and mean sound pressure threshold range = 146 to 150 dB re: 1 μPa) (Figure 2.7).

In addition, utricular potentials were consistently ($\geq$ 95%) recorded at sound levels [relative to particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa)] above threshold at frequencies from 105 to 705 Hz in the 31 recordings collected from the 15 non-reproductive type I males (Figure 2.8). The percentage of recordings with evoked utricular potentials at sound levels above threshold from 805 Hz to 1005 Hz decreased from 84% to 65%. In sum, relatively high percentages (84–95%) of evoked utricular potentials were recorded across a range of frequencies from 105 to 805 Hz.

Discussion

The goal of this study was to characterize the auditory evoked potentials of hair cells in the utricle of non-reproductive type I male midshipman to test the hypothesis that the utricle is sensitive to behaviorally relevant acoustic stimuli. We show, based on the utricular tuning profiles for particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa), that the utricle is highly sensitive to a broad range of behaviorally relevant, particle motion stimuli in the horizontal plane and that the midshipman utricle is capable of detecting the dominant higher frequencies contained within conspecific social signals.

The utricle of the midshipman, like the other inner ear end organs, contains a dense calcium carbonate otolith that rests on a sensory bed of hair cells, which acts as an inertial accelerometer that is sensitive to particle motion and responds to linear acceleration. We show that the utricle is relatively sensitive to particle motion across a broad range of frequencies with lowest particle acceleration (dB re: 1 ms^{-2}) thresholds occurring at 105 Hz (mean threshold = -32 dB re: 1 ms^{-2}) and highest thresholds between 605 and 1005 Hz (mean threshold range = -5 to -4 dB re: 1 ms^{-2}) (Figure 2.7). Surprisingly, utricular particle motion (dB re: 1 ms^{-2}) sensitivity of type I males is remarkably similar to that of the saccule in type I males at frequencies $\leq$ 305 Hz; however, at frequencies > 305 Hz the utricle may be even more particle motion (dB re: 1 ms^{-2}) sensitive than the saccule, at least in type I males (Colleye et al. 2019) (Figure 2.9). Another important difference in particle motion (dB re: 1 ms^{-2}) sensitivity between the utricle and saccule is the directional axis of sensitivity. The utricle is oriented in the horizontal plane [see Figure 2.1; but also see CT scans for *P. notatus* in the Virtual Natural History Museum: http://131.220.133.140/VNHM/) with the utricular hair cells also oriented in the horizontal plane (x- and y-axes); and see Figure 6 in Coffin et al., 2012]; thus, the utricle is likely to be highly directionally sensitive to particle motion stimuli in the horizontal plane. Here, we show based on utricular potential thresholds that the utricle was highly sensitive to the particle motion stimuli produced by the underwater speaker positioned in the horizontal plane, which emitted the majority of the particle motion magnitude in the x- and y-axis (Figure 2.3). In contrast, the midshipman saccule is primarily oriented in the vertical plane (z-axis), with hair cell orientation patterns in both the vertical and horizontal planes (x- and y-axis) with a corresponding directional sensitivity in both the vertical and horizontal planes (Coffin et al. 2012; Mohr et al. 2017). Thus, the horizontal directional sensitivity and high gain of the utricle likely complements the directional sensitivity and gain of the saccule to enhance the ability of the midshipman inner ear to detect and localize biologically relevant acoustic stimuli, including conspecific vocalizations. Previous work has shown that reproductive state-dependent changes occur in the saccular sensitivity of males (type I and II) and females such that reproductive animals become better suited than non-reproductive animals to detect conspecific vocalizations (Sisneros and Bass 2003; Sisneros 2009b; Rohmann and Bass 2011; Bhandiwad et al. 2017). Our data suggests that the particle motion (dB re: 1 ms^{-2}) sensitivity of the utricle in non-reproductive type I males is already well suited to detect conspecific type I male vocal signals, including the broadband agonistic growls and multiharmonic advertisement calls. Future studies that employ a shaker table system, such as that used by Fay (1984), will be needed to verify the directional sensitivity of the utricle. In addition, future investigations that examine reproductive state-dependent changes in utricular sensitivity will be instrumental in determining whether midshipman also exhibits seasonal enhancement of utricular sensitivity for the detection of socially relevant acoustic stimuli.

The lowpass filter tuning characteristics of the midshipman utricle reported in this study were similar to that reported for other teleost fishes. Lu et al. (2004) showed using a shaker table system that the non-soniferous sleeper goby (*Dormitator latifrons*, Richardson 1844) had similar low-pass tuning characteristics for the utricular afferents, which exhibited CFs ranging from ≤ 50 – 400 Hz and a median CF of 80 Hz. Lu et al. (2004) also reported that the best sensitivity of the utricular afferents occurred along the horizontal axis and ranged from -70 to -40 dB re: 1 g with a mean particle acceleration threshold of -52 dB re: 1 g, which was about 30 dB less sensitive than that reported for sleeper goby saccular afferents (Lu et al. 2010). In the sleeper goby, the mean particle acceleration threshold (mean = -52 dB re: 1 g) of utricular afferents was similar to the mean particle acceleration threshold for utricular potentials in midshipman (most sensitive frequency: 105 Hz, mean threshold = -32 dB re: 1 ms^{-2}, or approx. -52 dB re: 1 g). In addition, the range of CFs reported for midshipman utricular potentials (105-205 Hz) was similar and overlapped with that reported for the sleeper goby (Lu et al. 2004). Similarly, Maruska and Mensinger (2015) showed that utricular afferents in free-swimming oyster toadfish (*Opsanus tau*, Linnaeus 1766) responded best to low frequencies from 80 to 200 Hz and were sensitive to the playbacks of conspecific boatwhistles and grunts, which had fundamental frequencies that ranged from 80–180 Hz. Although there is very limited data regarding the response characteristics of the utricle in fishes, our midshipman data and that of the toadfish and sleeper goby suggest the auditory utricle is highly sensitive to low-frequency linear acceleration in the horizontal plane and that the utricle is capable of detecting conspecific vocalizations. Whether these utricular response characteristics are conserved in other various fish species needs to be examined in future work.

Results from our study also indicate that the midshipman utricle has a similar frequency response range to that of the midshipman lagena; however, the auditory thresholds based on particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa) are considerably lower for the utricle (i.e., more sensitive) than that reported for the midshipman lagena (Vetter et al. 2019; Vetter and Sisneros In Review). Lowest particle acceleration (dB re: 1 ms^{-2}) thresholds for the lagena in non-reproductive type I males occurred at 85 Hz (mean threshold = -9.7 dB re: 1 ms^{-2}) and 125 Hz (mean threshold = -4.3 dB re: 1 ms^{-2}), while the highest thresholds for the lagena occurred at 165 Hz (mean threshold = 7.3 dB re: 1 ms^{-2}) with particle acceleration (dB re: 1 ms^{-2}) thresholds decreasing to mean threshold levels of 0.05 to 3.4 dB re: 1 ms^{-2} from 205 to 505 Hz (Vetter et al. 2019). The recent work by Vetter et al. (2019) suggests that the relatively high thresholds of the lagena may be important for the detection of high-intensity levels of behaviorally relevant acoustic stimuli close to a sound source when the saccule and its afferents are likely overstimulated and saturated. In contrast, the particle acceleration thresholds (dB re: 1 ms^{-2}) of the utricle in non-reproductive type I males was very similar to saccule in reproductive type I males at frequencies $\leq$ 305 Hz, but at frequencies > 305 Hz, the utricle may be even more sensitive (Colleye et al. 2019) (Figure 2.9). One possible explanation for this difference in particle motion sensitivity at frequencies > 305 Hz between the utricle in non-reproductive type I males and the saccule of reproductive type I males may be due to the times at which saccular recordings from reproductive type I males were made. In the study by Colleye et al. (2019), reproductive type I males were collected during the summer but were held in captivity for greater than 2 months before the auditory thresholds of the saccule were measured. Sisneros and Bass (2003) showed that reproductive midshipman maintained in captivity longer than 25 days exhibit decreased saccular sensitivity to frequencies greater than 300 Hz. Thus, the saccular thresholds for type I males reported by Colleye et al (2019) may actually be higher (i.e., less sensitive) than saccular thresholds from recently collected summer reproductive type

I males. Alternatively, the differences in particle acceleration (dB re: 1 ms^{-2}) thresholds between the utricle and saccule at frequencies > 305 Hz in type I males may be related to differences in the intrinsic response properties of the hair cells in the two different auditory end organs. We show, based on iso-intensity response curves, that the utricles of non-reproductive type I males exhibited BFs that ranged from 105 to 205 Hz, with the majority of BFs (52%) occurring at 105 Hz. In addition, a number of the utricle recordings from type I males exhibited a prominent secondary peak in the evoked potentials at frequencies that ranged from 185 to 505 Hz, with the majority of the secondary peaks occurring at 205 Hz (Figure 2.5). In contrast, the saccules of non-reproductive midshipman (females and type I males) exhibited BFs that ranged from 75 to 145 Hz, with the majority of BFs (>63%) occurring at 75 Hz (Sisneros 2007; Colleye et al. 2019). A prominent secondary peak in the iso-intensity response curves was also observed in the saccular recordings of non-reproductive midshipman that ranged from 95 to 205 Hz, with the majority of secondary peaks occurring at 135 to 145 Hz (mean = 140 Hz) (Sisneros 2007). Future studies that investigate the intracellular recordings of hair cells from the midshipman inner ear will provide valuable insight into whether the electrical tuning properties of hair cells are different between the utricle and saccule.

The recent work by Colleye et al. (2019) showed that the plainfin midshipman is capable of pressure-mediated hearing through the use of its swim bladder, which can aid in the reception of sound pressure components of acoustic signals. Both female and type II male midshipman possess prominent horn-like extensions on the rostral ends of their swim bladders that decrease the distance between the swim bladder and the individual auditory end organs (saccule, lagena, and utricle) (Mohr et al., 2017). The mean distance between the swim bladder and the saccule was less than 3 mm (mean distance in females = 2.6 mm; mean distance in type II males = 2.0 mm), which was half the distance for the same measurement in type I males (mean swim bladder-to-saccule distance = 5.2 mm) (Mohr et al., 2017). In addition, the mean distance between the swim bladder and the lagena was also less than 3 mm (mean distance in females = 2.9 mm; mean distance in type II males = 2.3 mm), which was also approximately half the distance between the swim bladder and lagena in type I males (mean distance = 4.7 mm) (Mohr et al., 2017). This decreased distance between the swim bladder and the inner ear end organs allows the sound pressure-induced vibrations of the swim bladder to be detected by the particle motion sensitive otolithic end organs. Colleye et al. (2019) showed that in females, the rostral swim bladder extensions enhance saccular and lagenar sensitivity to sound pressure (dB re: 1 μPa) and extend the upper bandwidth limit of frequency sensitivity to 1005 Hz. In other pressure-sensitive fishes, increased sensitivity to sound pressure (dB re: 1 μPa) and higher frequencies is often associated with the swim bladder being in close proximity (< 3 mm) to the otic capsule that contains the auditory end organs (Ramcharitar and Popper 2004; Schulz-Mirbach et al. 2012b; Kéver et al. 2014). Although the mean distance between the swim bladder and the utricle is much greater than 3 mm in type I male midshipman (mean distance = 8.8 mm), the mean distance between the swim bladder and the utricle in females and type II males is considerably closer [mean swim bladder-to-utricle distance = 5.2 mm (females) and 5.0 mm (type II males)] (Mohr et al., 2017). Interestingly, the mean swim bladder-to-utricle distance in females and type II males is approximately the same distance between the saccule and swim bladder in type I males (mean distance = 5.2 mm), which lack swim bladder horn extensions (Mohr et al., 2017). Whether the utricle of females and type II males is close enough to detect pressure-induced vibrations of the swim bladder and enhance utricular sensitivity to acoustic sound pressure and higher frequencies remains to be determined.

The ability to perceive behaviorally relevant social acoustic signals is critical for the reproductive success and bioacoustic ecology of the plainfin midshipman. Nocturnally active male and female midshipman rely upon their auditory sense to detect and locate vocally active conspecifics during social behaviors. Previous work showed that the auditory system of the midshipman is highly adapted to detect and encode socially relevant acoustic stimuli. Males (type I and II) and females exhibit an adaptive form of auditory plasticity whereby reproductive state-dependent changes in gonadal steroids (testosterone and estrogen) act to lower the auditory thresholds (i.e., increase sensitivity) of saccular hair cells and their afferents by 7-14 dB (re: 1 μPa) over a broad range of frequencies that include the dominant higher harmonic components of advertisement and agonist calls produced by type I males (Sisneros and Bass 2003; Sisneros et al. 2004a; Sisneros 2009a; Rohmann and Bass 2011). The detection of the dominant high-frequency components of midshipman vocalizations is important for acoustic communication because primarily only the higher acoustic frequencies (above the fundamental frequency of most midshipman vocal signals) propagate in shallow water breeding environments (Bass and Clark 2003; Sisneros 2009b; Forlano et al. 2016). As previously mentioned, work by Vetter et al. (2019) showed that the lagena is also well adapted to detect and encode a broad range of frequencies similar to that of the saccule, but with much higher thresholds across the same bandwidth of frequency sensitivity. The relatively high auditory thresholds of the lagena may extend the dynamic sensitivity range of the inner ear and be useful for detecting high-intensity levels of behaviorally-relevant acoustic stimuli when close to a sound source (Lu et al. 2003, p. 200; Khorevin 2008; Vetter et al. 2019; Vetter 2019). In contrast to the lagena, the midshipman utricle is highly sensitive to particle acceleration (dB re: 1 ms^{-2}), and its particle motion sensitivity is similar to that of saccule at frequencies < 305 Hz, and potentially even more sensitive than the saccule at frequencies from 305 to 1005 Hz (Colleye et al., 2019). The high gain and broadband frequency sensitivity of the utricle suggests that the midshipman utricle is also well suited to detect conspecific vocal signals, including broadband agonistic signals and the multiharmonic advertisement calls produce by reproductive type I males (Figure 2.10). Although the lagena and utricle were previously thought to serve as accessory end organs to the “more sensitive” saccule, results from our study suggest that the utricle may serve a more important auditory function in the midshipman and be complementary to the saccule for detecting behaviorally relevant acoustic stimuli including social acoustic signals.

Conflict of interest: The authors declare no competing financial interests.

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Figures

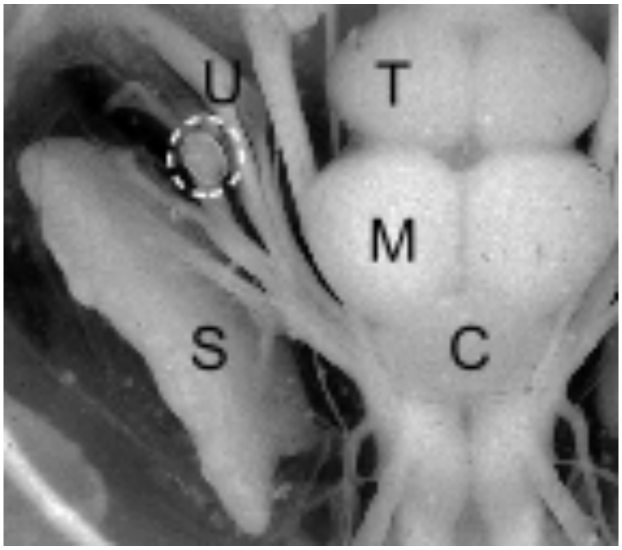

Figure 2.1: Dorsal view of the brain and inner ear of the plainfin midshipman. Dashed circle highlights the utricle (U). Abbreviations: *T* telencephalon, *M* midbrain, *C* cerebellum, and *S* saccule. Image from a type I male midshipman (SL = 20.4 cm; BM = 99.7 g). Note that the position of the lapillus (utricular otolith) is positioned in the horizontal plane relative to the brain/head of the fish and is approximately orthogonal to the sagitta (saccular otolith), which has been slightly deflected laterally in the photo in order to better view the utricle and the auditory afferents of the saccule and utricle.

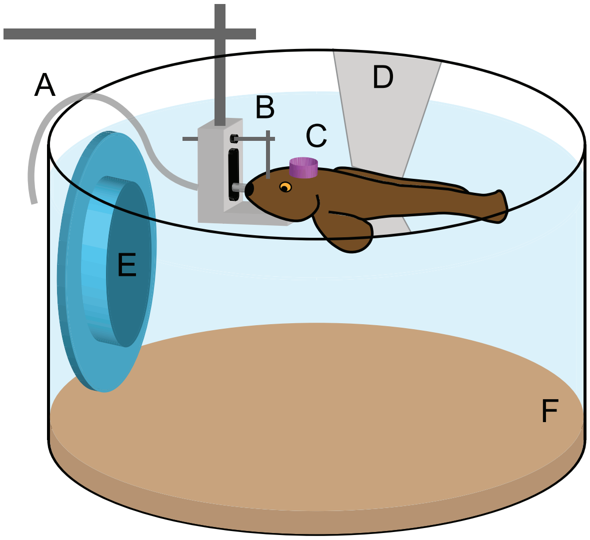

Figure 2.2: Schematic representation of experimental physiology tank. For each physiology experiment, a fish was affixed and suspended 4 cm below the water's surface with the otic capsule 10 cm perpendicular to the face of the underwater speaker (E). Labels are as follows: *A* respiration tube, *B* head holder, *C* hydrophobic water dam, *D* parafilm sling, *F* sediment. Physiology tank dimensions: 40 cm diameter, 20 cm water depth.

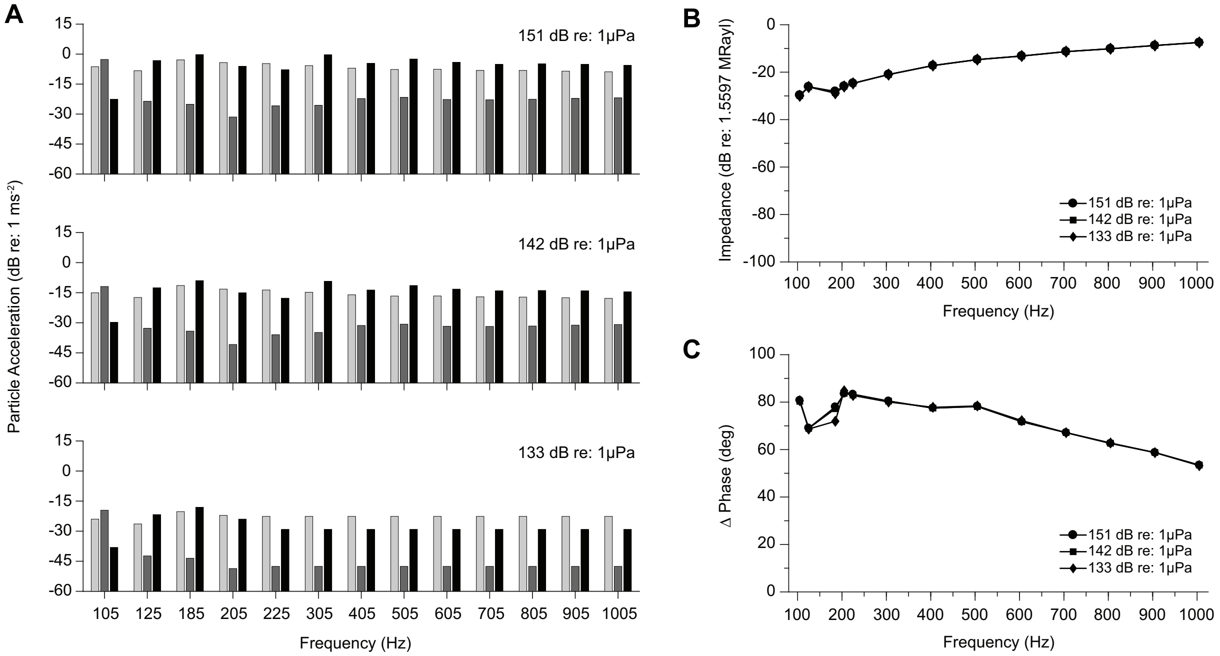

Figure 2.3: Acoustic characteristics of the experimental speaker and tank. A, Particle acceleration levels (dB re: 1 ms^{-2}) from the underwater speaker measured along each axis (x-axis: light grey; y-axis: dark grey; z-axis: black). **B**, Acoustic impedance (dB re: 1.5597 MRayl), which is the complex ratio of sound pressure to particle velocity and is expressed in Rayls [1 Rayl = 1 (Pa s)/m]. **C**, Phase difference (Δ) between the pressure and particle velocity wave. All measurements were made using a triaxial accelerometer placed in the center of the tank at the position of the fish head during testing. Additionally, measurements were made at three sound pressure levels (151, 142, and 133 dB re: 1 μPa) for all tested frequencies (105, 125, 185, 205, 225, 305, 405, 505, 605, 705, 805, 905, and 1005 Hz).

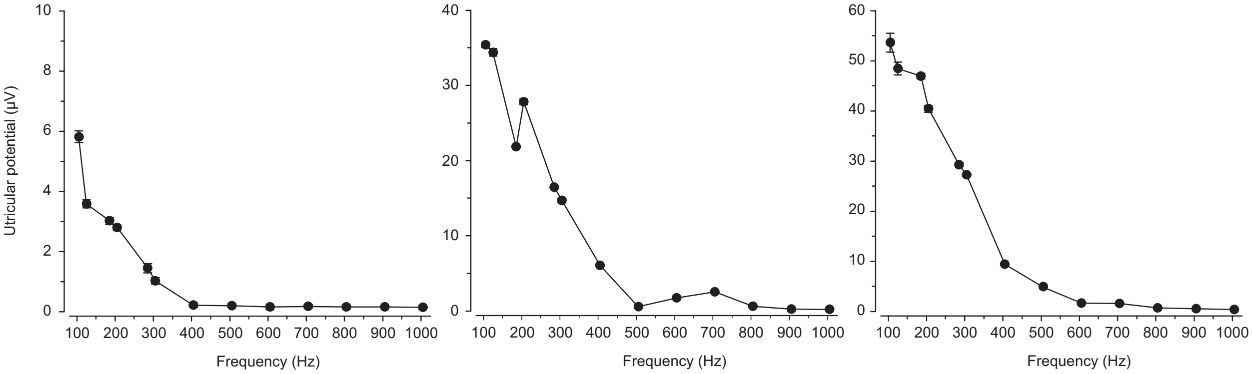

Figure 2.4: Representative utricular hair cell iso-intensity curves. Evoked responses were recorded from in response to single-tone playbacks at a sound pressure level of 154 dB re. 1 μPa. Thresholds were defined as the lowest sound pressure level (dB re: 1 μPa) needed to evoke receptor potentials at least 2 SD above the background electrical noise level. Data are represented as mean ± 95% confidence interval; note that the confidence intervals are very small, and the bars may be obscured by the symbols.

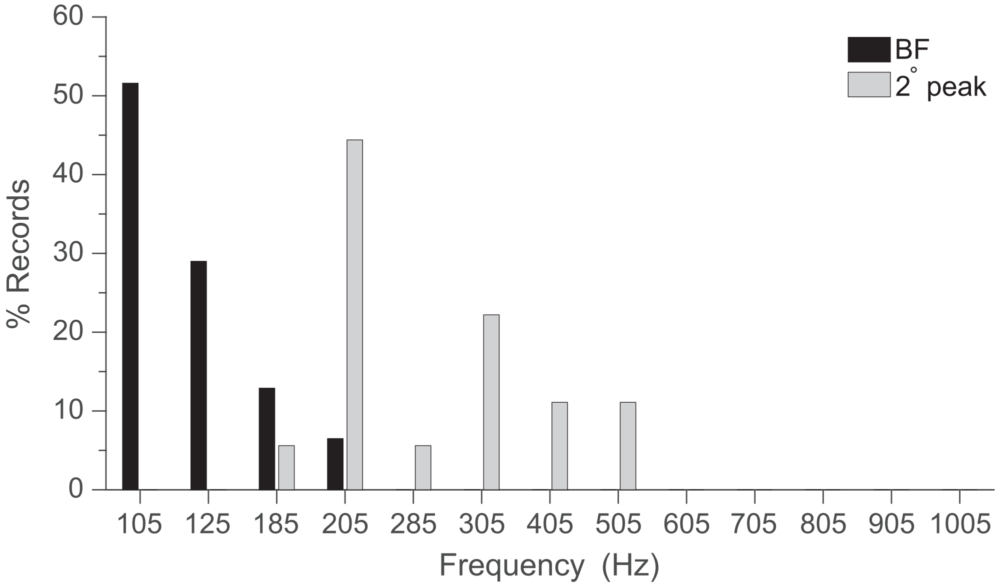

Figure 2.5: Best frequency (BF) and secondary peak (2° peak) histograms of the midshipman utricular potentials evoked in response to 154 dB re: 1 μPa pure tones. Best frequency (BF) was defined as the frequency that elicited the highest utricular potential voltage in the iso-intensity analyses, while the secondary peak (2° peak) was characterized as the second highest utricular potential for recordings that had more than one prominent peak present in their iso-intensity level curve.

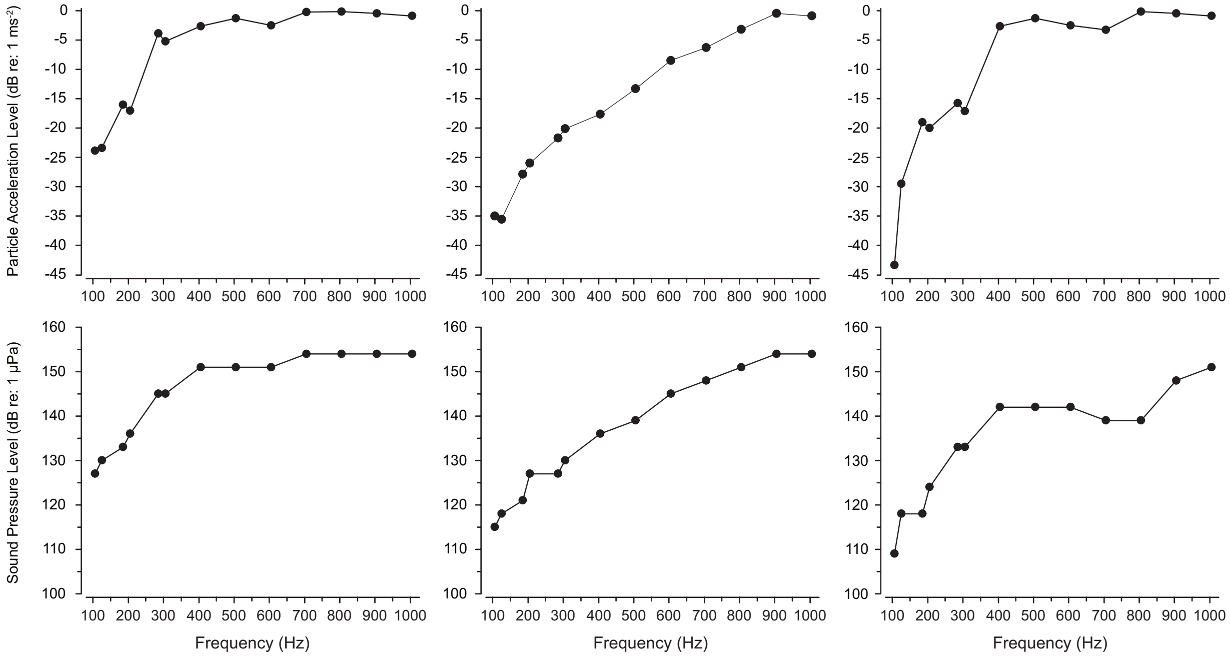

Figure 2.6: Representative utricle auditory threshold tuning curves relative to particle acceleration (dB re: 1 ms^{-2}; top) and sound pressure (dB re: 1 μPa; bottom) level. Tuning curves were constructed using the non-reproductive type I male midshipman utricular evoked response. Thresholds were defined as the lowest sound pressure level (dB re: 1 μPa) needed to evoke a utricular potential that was at least 2 SD above the background electrical noise level.

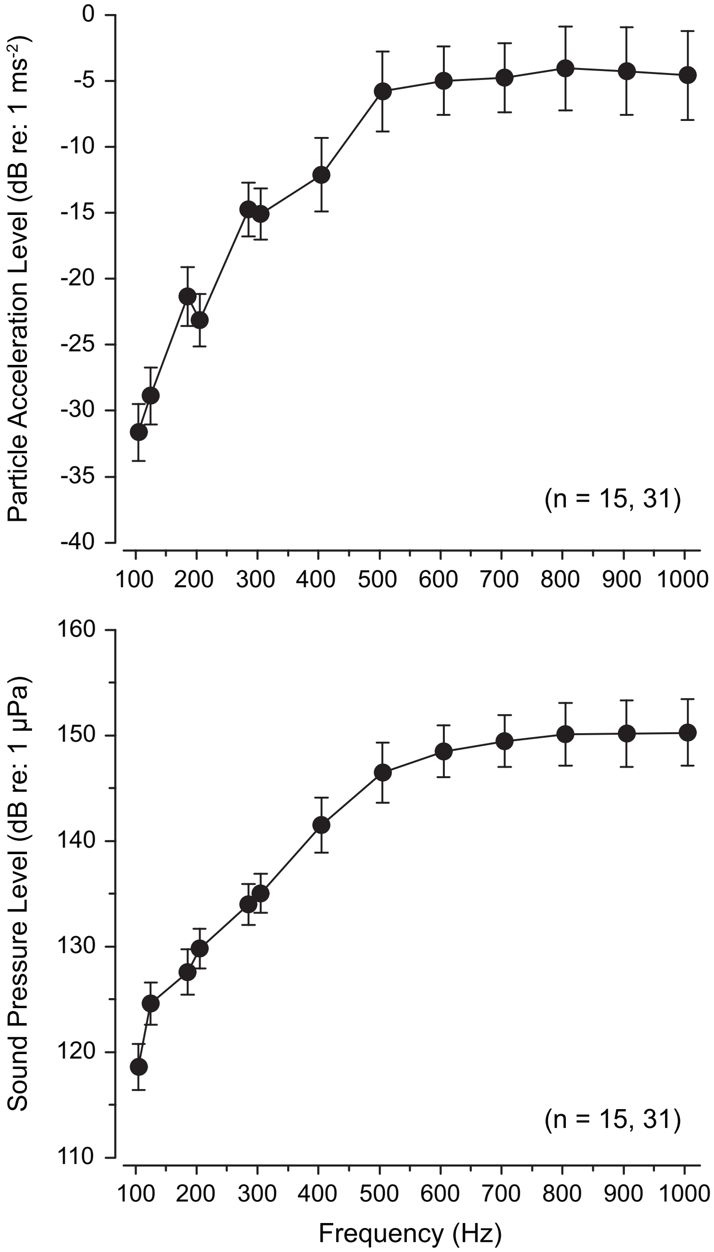

Figure 2.7: Particle acceleration (dB re: 1 ms^{-2}; top) and sound pressure (dB re: 1 µPa; bottom) level auditory threshold tuning curves recorded from midshipman utricular hair cells. The auditory thresholds were defined as the lowest auditory stimulus level needed to evoke utricular potentials that were at least 2 SD above the background electrical noise level. All data are plotted as mean ± 95% confidence interval. The number of animals and records is indicated in parentheses.

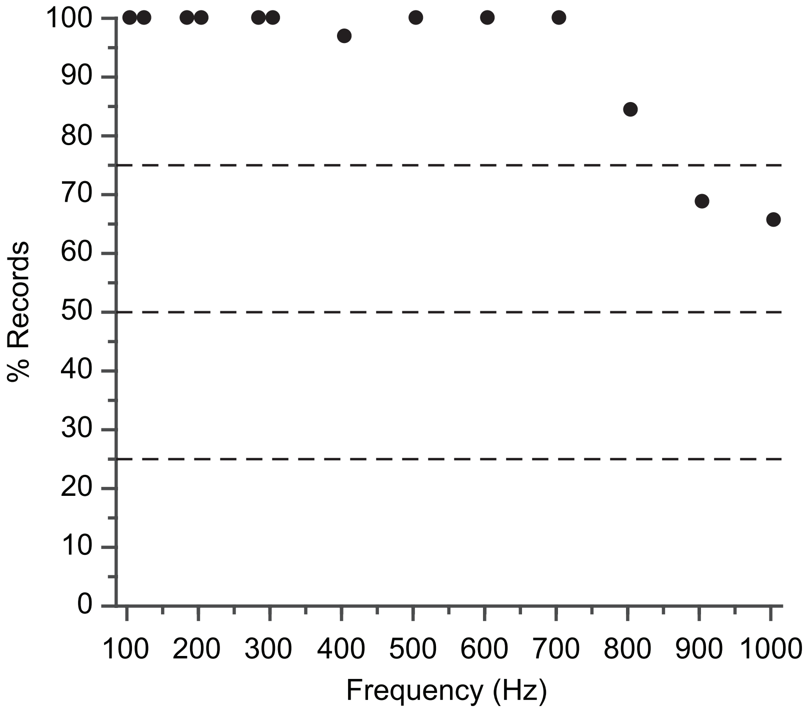

Figure 2.8: Distribution of the percentage of utricular potential recordings that displayed evoked potential thresholds above background electrical noise levels for each frequency tested. Note that utricular potentials were consistently (≥ 95%) recorded at sound levels [relative to particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa)] above threshold at frequencies from 105 to 705 Hz in the 31 recordings collected from the 15 non-reproductive type I males; however, were not always detected, even at the highest sound pressure levels (dB re: 1 μPa) tested, for test frequencies > 705 Hz.

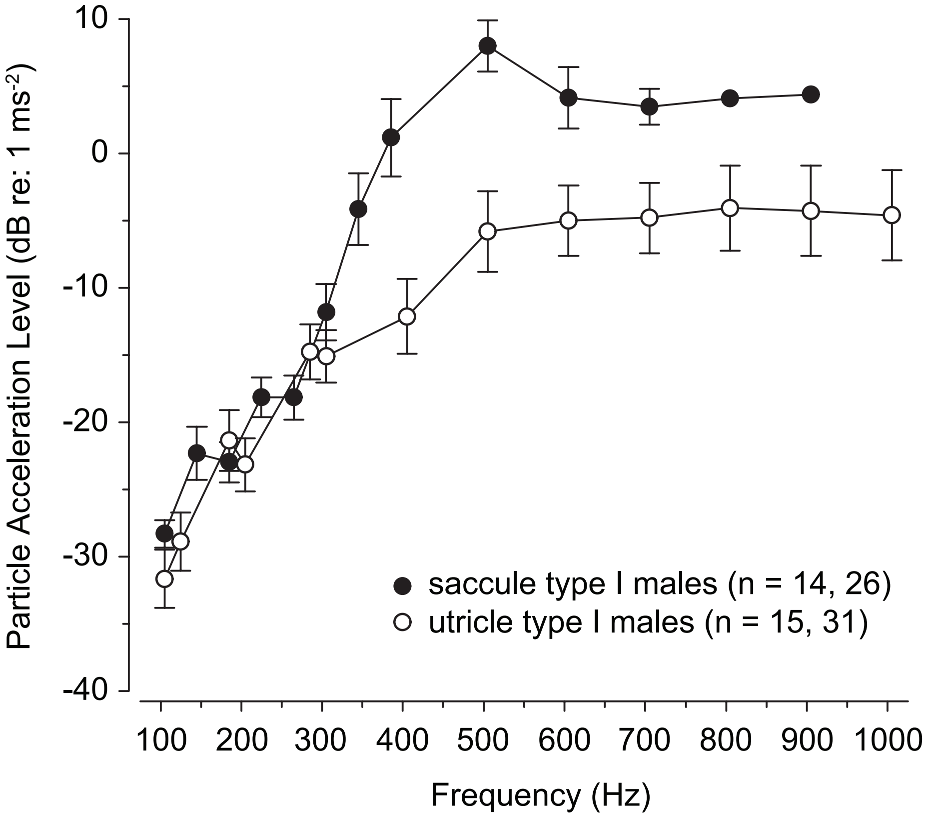

Figure 2.9: Particle acceleration (dB re: 1 ms^{-2}) sensitivity comparison of type I male plainfin midshipman saccular (•) and utricular (○) hair cell auditory threshold tuning curves. Saccular auditory thresholds were recorded from reproductive type I males that were collected during the reproductive summer but were held in captivity for greater than 2 months before the auditory thresholds of the saccule were measured (Colleye et al., 2019). Therefore, the saccular thresholds for reproductive type I males may be less sensitive than saccular thresholds recorded from recently collected summer reproductive type I males. The auditory thresholds were defined as the lowest auditory stimulus level needed to evoke utricular potentials that were at least 2 SD above the background electrical noise level. All data are plotted as mean ± 95% confidence interval. The number of animals and records is indicated in parentheses. Auditory saccular threshold tuning data for reproductive type I males was adapted from Colleye et al. (2019).

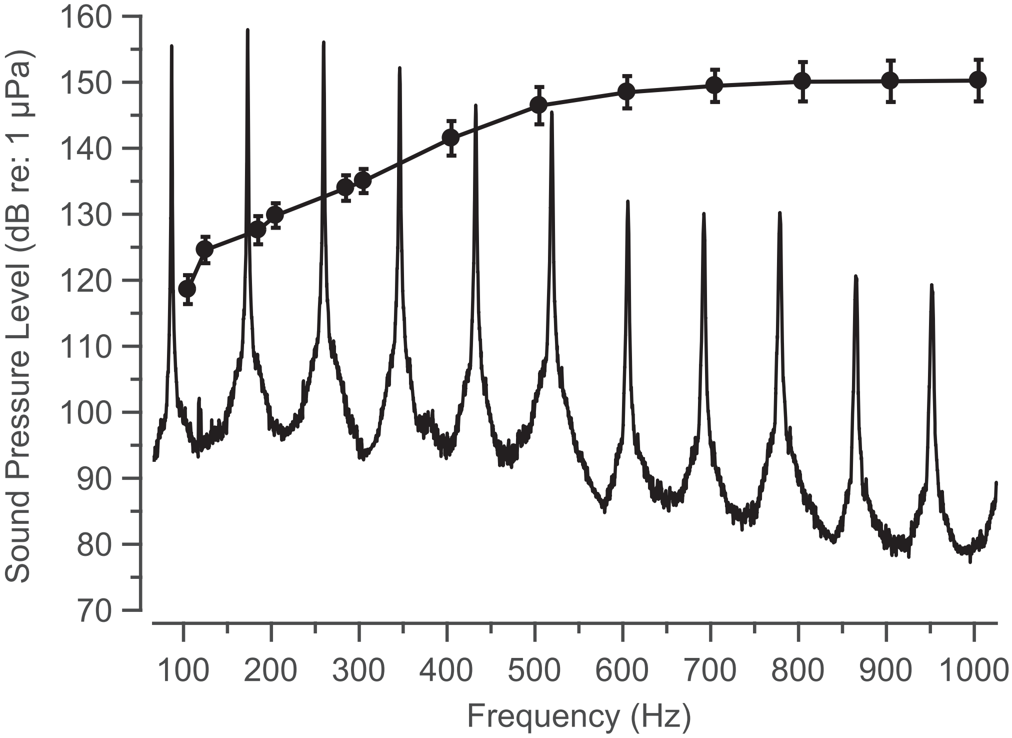

Figure 2.10: Comparison of the type I male midshipman advertisement call and utricular hair cell threshold tuning curve. Advertisement calls were recorded from a reproductive type I male midshipman (SL = 19.2 cm; BM = 101.2 g) collected during the summer at Seal Rock near Brinnon, WA, at low tide. Recordings of the male advertisement call were made at night in a large, indoor concrete tank (3 m diameter; 14.1 °C) at the University of Washington Friday Harbor Laboratories. Source-level recordings were made using a mini-hydrophone that was placed directly in front of the entrance of an artificial nest. The fundamental frequency of the advertisement call was 87 Hz (155 dB re: 1 μPa), with dominant harmonics occurring at the following frequencies: 173 Hz (158 dB re: 1 μPa), 260 Hz (156 dB re: 1 μPa), 346 Hz (152 dB re: 1 μPa), 433 Hz (147 dB re: 1 μPa), 519 Hz (146 dB re: 1 μPa), 606 Hz (132 dB re: 1 μPa), 692 Hz (130 dB re: 1 μPa), 779 Hz (130 dB re: 1 μPa), 865 Hz (121 dB re: 1 μPa), and 952 Hz (119 dB re: 1 μPa).

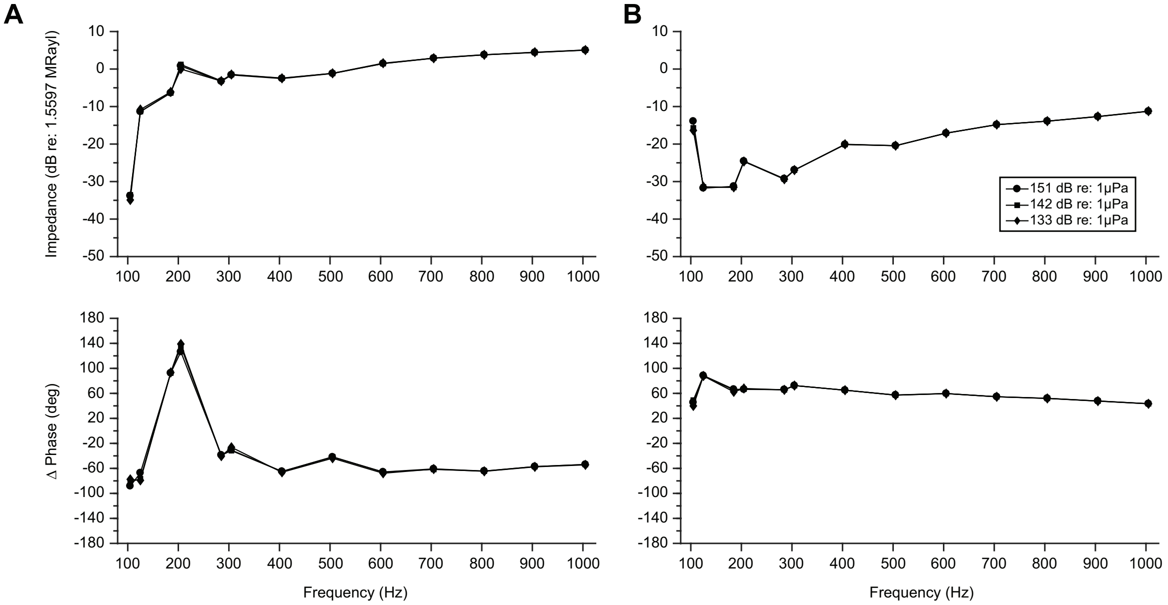

Figure S2.1: Acoustic characteristics of the experimental speaker and tank. The acoustic characteristics were characterized along the along the **A**, y- and **B**, z-axes. Top: acoustic impedance (dB re: 1.5597 MRayl) which is the complex ratio of sound pressure to particle velocity and is expressed in Rayls [1 Rayl = 1 (Pa s)/m]. Bottom: phase difference (Δ) between the pressure and particle velocity wave. All measurements were made using a triaxial accelerometer placed in the center of the tank at the position of the fish head during testing. Additionally, measurements were made at three sound pressure levels (151, 142, and 133 dB re: 1 μPa) for all tested frequencies (105, 125, 185, 205, 225, 305, 405, 505, 605, 705, 805, 905, and 1005 Hz).

CHAPTER 3

Utricular sound pressure sensitivity in the vocal plainfin midshipman

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This chapter has been prepared in publication format.

Abstract

The plainfin midshipman, *Porichthys notatus*, is a seasonally breeding fish that relies on acoustic communication to mediate intraspecific social and reproductive communication. Reproductive females rely on their auditory sense to detect and localize nesting males, which produce long-duration multiharmonic advertisement (mate) calls during the breeding season. Female midshipman have evolved multiple adaptive auditory mechanisms, including rostral “horn-like” swim bladder extensions that are in close proximity to the inner ear otic capsule, which has been shown to result in enhanced auditory sensitivity to sound pressure and frequency sensitivity in both the inner ear saccule and lagena. It has been suggested that the utricle may not serve a primary role in sound pressure sensitivity due to it being the inner ear end organ located furthest from the swim bladder. Here, we characterized the evoked potentials of utricular hair cells in female midshipman with intact, sham removed, or experimentally removed (treated) swim bladders in response to behaviorally-relevant auditory stimuli to determine if the utricle is sensitive to sound pressure We show that intact, sham, females are approximately 3 – 11 dB re: 1 µPa more sensitive to sound pressure and exhibit a greater frequency sensitivity than treated females across a broad range of frequencies, which include the dominant harmonics of male advertisement calls. This enhanced sound pressure and frequency sensitivity may facilitate the acquisition of auditory information needed for conspecific localization and mate choice decisions in females during the breeding season.

Introduction

The auditory system of fishes is composed of three paired otolithic end organs (saccule, utricle, and lagena) that are inherently sensitive to acoustic particle motion. However, it is well established that acoustic sound pressure sensitivity across fishes falls along a continuum, with a subset (e.g., ostariophysan fishes) of fishes mediating acoustic sound pressure sensitivity via the evolution of hearing specializations (Popper and Fay 2011; Putland et al. 2018b; Popper et al. 2021). One of the most common specializations involves the swim bladder, which in addition to facilitating sound production functions to regulate buoyancy and serves as an oxygen reservoir [For review, see (Alexander 1966; Braun and Grande 2008; Fine and Parmentier 2015)]. For this subset of fishes, sound pressure is transduced via indirect mechanisms to the auditory inner ear through volumetric changes of the swim bladder in response to received sound pressure, which introduces local particle motion vectors that are detectable by peripheral auditory structures (Popper and Fay 1999; Ladich and Popper 2004; Braun and Grande 2008). Thus, the coupling of the swim bladder to the inner ear (e.g., otophysan fishes) and the relative positioning of the swim bladder to the auditory inner ear play a major role in mediating sound pressure sensitivity (Popper and Fay 2011; Putland et al. 2018b; Popper et al. 2021). However, in addition to the swim bladder-inner ear variation observed across fishes, the swim bladders position also varies relative to each of the three paired auditory end organs, with the saccule and lagena being the closest and the utricle being the furthest across most fishes. Interestingly, few comparative studies have investigated differential sound pressure sensitivity across the otolithic end organs.

Plainfin midshipman, *Porichthys notatus*, are seasonally breeding vocal fish that are highly dependent upon the production and reception of acoustic signals for intraspecific communication during the reproductive season, thus making them an excellent model for investigating the neural mechanisms of acoustic communication (Bass et al. 1999; Bass and McKibben 2003; Forlano et al. 2015). During late spring and summer, courting (type I) males will migrate from deep-offshore waters and establish nest sites in the rocky substrate where they produce long-duration multiharmonic advertisement calls to attract gravid females for reproduction (Sisneros 2009a). To facilitate successful sound source localization and reproductive behaviors, females have evolved numerous auditory adaptations, including hormonal and seasonal state-dependent modulation of the auditory system (Sisneros et al. 2004a; Sisneros 2009b; Coffin et al. 2012; Perelmuter et al. 2019, 2021; Lozier and Sisneros 2019; Rogers et al. 2022a).

Recently, through morphological and physiological approaches, it has also been observed that the midshipman saccule and lagena, which are in close proximity to the swim bladder (mean distance: saccule = 2.59 mm; lagena = 2.89 mm), are capable of detecting sound pressure via the swim bladder (Mohr et al. 2017; Colleye et al. 2019; Vetter and Sisneros 2020). Whereby the presence of the swim bladder enhances female auditory sensitivity 5 – 11 dB re. 1 μPa and 3 – 6 dB re. 1 μPa, respectively, and extends the upper bandwidth of detectable frequencies to 1000 Hz (Colleye et al. 2019; Vetter and Sisneros 2020). These results are similar to those observed in other pressure-sensitive fishes, which exhibit enhanced sound pressure and frequency when the swim bladder is in close proximity (< 3 mm) to the auditory inner ear (Ramcharitar and Popper 2004; Schulz-Mirbach et al. 2012b; Kéver et al. 2014). However, the utricle of female midshipman is positioned at a much greater distance relative to the swim bladder (mean distance = 5.2 mm) (Mohr et al. 2017). Thus, whether the female midshipman utricle is close enough to mediate sound pressure detection pressure-induced vibrations of the swim bladder and enhance utricular sensitivity to acoustic sound pressure and higher frequencies is unknown.

The objective of this study was to determine if the utricle of female midshipman is sensitive to acoustic sound pressure. Based on the large swim bladder-to-utricle distance (> 3 mm), we hypothesized that the swim bladder does not function to transduce sound pressure to the utricle as has been observed in the midshipman saccule and lagena. Using a well-established electrophysiological approach, we compare the auditory evoked utricular hair cell response of female midshipman with intact or ablated swim bladders. We show that removal of the swim bladder results in a 3 – 12 dB re: 1 μPa decrease in auditory sensitivity and decreases the bandwidth of detectable frequencies. Furthermore, we interpret our results relative to those of our previous studies on the saccule and lagena and interpret our findings as they relate to the detection and reception of conspecific acoustic communication signals.

Methods

Animals

Adult female plainfin midshipman were collected from the rocky intertidal area at Seal Rock near Brinnon, WA, during low tide. Fish were housed at the University of Washington in recirculating artificial saltwater tanks and kept on a 12/12-hr light/dark photoperiod. All experiments were performed within 14 days of collection to minimize the effects of prolonged captivity on midshipman auditory sensitivity.

Prior to experimental data collection, standard length (SL; cm) and body mass (BM; g) were recorded. Sex was determined following each experiment via visual inspection of the gonads and reproductive state via gonadosomatic index [GSI; defined here as 100 * (gonad mass / (BM – gonad mass))]. All animal care and experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee and conformed to the NIH *Guide for the Care and Use of Laboratory Animals.*

Acoustic stimulus and calibration

The methodology of acoustic stimulus presentation and calibration is similar to that of previously published work from in the utricle (Rogers and Sisneros 2020; Rogers et al. 2022a). Briefly, calibrated acoustic stimuli were generated using a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA), which sent pure tone signals to an audio amplifier (BG-1120, TOA Corporation, Hyogo, Japan) and then to an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA). The underwater speaker was positioned such that its face was in the horizontal plane and was fully submerged 2 cm below the water's surface, which corresponds with the horizontal orientation of both the utricular end organs [See Figure S1 in (Rogers et al. 2022a)]. single tones (500 ms duration, 0.25 Hz duty cycle, 8 repetitions) at the following frequencies: 105, 125, 145, 165, 185, 205, 245, 285, 305, 405, 505, 605, 705, 805, 905, and 1005 Hz, which encompasses the dominant bandwidth frequencies contained within the male midshipman advertisement call and avoids frequencies that could potentially cause interference associated with resonance frequencies of the experimental tank [see Rogers and Sisneros, 2020 for tank acoustic properties].

Swim bladder manipulations

Fish were first anesthetized by immersion in a 0.025% ethyl *p*-aminobenzoate (benzocaine) buffered saltwater bath and then given an intramuscular injection of bupivacaine HCL (~1 mg/kg of BM) and cisatracurium besylate (~3 mg/kg of BM) for analgesia and immobilization, respectively. Similar to previous swim bladder manipulation studies in the midshipman (Colleye et al. 2019; Vetter and Sisneros 2020), a small incision (~1.5 cm) was then made on the ventral surface, approximately 1 cm rostral the vent. Using sterile forceps, the connective tissue and the swim bladder were then removed from the body cavity and the incision was closed with running sutures. Alternatively, to ensure that all experimental fish were exposed to the same surgery-related stress, control fish underwent a sham surgery, which followed the same surgical protocol, except for the removal of the swim bladder.

Utricular potential measurements

The methodology for recording *in vivo* auditory evoked utricular hair cell potentials in midshipman follows the techniques used in previous studies (Rogers and Sisneros 2020; Rogers et al. 2022a). Following surgical manipulations, a craniotomy was performed lateral the sagittal crest of the skull to expose bilateral inner ear utricles, and a hydrophobic barrier (approximately 3 cm dia. x 5 cm height; Fixodent, Proctor and Gamble Company, Cincinnati, OH, USA) was constructed around the craniotomy to prevent saltwater contamination during experimental testing. Fish were then transferred to the experimental tank (40 cm diameter, 20 cm water depth), which was maintained on a vibration-isolation table (TMC Vibration Control, Peabody, MA, USA) inside a sound attenuation chamber (Industrial Acoustics, New York, NY, USA), suspended in the center of the experimental tank using acoustically transparent film, and head-fixed 4 cm below the water's surface via a custom-built acrylic head holder and perfused with chilled saltwater (13 – 15°C) throughout experimental testing.

Auditory evoked utricular hair cell potentials were recorded using borosilicate glass microelectrodes (2 mm outer diameter; 1.16 mm inner diameter; A-M Systems, Sequim, WA) filled with 3 M KCl (impedance: 4.0 – 8.0 MΩ) that were positioned in close proximity (≤ 2 mm) to the utricular sensory epithelia. Amplified (100×; Model 5A, Getting Instruments, San Diego, CA, USA) and bandpass filtered (0.07 to 3 kHz; model SR650, Stanford Research Systems, Sunnyvale, CA, USA) analog evoked potential signals were recorded in response to pure tone auditory stimuli (105 – 1005 Hz). Since the auditory end organs of the inner ear are composed of sensory hair cells with orientations in opposing directions, the maximum hair cell evoked potential occurs at twice the sound stimulus frequency (Cohen and Winn 1967; Furukawa and Ishii 1967; Sisneros 2007). Therefore, for all recordings, utricular potentials were defined as the amplitude of the hair cell response at the second harmonic of the stimulus frequency.

Prior to each physiology recording session, background utricular potential levels were measured (n = 8) under ambient sound levels in the absence of auditory stimuli. After determining background levels, iso-intensity level responses were measured in response to randomly presented pure tone auditory stimuli (105 – 1005 Hz). All experimental trials were carried out using custom MATLAB scripts, which controlled stimulus timing and data acquisition, and all data were stored on a desktop computer. Utricular hair cell auditory threshold tuning curves relative to sound pressure (dB re: 1 μPa) were determined via input-output measurements of the evoked receptor potentials from bilateral utricular over the range of tested frequencies (105–1005 Hz) and sound levels (103 – 154 dB re: 1 μPa, 3 dB increments). Auditory threshold levels were defined as the lowest stimulus level that yielded the lowest mean utricular evoked potential that was at least two standard deviations above background levels.

Analyses

All statistical analyses were performed using MATLAB (MathWorks Inc., Natick, MA) or R software (R Core Team, Vienna, Austria) using the following packages: car, dplyr, ggpubr, lme4, rstatix, tidyr, and tidyverse. For all statistical tests, a significance level of 0.05 was defined. To determine if the presence of the swim bladder plays a role in modulating utricular hair cell auditory sensitivity, the effects of swim bladder condition (i.e., sham or removal) were analyzed via a repeated-measures analysis of variance (ANOVA, between-subject factor: swim bladder condition, within-subject factor: frequency * swim bladder treatment). As we were only interested in how swim bladder condition modulates frequency sensitivity, *a priori* pairwise t-tests compared the frequency-dependent auditory sensitivity of sham and removal groups at the same frequency across the stimulus frequency bandwidth (105 – 1005 Hz).

Results

Auditory evoked potentials were recorded from the utricular hair cells of 33 reproductive adult female plainfin midshipman. Sham (n = 16) and removal (n = 17) fish had similar standard lengths (Sham = 16.9 ± 1.6; mean ± s.d.; Removal = 16.9 ± 1.3; two-sample t-test, $t_{1,31} = -0.134$, $p = 0.894$), body mass (Sham = 66.9 ± 15.6; Removal = 65.3 ± 16.7; two-sample t-test, $t_{1,31} = -0.289$, $p = 0.775$), and gonadosomatic indicies (Sham = 21.2 ± 8.1; Removal = 25.2 ± 5.3; two-sample t-test, $t_{1,31} = 1.683$, $p = 0.102$).

Utricular hair cell iso-level response profiles across the bandwidth of test frequencies (105 – 1005 Hz) were generated at three representative sound pressures, 154, 142, and 130 dB re: 1 µPa, which corresponded to a broad range of biologically relevant sound levels. Figure 3.1 displays representative iso-level response curves from females with intact (i.e., sham removal) or removed swim bladders. Across recordings, the magnitude of the auditory evoked potentials recorded from utricular hair cells in response to pure tone stimuli was greatest in females with intact swim bladders when compared to the removal group. Figure 3.2 illustrates the mean iso-level response profiles of the evoked utricular potentials from these two treatment groups at 154, 142, and 130 dB re: 1 µPa, respectively. Females with intact swim bladders (i.e., sham) had significantly higher evoked utricular potentials than females that had their swim bladders removed across a range of biologically-relevant sound levels [One-way repeated measures ANOVA, between-subject factor: swim bladder treatment] at 154 dB re: 1 µPa ($F_{1, 928} = 128.95$, $p < 0.001$), 142 dB re: 1 µPa ($F_{1, 928} = 108.22$, $p < 0.001$), and 130 dB re: 1 µPa ($F_{1, 928} = 128.95$, $p < 0.001$)], and exhibited a significant interaction of frequency and swim bladder treatment at 154 dB re: 1 µPa ($F_{1, 15} = 5.59$, $p < 0.001$), 142 dB re: 1 µPa ($F_{1, 15} = 7.64$, $p < 0.001$), and 130 dB re: 1 µPa ($F_{1, 15} = 5.89$, $p < 0.001$). Furthermore, frequency-specific differences in utricular hair cell evoked magnitude response were observed between swim bladder treatment groups within each sound level tested, with the presence of the swim bladder resulting in evoked potentials that were up to 4.1, 4.0, and 7.8 times greater at sound levels of 154, 142, and 130 dB re: 1 μPa, respectively (Figure 3.2; Table S3.1-S3.3).

Concurrent with the dramatic increase in utricular hair cell evoked potential magnitude, auditory threshold tuning curves of females with intact swim bladders were lower (i.e., more sensitive) than females that had their swim bladders removed (Figure 3.3; Figure 3.4A; One-way repeated measures ANOVA, between-subject factor: swim bladder treatment, $F_{1, 785} = 144.97$, $p < 0.001$) and a significant interaction was observed between swim bladder treatment and frequency (One-way repeated measures ANOVA, within-subject factor: frequency * swim bladder treatment, $F_{1, 15} = 3.41$, $p < 0.001$). Additionally, frequency-specific differences in auditory thresholds were observed between females with intact (i.e., sham) and removed swim bladders, with females that underwent sham swim bladder ablations being more sensitive at frequencies from 105 to 605 Hz (Figure 3.4A; *a priori* t-tests for pairwise comparisons across frequency, $p < 0.001$) that resulted in an approximately four-fold difference in tuning (Figure 3.4B). Furthermore, differences in the highest detectable frequency were observed, with sham females exhibiting greater frequency bandwidth sensitivity than removal females at frequencies ranging from 605 – 1005 Hz (Figure 3.4C).

Discussion

The goal of this study was to determine if the swim bladder of female plainfin midshipman functions to indirectly enhance utricular hair cell auditory and frequency sensitivity, via transduction of a sound field's received sound pressure wave. We show, using a well-characterized electrophysiological approach, that the utricular hair cells of female plainfin midshipman exhibit up to an 8-fold decrease in evoked potential magnitude when the swim bladder is removed. Additionally, we show that removal of the swim bladder results in a 3 – 12 dB re: 1 μPa decrease in auditory sensitivity and decreases the bandwidth of detectable frequencies. Taken together, these results suggest that the utricle, similar to the saccule and lagena, is sensitive to acoustic sound pressure. In this discussion, we consider how our results relate to those observed in the midshipman inner ear saccule and lagena and how the swim bladder may function to enhance the detection and localization of acoustic courtship calls by males during the breeding season.

The teleost swim bladder serves the primary function of regulating buoyancy and acting as an oxygen reservoir [For review, see (Alexander 1966; Braun and Grande 2008; Fine and Parmentier 2015)]. In addition, for a subset of fishes, the swim bladder has been secondarily adapted to facilitate sound production and/or reception. To mediate the reception of sound, the swim bladder acts to indirectly stimulate the inner ear in response to a sound field's pressure wave, whereby reverberation of the swim bladder reradiates sound energy in the form of local particle motion, which then stimulates the auditory inner (Popper and Fay 1999; Ladich and Popper 2004; Braun and Grande 2008).

It was first posited that midshipman might encode sound pressure information based on behavioral sound source localization experiments carried out in reproductive female midshipman by Coffin and colleagues (2014). Later, Mohr et al. (2018) conducted microCT scanning to morphologically characterize female swim bladder-inner ear relationships and showed that the utricle is positioned the furthest from the inner ear (5.24 ± 0.76 mm) when compared to the saccule (2.59 ± 0.52 mm) and lagena (2.89 ± 0.16 mm). More recently, Colleye et al. (2019) and Vetter and Sisneros (2020) showed that following swim bladder removal, the saccule and lagena of female midshipman exhibit up to a 12 and 6 dB re: 1 μPa decrease in auditory sensitivity, respectively, and decreased frequency sensitivity to high frequencies (> 400 Hz). However, based on the increased swim bladder-inner ear distance in midshipman and pressure sensitivity observed in other fishes, it has been suggested that the utricle may not be sensitive to sound pressure.

The results from our study show that despite female midshipman's enhanced swim bladder-to-inner ear utricle distance, the swim bladder functions to enhance auditory sensitivity to sound pressure and high frequencies. We show that females with intact swim bladders (i.e., sham removal) were approximately 4 – 12 dB re: 1 μPa more sensitive than females that had their swim bladders removed across a broad range of behaviorally relevant acoustic stimuli (105 – 705 Hz). Additionally, females that underwent sham swim bladder removals displayed a greater number of evoked potential responses to higher frequencies ranging from 605 – 1005 Hz when compared to females that underwent swim bladder removals. Given these results, we suggest that enhanced sensitivity to sound pressure and higher frequencies in females with intact swim bladders is directly related to the presence of swim bladders, and that enhancement of auditory sensitivity aids in facilitating the detection of courting male vocalizations, especially high-frequency components of the multiharmonic call, and the distance at which females are able to begin localizing male nest sites during the reproductive season.

Given the diversity in fish swim bladder-inner ear relationships, it is suggested that sound pressure sensitivity falls along a continuum. In other pressure-sensitive fishes, increased sound pressure (dB re: 1 μPa) and frequency sensitivity is often associated with the swim bladder being in close proximity (< 3 mm) to the inner ear (Ramcharitar and Popper 2004; Schulz-Mirbach et al. 2012b; Kéver et al. 2014). Here, we showed that while the utricle is positioned the furthest from the inner ear (5.24 ± 0.76 mm), it is more sensitive to sound pressure than lagenar hair cells and is as pressure sensitive as saccular hair cells, both of which are positioned closer to the swim bladder. Given that the saccule is considered the main organ for hearing in most fishes, the similarity in saccular and utricular hair cell sound pressure sensitivities. One possible explanation for the observed results is that reverberation of the swim bladder in response to a sound field's received pressure wave differentially excites the saccule and utricle, which are positioned in approximately orthogonal axes. Recently, Schulz-Mirbach and colleagues (2020), showed that the inner ear otolithic end organs of the cichlid *Etroplus canarensis,* which has a swim bladder that is in close proximity to the inner ear, exhibit differential motion in response to acoustic sound pressure generated by a standing wave tube-like tank. However, whether the otolithic end organs of this species exhibit differential sound pressure sensitivity has yet to be determined. Development or use of a similar standing wave tube-like system, which uses relatively high speed (~100 fps) x-ray phase contrast imaging to track otolith motion over time, in the midshipman could provide insight into how differential sound pressure sensitivity arises in the midshipman fish. Furthermore, it is possible that female midshipman rely upon both the highly pressure-sensitive saccule and utricle, which contain hair cells that are directionally sensitive along orthogonal axes, to aid in the detection of their auditory scene and localization of courting males during the reproductive season.

Sexually dimorphic differences in swim bladder morphology have been noted in a number of fishes. Previously, Mohr et al. (2018) showed that there were stark differences in reproductive female and male swim bladder morphology, with the most notable difference being that males have reduced rostral swim bladder extensions likely due to the presence of the sonic muscles, which facilitate social and reproductive communication during the reproductive season. Thus, reproductive males have a swim bladder that is positioned approximately 1.5 – 2 times the distance relative to the otic capsule. Whether the utricle and the other midshipman auditory end organs (i.e., saccule and lagena) of reproductive male midshipman exhibit sound pressure sensitivity has yet to be considered but would provide valuable insight into the potential adaptations of the midshipman swim bladder as an acoustic organ for sound production and reception.

Conclusions

The inner ear of all fishes is inherently sensitive to acoustic particle motion. In contrast, a subset of fishes exhibit sound pressure sensitivity via direct linkage or the close positioning of the swim bladder to the inner ear. Here, we show that the utricle of female plainfin midshipman, which has a swim bladder that is positioned near (~ 5 – 6 mm) the inner ear, is sensitive to sound pressure via the swim bladder's response to a sound field's received pressure wave. Taken together, we show that utricular sound pressure sensitivity functions to effectively enhance auditory sensitivity to biologically relevant auditory stimuli, including conspecific acoustic signals important for facilitating social and reproductive interactions.

Conflict of interest: The authors declare no competing financial interests.

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Figures

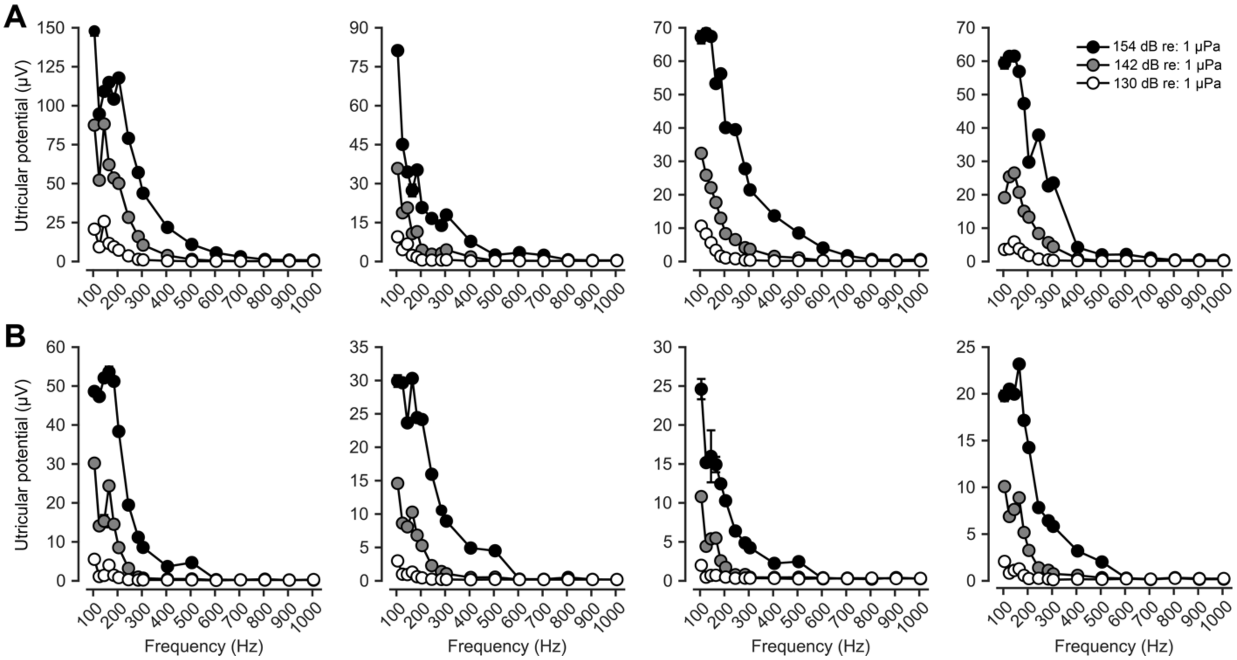

Figure 3.1: Representative examples of iso-intensity level curves recorded from utricular hair cells of female plainfin midshipman with (A) sham and (B) removed swim bladders. Iso-intensity responses were recorded in response to single-tone playbacks at sound pressure levels of 154 (black), 142 (gray), and 130 (white) dB re: 1 μPa. Data are represented as mean ± 1 SD; note that some error bars are minimal, and the symbols may obscure the bars.

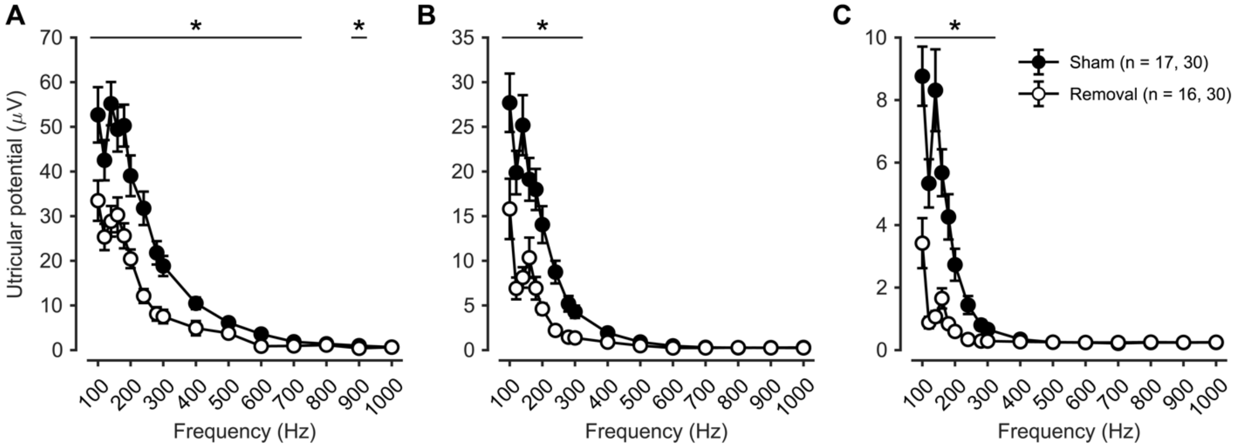

Figure 3.2: Iso-level response curves recorded from utricular hair cells of female plainfin midshipman with sham (black) and removed (gray) swim bladders in response to single-tone playbacks at sound pressure levels of (A) 154, (B) 142, and (C) 130 dB re: 1 μPa. Data are represented as mean ± 1 SE; note that some error bars are minimal, and the symbols may obscure the bars. The number of animals and records for each group is indicated in parentheses.

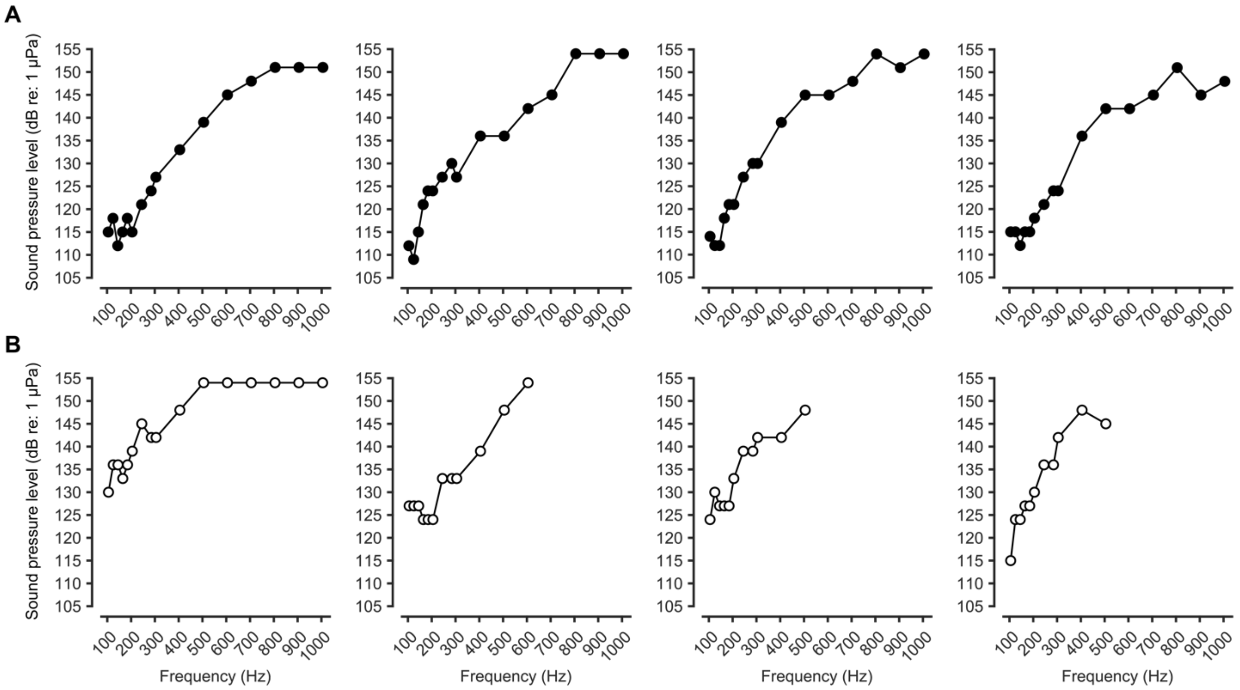

Figure 3.3: Representative sound pressure (dB re: 1 μPa) level auditory threshold tuning curves recorded from female plainfin midshipman with (A) intact (black) and (B) removed (white) swim bladders. Tuning curves were constructed using utricular hair cell evoked responses, with thresholds defined as the lowest sound pressure level (dB re: 1 μPa) needed to evoke a utricular potential at least 2 SD above the background electrical noise level.

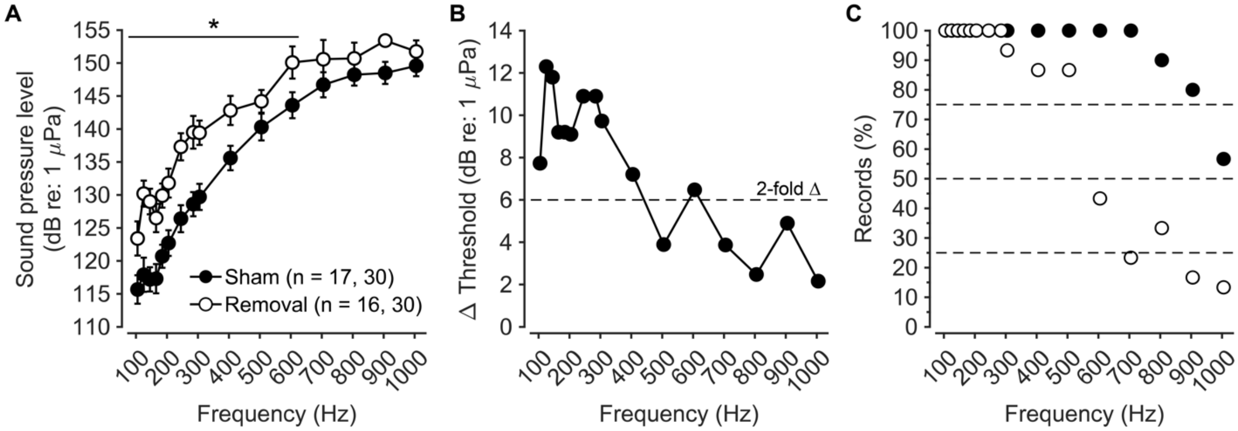

Figure 3.4: Utricular hair cell sound pressure (dB re: 1 µPa) sensitivity. (A) Auditory tuning curves of female midshipman with swim bladders intact (sham; black) or removed (removal; white). The auditory thresholds were defined as the lowest auditory stimulus level needed to evoke utricular potentials at least 2 SD above the background electrical noise level. All data are plotted as mean ± 95% confidence interval. The number of animals and records for each group is indicated in parentheses, respectively.

Table S3.1: Summary of mean evoked potentials (μV) recorded from utricular hair cells of reproductive females with swim bladders that were intact (control) or removed (removal) in response to pure tone stimuli at 154 dB re. 1 μPa. Evoked potential (μV) responses are displayed as mean ± SE Fold change was calculated as the ratio of the frequency-specific mean evoked response (μV) of control to removal females. Frequency-specific mean evoked potentials (μV) were compared via *a priori* t-tests with Bonferroni corrections, and the *p*-values are shown. *Significant differences between the two groups (*p*<0.05).

	Evoked potential (μV)			
Frequency (Hz)	Control	Removal	Fold change	*p*-value
105	52.69 ± 6.20	33.47 ± 4.52	1.6	0.031*
125	42.53 ± 4.5	25.34 ± 2.94	1.7	0.008*
145	55.19 ± 4.85	28.85 ± 3.43	1.9	<0.001*
165	49.45 ± 4.99	30.29 ± 3.90	1.6	0.010*
185	50.27 ± 4.70	25.60 ± 2.81	2.0	<0.001*
205	39.04 ± 4.56	20.43 ± 2.09	1.9	0.002*
245	31.76 ± 3.74	12.12 ± 1.57	2.6	<0.001*
285	21.79 ± 2.62	8.11 ± 1.52	2.7	<0.001*
305	18.84 ± 2.25	7.52 ± 1.52	2.5	<0.001*
405	10.48 ± 1.32	4.92 ± 1.61	2.1	0.020*
505	6.14 ± 0.76	3.78 ± 0.79	1.6	0.036*
605	3.59 ± 0.42	0.87 ± 0.30	4.1	<0.001*
705	1.86 ± 0.27	0.94 ± 0.36	2.0	0.016*
805	1.41 ± 0.26	1.13 ± 0.42	1.3	0.556
905	0.99 ± 0.19	0.41 ± 0.10	2.4	0.010*
1005	0.67 ± 0.10	0.65 ± 0.25	1.0	0.914

Table S3.2: Summary of mean evoked potentials (μV) recorded from utricular hair cells of reproductive females with swim bladders that were intact (control) or removed (removal) in response to pure tone stimuli at 142 dB re. 1 μPa. Evoked potential (μV) responses are displayed as mean ± SE Fold change was calculated as the ratio of the frequency-specific mean evoked response (μV) of control to removal females. Frequency-specific mean evoked potentials (μV) were compared via *a priori* t-tests with Bonferroni corrections, and the *p*-values are shown. *Significant differences between the two groups (*p*<0.05).

	Evoked potential (μV)			
Frequency (Hz)	Control	Removal	Fold change	*p*-value
105	27.70 ± 3.27	15.81 ± 3.38	1.8	0.026*
125	19.88 ± 2.44	6.91 ± 1.22	2.9	<0.001*
145	25.18 ± 3.36	8.13 ± 1.15	3.1	<0.001*
165	19.12 ± 2.39	10.34 ± 2.26	1.9	0.018*
185	17.99 ± 2.3	6.93 ± 1.26	2.6	<0.001*
205	14.05 ± 2.06	4.61 ± 0.62	3.1	<0.001*
245	8.74 ± 1.27	2.21 ± 0.52	4.0	<0.001*
285	5.19 ± 0.87	1.45 ± 0.49	3.6	0.002*
305	4.29 ± 0.69	1.36 ± 0.57	3.2	0.005*
405	1.92 ± 0.35	0.89 ± 0.44	2.2	0.086
505	0.90 ± 0.17	0.50 ± 0.13	1.8	0.063
605	0.47 ± 0.12	0.24 ± 0.01	2.0	0.071
705	0.31 ± 0.05	0.24 ± 0.02	1.3	0.225
805	0.26 ± 0.02	0.28 ± 0.06	0.9	0.736
905	0.27 ± 0.04	0.25 ± 0.01	1.1	0.639
1005	0.30 ± 0.05	0.25 ± 0.01	1.2	0.366

Table S3.3: Summary of mean evoked potentials (μV) recorded from utricular hair cells of reproductive females with swim bladders that were intact (control) or removed (removal) in response to pure tone stimuli at 130 dB re. 1 μPa. Evoked potential (μV) responses are displayed as mean ± SE Fold change was calculated as the ratio of the frequency-specific mean evoked response (μV) of control to removal females. Frequency-specific mean evoked potentials (μV) were compared via *a priori* t-tests with Bonferroni corrections, and the *p*-values are shown. *Significant differences between the two groups ($p<0.05$).

	Evoked potential (μV)			
Frequency (Hz)	Control	Removal	Fold change	*p*-value
105	8.76 ± 0.95	3.42 ± 0.80	2.6	<0.001*
125	5.33 ± 0.77	0.88 ± 0.18	6.1	<0.001*
145	8.31 ± 1.31	1.07 ± 0.18	7.8	<0.001*
165	5.68 ± 0.75	1.65 ± 0.32	3.4	<0.001*
185	4.26 ± 0.72	0.84 ± 0.14	5.1	<0.001*
205	2.73 ± 0.51	0.60 ± 0.07	4.6	<0.001*
245	1.44 ± 0.29	0.34 ± 0.05	4.2	<0.001*
285	0.80 ± 0.16	0.29 ± 0.04	2.8	0.006*
305	0.66 ± 0.15	0.29 ± 0.04	2.3	0.025*
405	0.35 ± 0.06	0.27 ± 0.04	1.3	0.289
505	0.25 ± 0.03	0.25 ± 0.02	1.0	0.955
605	0.24 ± 0.02	0.25 ± 0.02	1.0	0.897
705	0.22 ± 0.01	0.24 ± 0.01	0.9	0.208
805	0.24 ± 0.02	0.26 ± 0.01	1.0	0.731
905	0.24 ± 0.01	0.25 ± 0.01	1.0	0.635
1005	0.24 ± 0.02	0.25 ± 0.01	1.0	0.789

CHAPTER 4

Reproductive state modulates utricular auditory sensitivity in a vocal fish

Running title: Reproductive state modulates utricular sensitivity

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Abstract

The plainfin midshipman, *Porichthys notatus*, is a seasonally breeding vocal fish that relies on acoustic communication to mediate nocturnal reproductive behaviors. Reproductive females use their auditory sense to detect and localize “singing” males that produce multiharmonic advertisement (mate) calls during the breeding season. Previous work showed that the midshipman saccule, which is considered the primary end organ used for hearing in midshipman and most other fishes, exhibit reproductive state and hormone-dependent changes that enhanced saccular auditory sensitivity. In contrast, the utricle was previously posited to serve primarily a vestibular function, but recent evidence in midshipman and related toadfish suggests that it may also serve an auditory function and aid in the detection of behaviorally relevant acoustic stimuli. Here, we characterized the auditory evoked potentials recorded from utricular hair cells in reproductive and non-reproductive female midshipman in response to underwater sound to test the hypothesis that variation in reproductive state affects utricular auditory sensitivity. We show that utricular hair cells in reproductive females exhibit up to a 6-fold increase in the utricular potential magnitude and have thresholds based on measures of particle acceleration (re: 1 ms^{-2}) that are 7-10 dB lower than non-reproductive females across a broad range of frequencies, which include the dominant harmonics of male advertisement calls. This enhanced auditory sensitivity of the utricle likely plays an essential role in facilitating midshipman social and reproductive acoustic communication.

Introduction

Seasonal changes in sensory processing related to an animal's reproductive state occur in many non-mammalian vertebrates, including songbirds, amphibians, and fishes [For review, see (Ball et al. 2003; Bass and Zakon 2005; Wilczynski et al. 2005)]. Furthermore, reproductive-related changes in sensory processing of auditory information occur in a number of seasonally breeding species that rely on acoustic communication to mediate social interactions in a reproductive context [e.g., birds: (Henry and Lucas 2009; Caras et al. 2010, 2012, 2015; Gall et al. 2013; Vélez et al. 2015); amphibians: (Penna et al. 1992; Goense and Feng 2005; Miranda and Wilczynski 2009); and fishes: (Sisneros and Bass 2003; Sisneros 2009b)]. However, previous work has primarily focused on reproductive state-dependent changes in sensitivity of the central auditory system (Penna et al. 1992; Goense and Feng 2005; Miranda and Wilczynski 2009; Caras et al. 2012, 2015) or primary hearing organs of the peripheral auditory system (Sisneros and Bass 2003; Sisneros 2009b; Caras et al. 2010). Here, we consider reproductive state-dependent changes in the frequency sensitivity and auditory gain of the utricle, an end organ not often associated with an auditory function, in a seasonally breeding vertebrate for which the detection and localization of conspecific acoustic signals is critical to its reproductive success.

The plainfin midshipman fish (*Porichthys notatus*) is a seasonally breeding vocal fish that produces social acoustic signals for intraspecific communication during the reproductive season. The social behaviors of this nocturnally-active species are highly dependent upon the production and reception of acoustic signals, which makes the midshipman an excellent model for investigating the neural mechanisms of acoustic communication, especially those related to seasonal changes in vocal-acoustic behavior and auditory reception (Bass et al. 1999; Bass and McKibben 2003; Forlano et al. 2015). During the late spring and summer, midshipman migrate into the shallow intertidal zone to reproduce and care for their offspring. Courting (type I) males establish nest sites in the rocky substrate where they produce long-duration multiharmonic advertisement calls to attract gravid females for reproduction (Sisneros 2009a). Previous work has shown that females exhibit reproductive state- and hormone-dependent changes in the auditory sensitivity of the saccule, such that reproductive females are better suited than non-reproductive females to detect conspecific vocalizations (Sisneros et al. 2004a; Sisneros 2009b; Forlano et al. 2016). This steroid-, reproductive state-dependent modulation of auditory saccular sensitivity is thought to enhance the coupling of sender and receiver in the midshipman acoustic communication system.

In most fishes, the inner ear saccule is often the largest otolithic end organ and most associated with hearing (Popper and Fay 1993, 2011), while the smaller utricle has been posited to serve primarily a vestibular function as a gravistatic organ (Riley and Moorman 2000; Boyle et al. 2001, 2018; Bianco et al. 2012; Inoue et al. 2013). However, recent evidence in toadfish and midshipman (Family Batrachoididae) suggests that the utricle is capable of detecting and encoding behaviorally-relevant acoustic stimuli, including conspecific vocalizations (Maruska and Mensinger 2015; Rogers and Sisneros 2020; Rogers et al. 2022b). Yet, the extent to which the utricle may exhibit reproductive-related changes in auditory sensitivity to social acoustic signals remains unknown.

Here, we test the hypothesis that seasonal variation in reproductive state modulates the auditory sensitivity of the utricle in female plainfin midshipman. We compare the auditory evoked utricular potentials of reproductive and non-reproductive females to determine whether there are differences related to reproductive state in the frequency response and auditory threshold of utricular hair cells to behaviorally relevant auditory stimuli. We show that the utricle serves an auditory function that is seasonally plastic and highly adapted in reproductive females to detect the dominant frequencies of conspecific vocalizations.

Materials and Methods

Animal collection and husbandry

Non-reproductive adult female plainfin midshipman fish, *Porichthys notatus* Girard 1854, were collected via otter trawls (*R/V Kittiwake*, Friday Harbor Laboratories) in January 2021 from the Puget Sound near Edmonds, WA, at depths ranging from 85 to 100 m. Reproductive adult female plainfin midshipman were collected during their breeding season (May – June 2021) by hand at low tide from exposed nest sites in the rocky intertidal area at Seal Rock near Brinnon, WA. Following collection, fish were transported to the University of Washington and housed in a 350 L recirculating artificial saltwater tank maintained at 15 ± 2 °C and kept on either a winter (9/15-h) or summer (12/12-h) light/dark photoperiod, which corresponds with the non-reproductive and reproductive ambient photoperiods, respectively. Before each physiology experiment, standard length (SL; cm) and body mass (BM; g) were recorded and sex was determined by visual inspection of the gonads. The gonadosomatic index [GSI; defined here as 100 * (gonad mass / (BM – gonad mass))] for each fish was recorded following each experiment. Utricular hair cell potential recordings were performed within 17 days after trawl collection in the winter and 14 days after hand-collection during the summer to minimize any effects of prolonged captivity on midshipman auditory sensitivity while still allowing the animals to recover from capture-related stress.

Acoustic stimulus and calibration

The methodology used for acoustic stimulus presentation and calibration was similar to that of previously published work (Sisneros 2007, 2009b; Alderks and Sisneros 2011; Bhandiwad et al. 2017; Vetter 2019; Rogers and Sisneros 2020). Acoustic stimuli were generated by a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA), which sent pure tone signals to an audio amplifier (BG-1120, TOA Corporation, Hyogo, Japan) and then to an underwater speaker (UW-30, Telex Communications, Burnsville, MN, USA). The midshipman utricle is likely highly sensitive to particle motion along the horizontal plane as both the otolith (i.e., lapillus) and hair cells are oriented along the horizontal plane [Figure 4.1b, Figure S4.1 https://figshare.com/s/e17c71d36f441dce06e4; but also see figure 6 in (Coffin et al. 2012)]. Therefore, the underwater speaker was positioned such that the speaker's face resided along the horizontal plane and was fully submerged 2 cm below the water's surface (Figure 4.1a – 1). Acoustic stimuli consisted of single 500 ms pure tones repeated 8 times at a rate of one every 1.5 s. Acoustic stimuli were randomly presented at the following frequencies 105, 125, 145, 165, 185, 205, 245, 285, 305, 405, 505, 605, 705, 805, 905, and 1005 Hz, which encompasses the dominant bandwidth frequencies contained within the male midshipman advertisement call and avoids frequencies that could potentially cause interference associated with resonance frequencies of the experimental tank [see Rogers and Sisneros, 2020 for tank acoustic properties].

All acoustic stimuli were calibrated relative to the stimuli's sound pressure (dB re: 1 μPa) via a mini-hydrophone (model 8103, Bruel and Kjaer, Naerum, Denmark) connected to a conditioning amplifier (gain = 100 mV/Pa, Nexis 2692-0S1, Bruel and Kjaer, Naerum, Denmark). However, only certain groups of fishes can detect sound pressure via secondary structures that are close in proximity or connect to the inner ear, and function to convert the received sound pressure wave into local particle motion that stimulates the inner ear. Previous midshipman studies showed that both the saccule and lagena are sound pressure sensitive based on their proximity to the swim bladder (Colleye et al. 2019; Vetter and Sisneros 2020). However, it remains to be determined if the utricle is sensitive to sound pressure; therefore, we also report the equivalent particle acceleration levels (dB re: 1 ms^{-2}) that corresponded to the sound pressure levels (dB re: 1 μPa) used in this study, based on our calibration procedures (detailed below).

Particle acceleration levels (dB re: 1 ms^{-2}) were determined by suspending a neutrally buoyant waterproofed triaxial accelerometer (Model VW3567A12; Sensitivity at 100 Hz: 10.42 mV/ms^{-2} (*x*-axis), 10.03 mV/ms^{-2} (*y*-axis), 10.37 mV/ms^{-2} (*z*-axis); PCB Piezotronics, Depew, NY, USA) that connected to a signal conditioner (gain = ×100/axis; Model: 482A16; PCB Piezotronics, Depew, NY, USA). For both sound pressure (dB re: 1 μPa) and particle acceleration (dB re: 1 ms^{-2}) measurements, the mini-hydrophone and particle accelerometer, respectively, were suspended 10 cm perpendicular to the face of the underwater speaker and 4 cm below the water's surface to coincide with the position of the midshipman inner ear during auditory evoked hair cell potential measurements. Sound pressure level (dB re: 1 μPa) measurements were calibrated by measuring the peak-to-peak (pk-pk) voltage (V_{pk-pk}) amplitude on an oscilloscope (Tektronix, Beaverton, OR, USA) and then equalized in sound pressure level (dB re: 1 μPa) using a custom MATLAB (MathWorks Inc., Natick, MA, USA) script, which measured the power spectral density for all tested frequencies. The signal (V_{pk-pk}) sent to the speaker was scaled until a reference peak-to-peak sound pressure level (SPL_{pk-pk}) output from the speaker of 130 ± 0.5 dB re: 1 μPa was achieved. Particle acceleration level (dB re: 1 ms^{-2}) measurements were acquired by measuring the particle motion amplitude (V_{pk-pk}) of each tested frequency across the entire range of sound levels using a National Instruments data acquisition system (Model: NI USB-6009, National Instruments, Austin, TX, USA) and visualized using LabVIEW software (National Instruments, Austin, TX, USA). Using a custom LabVIEW (National Instruments, Austin, TX, USA) script, particle motion amplitude measurements (V_{pk-pk}) for each axis (*x*-, *y*-, and *z*-axis) were corrected for the gain (sensitivity) of the accelerometer. Particle motion values (dB re: 1 ms^{-2}) for each test frequency at three representative sound levels (130, 142, and 154 dB re: 1 μPa) are displayed in figure S4.2 (https://figshare.com/s/e17c71d36f441dce06e4).

Utricular potential measurements

The methodology for recording utricular hair cell potentials follows the techniques used in our previous study, which measured the auditory evoked potentials from the utricular hair cells of adult male plainfin midshipman (Rogers and Sisneros 2020). Midshipman were anesthetized by immersion in a 0.025% ethyl *p*-aminobenzoate (benzocaine) buffered saltwater bath and then given an intramuscular injection of bupivacaine HCL (~1 mg/kg of BM) and cisatracurium besylate (~3 mg/kg of BM) for analgesia and immobilization, respectively. A craniotomy was then performed lateral the sagittal crest of the skull to expose the inner ear saccule and utricle and the brain (Figure 4.1b) and a hydrophobic barrier (approximately 2.5 cm dia. x 5 cm height) made of denture adhesive cream (Fixodent, Proctor and Gamble Company, Cincinnati, OH, USA) was constructed around the craniotomy to prevent saltwater contamination during experimental testing (Figure 4.1a – 2). Fish were then transferred to the experimental tank (40 cm diameter, 20 cm water depth), which was maintained on a vibration-isolation table (TMC Vibration Control, Peabody, MA, USA) inside a sound attenuation chamber (Industrial Acoustics, New York, NY, USA), suspended in the center of the experimental tank using acoustically transparent film (Figure 4.1a – 3), head-fixed 4 cm below the water's surface via a custom-built acrylic head holder (Figure 4.1a – 4) and perfused with chilled saltwater (13 – 15°C) throughout experimental testing (Figure 4.1a – 5).

Auditory evoked utricular hair cell potentials were recorded using borosilicate glass microelectrodes (2 mm outer diameter; 1.16 mm inner diameter; A-M Systems, Sequim, WA) that were pulled using a Narishige puller (Model: PE-21) and filled with 3 M KCl (impedance: 4.0 – 8.0 MΩ). Electrodes were positioned in close proximity (≤ 2 mm) to the medial region of the utricle near the sensory epithelia (Figure 4.1a – 6). The analog evoked potential signals were pre-amplified (10×; Model 5A, Getting Instruments, San Diego, CA, USA), bandpass filtered (0.07 to 3 kHz), and then amplified (10×) again via a digital filter (model SR650, Stanford Research Systems, Sunnyvale, CA, USA). Using a lock-in amplifier (SR830, Stanford Research Systems, Sunnyvale, CA, USA), the output signal, which was proportional to the utricular hair cell evoked response to the stimulus fundamental frequency, was locked to a reference frequency set to the second harmonic of the pure tone stimulus frequency (i.e., 2 $*$ fundamental frequency), which due to populations of oppositely oriented hair cells in the teleost inner ear corresponds to the greatest evoked potential amplitudes (Cohen and Winn 1967; Furukawa and Ishii 1967; Sisneros 2007; Lozier and Sisneros 2019) (Figure 4.1c). At the start of each experimental recording session, control trials (i.e., no sound stimulus) were conducted to measure background utricular potential levels (n = 8 measurements) under ambient sound levels (−71 ± 1 dB re: 1 ms^{-2}; 76 ± 1 dB re: 1 μPa). After determining background levels, stimulus trials across the experimental frequency bandwidth were carried out to construct iso-intensity level responses at various sound levels (Figure 4.1d). All experimental trials were carried out using a custom MATLAB script, which controlled stimulus timing and acquired data, and all data were stored on a desktop computer.

Analyses

Utricular hair cell auditory threshold tuning curves relative to particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa) were determined via input-output measurements of the evoked receptor potentials over the range of tested frequencies (105–1005 Hz) and sound levels (-46.1 – 1.8 dB re: 1 ms^{-2}; 103 – 154 dB re: 1 μPa). The auditory threshold level was defined as the lowest stimulus level that yielded the lowest mean utricular evoked potential that was at least two standard deviations above the background electrical noise measurement. The frequency that evoked the lowest utricular threshold was defined as the characteristic frequency (CF), while the frequency that elicited the highest evoked utricular hair cell potential response was defined as the best frequency (BF). Particle acceleration level (dB re: 1 ms^{-2}) thresholds were calculated as the combined magnitude vector of particle acceleration in dB scale (Eq. 1) (Wysocki et al.

2009; Vasconcelos et al. 2010; Bhandiwad et al. 2017; Vetter 2019; Colleye et al. 2019; Rogers et al. 2020) as follows:

$$\mathrm{dB\ re{:}\ 1\ ms^{-2}} = 20\ \mathrm{Log}_{10}(\sqrt{x^2 + y^2 + z^2}) \qquad \text{(Eq. 1)}$$

For all statistical tests, the significance level was defined at 0.05. To determine if reproductive state plays a role in modulating utricular hair cell auditory thresholds, the effects of reproductive state and stimulus frequency were analyzed via a repeated-measures analysis of variance (ANOVA, between-subject factor: reproductive state, within-subject factor: frequency * reproductive state). Since we were only interested in how reproductive state modulates frequency sensitivity, *a priori* pairwise t-tests compared the frequency-dependent auditory sensitivity of females from different reproductive states across the stimulus frequency bandwidth (105 – 1005 Hz). Additionally, separate two-sample t-tests were performed to determine significant differences between the SL, BM, and GSI of reproductive and non-reproductive fish. All statistical analyses were performed using MATLAB software (MathWorks Inc., Natick, MA, USA).

Results

Auditory evoked potentials were recorded from the utricle of 33 adult female plainfin midshipman fish: 16 non-reproductive females with standard lengths (SL) that ranged from 12.4 – 19.2 cm (15.0 ± 2.2 cm; mean ± SD), body masses (BM) that ranged from 27.3 – 55.9 g (36.1 ± 9.0 g) and gonadosomatic indices (GSI) that ranged from 0.4 – 4.0 (1.8 ± 1.1), and 17 reproductive females with SL that ranged from 11.6 – 20.2 cm (16.2 ± 2.4 cm), BM that ranged from 35.5 – 111.0 g (78.3 ± 16.4 g) and GSI that ranged from 15.2 – 40.6 (31.8 ± 5.9). When comparing the morphometrics of non-reproductive and reproductive female plainfin midshipman, there was no difference in SL (two-sample t-test, $t_{1,31} = -1.499$, $p = 0.144$); however, both BM (two-sample t-test, $t_{1,31} = -9.069$, $p < 0.001$) and GSI (two-sample t-test, $t_{1,31} = -19.916$, $p < 0.001$) were larger in the reproductive females, which is reflective of their reproductive status [i.e., gravid (full of eggs) vs. non-gravid females].

Auditory evoked potentials were recorded from utricular hair cells in response to particle acceleration and sound pressure levels that ranged from -46.1 to 1.8 dB re: 1 ms^{-2} and 103 to 154 dB re: 1 µPa, respectively. Iso-level response profiles of the utricular evoked potentials were generated from the presentation of single tone stimuli that ranged from 105 Hz to 1005 Hz for three sound levels: 154 dB re: 1 μPa (-0.8 – 10.8 dB re: 1 ms^{-2}), 142 dB re: 1 μPa (-12.9 – -0.9 dB re: 1 ms^{-2}), and 130 dB re: 1 μPa (-25.9 – -12.5 dB re: 1 ms^{-2}), which corresponded to a broad range of sound levels that have been recorded in the nest of calling type I males (i.e., 154 – 161 dB re: 1 μPa) (Vetter et al. 2019; Rogers and Sisneros 2020) and recorded at or within 1 m of a calling type I males nest (i.e., 130 – 142 dB re: 1 μPa) (Feng and Bass 2016; Vetter et al. 2019; Balebail and Sisneros 2022) [see Figure S4.2 for details regarding the frequency-specific particle acceleration levels (dB re: 1 ms^{-2}) for the three sound pressure levels: 154, 142, and 130 dB re: 1 μPa; https://figshare.com/s/e17c71d36f441dce06e4]. Figure 4.2 displays representative utricular evoked iso-level response curves of non-reproductive and reproductive females in response to the bandwidth of tested frequencies (105 – 1005 Hz) at 154, 142, and 130 dB re: 1 μPa. Iso-level response curves consisted of profiles that had best frequencies (BFs, defined as the frequency that evoked the greatest utricular evoked potential magnitude at a given iso-level) ranging from 105 – 205 Hz in non-reproductive and reproductive females. Both non-reproductive and reproductive females had median BFs of 145 Hz at each of the sound levels tested, with no difference in the median BFs observed between non-reproductive and reproductive females at 154 dB re: 1 μPa (0.4 dB re: 1 ms^{-2}) (Friedman test, $\chi^2 = 0$, df = 1, $p = 1$), 142 dB re: 1μPa (11.6 dB re: 1 ms^{-2}) (Friedman test, $\chi^2 = 0.2$, df = 1, $p = 0.6547$), and 130 dB re: 1μPa (23.6 dB re: 1 ms^{-2}) (Friedman test, $\chi^2 = 0$, df = 1, $p = 1$).

The magnitude of the auditory evoked potentials recorded from utricular hair cells in response to pure tone stimuli was greater in reproductive females compared to non-reproductive females. Figure 4.3 illustrates the mean iso-level response profiles of the evoked utricular potentials from non-reproductive and reproductive females in response to pure tones (105–1005 Hz) at 154, 142, and 130 dB re: 1 µPa. Reproductive females had significantly higher evoked utricular potentials than non-reproductive females within sound levels encompassing a range of biologically relevant sound levels [One-way repeated measures ANOVA, between-subject factor: reproductive state) at 154 dB re: 1 μPa ($F_{1, 912} = 235.4$, $p < 0.001$), 142 dB re: 1 μPa ($F_{1, 912} = 247.0$, $p < 0.001$), and 130 dB re: 1 μPa ($F_{1, 912} = 166.5$, $p < 0.001$)], and exhibited a significant interaction of frequency and reproductive state at 154 dB re: 1 μPa ($F_{1, 15} = 12.0$, $p < 0.001$), 142 dB re: 1 μPa ($F_{1, 15} = 16.7$, $p < 0.001$), and 130 dB re: 1 μPa ($F_{1, 15} = 19.5$, $p < 0.001$). Additionally, frequency-specific differences in the evoked magnitude response of the utricular hair cells were also observed between non-reproductive and reproductive females within each sound level tested (*a prior* t-tests for pair comparisons were used to determine frequency-specific differences in utricular potentials). The magnitudes of evoked utricular potentials were greater in reproductive females compare to non-reproductive females at frequencies ≤505 Hz at 154 dB re: 1μPa ($p<0.05$; see table S4.1; https://figshare.com/s/e17c71d36f441dce06e4), ≤ 805 Hz at 142 dB re: 1 μPa ($p < 0.05$; see table S4.2; https://figshare.com/s/e17c71d36f441dce06e4), and ≤ 305 Hz at 130 dB re: 1 μPa ($p < 0.05$; see table S4.3; https://figshare.com/s/e17c71d36f441dce06e4). The greatest evoked utricular potential magnitude change with respect to differences in reproductive state occurred at 105 Hz and 125 Hz at a sound pressure level of 130 dB re: 1 μPa (particle acceleration level at 105 Hz = -20.9 dB re: 1 ms^{-2} and 125 HZ = -23.2 dB re: 1 ms^{-2}); at this sound pressure level reproductive females had evoked potentials that were 6.3 and 6.2 times greater than in non-reproductive females, respectively (see table S4.3; https://figshare.com/s/e17c71d36f441dce06e4). In sum, reproductive females exhibited greater evoked utricular potentials than non-reproductive females across the frequency bandwidth tested here, with mean magnitudes that were 2.2, 2.7, and 4.1 times greater at sound pressure levels of 154 dB re: 1 μPa (for frequencies ≤ 505 Hz), 142 dB re: 1 μPa (frequencies ≤ 805 Hz) and 130 dB re: 1 μPa (frequencies ≤ 305 Hz), respectively (see tables S4.1-3; https://figshare.com/s/e17c71d36f441dce06e4).

Auditory threshold curves based on particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa) were constructed from the evoked utricular potential recordings. Figure 4.4 illustrates representative non-reproductive and reproductive female auditory threshold curves based on particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa). In general, the utricular auditory threshold tuning curves of both non-reproductive and reproductive females exhibited the lowest thresholds at frequencies ≤ 205 Hz and steadily increased to the highest thresholds at frequencies ≥ 705 Hz. Characteristic frequencies (CFs, defined as the frequency that evoked the lowest utricular threshold) for non-reproductive females ranged from 105 to 205 Hz (median CF = 105 Hz and 145 Hz based on particle acceleration and sound pressure level tuning profiles, respectively), while for reproductive females CFs ranged from 105 to 185 Hz (median CF = 105 Hz based on both particle acceleration and sound pressure level tuning profiles). The CFs based on particle acceleration did not differ with respect to reproductive state (Friedman test, $\chi^2 = 0.2$, df = 1, $p = 0.6547$); however, the CFs based on sound pressure were lower in reproductive females when compared to non-reproductive females (Friedman test, $\chi^2 = 6$, df = 1, $p = 0.01431$).

The threshold tuning curves of non-reproductive and reproductive females relative to particle acceleration (dB re: 1 ms^{-2}) and sound pressure (dB re: 1 μPa) levels are summarized in figure 4.5. In general, for females of both reproductive states, the lowest utricular thresholds occurred at the lowest frequency tested (i.e., 105 Hz) (non-reproductive females: mean particle acceleration level threshold = -28.9 ± 1.7 dB re: 1 ms^{-2}, mean sound pressure level threshold = 121.5 ± 1.7 dB re: 1 μPa; reproductive females: mean particle acceleration level threshold = -36.5 ± 1.9 dB re: 1 ms^{-2}; mean sound pressure level threshold = 113.4 ± 1.9 dB re: 1 μPa), while the highest auditory threshold levels occurred between 705 Hz to 1005 Hz (non-reproductive females: mean particle acceleration level threshold range = -4 to -1 dB re: 1 ms^{-2}, mean sound pressure level threshold range = 150 to 153 dB re: 1 μPa; reproductive females: mean particle acceleration level threshold range = -8 to -3 dB re: 1 ms^{-2}; mean sound pressure level threshold range = 148 to 151 dB re: 1 μPa). The auditory thresholds were lower (i.e., more sensitive) in reproductive females than in non-reproductive females (One-way repeated measures ANOVA, between-subject factor: reproductive state, particle acceleration level: $F_{1,\,893} = 472.6$, $p < 0.001$, sound pressure level: $F_{1,\,893} = 473.6$, $p < 0.001$) and a significant interaction was observed between reproductive state and frequency (One-way repeated measures ANOVA, within-subject factor: frequency * reproductive state, particle acceleration level: $F_{1,15} = 3.5$, $p < 0.001$, sound pressure level: $F_{1,15} = 3.7$, $p < 0.001$). Furthermore, frequency-specific differences in auditory thresholds were observed between non-reproductive and reproductive females with reproductive females being more sensitive than non-reproductive females at frequencies from 105 to 805 Hz (*a priori* t-tests for pairwise comparisons of non-reproductive and reproductive females across frequency, $p < 0.001$).

Discussion

The goal of this study was to determine whether seasonal changes in reproductive state modulate the auditory sensitivity of the utricle in female plainfin midshipman. We show that the utricular hair cells of reproductive females exhibit up to a 6-fold magnitude increase in their evoked response to auditory stimuli and have particle acceleration thresholds that are 7-10 dB re: 1 ms^{-2} lower (i.e., more sensitive) than non-reproductive females across a frequency bandwidth that includes the dominant frequencies contained within type I male vocalizations. To our knowledge, this is the first study to demonstrate reproductive state-dependent plasticity of the frequency sensitivity and auditory gain in the teleost utricle, an inner ear end organ not often associated with auditory function. In this discussion, we consider how changes in midshipman utricular auditory sensitivity may facilitate acoustic communication during social and reproductive behaviors.

Auditory sensitivity of the midshipman utricle

In mammals, the utricle primarily serves a vestibular function as it detects linear acceleration, senses horizontal translational movements, and plays an important role in static balance. However, in teleost fishes, the utricle is one of three inner ear otolithic end organs (along with the saccule and lagena) that acts as an inertial accelerometer and responds to direct displacement by acoustic particle motion and linear accelerations primarily in the horizontal plane (De Vries 1950; Fay 1984; Popper and Fay 1993). While the saccule and lagena are most often implicated in sound detection and directional hearing (Sand 1974; Sisneros and Rogers 2016; Hawkins and Popper 2018), the utricle is posited to serve primarily a vestibular role functioning to detect head/body position relative to gravity (i.e., acts as a gravistatic organ) (Riley and Moorman 2000; Inoue et al. 2013; Boyle et al. 2018).

In our current study, we show that the female midshipman utricle, especially in the reproductive state, is sensitive to a broad range of acoustic frequencies with a relatively high gain in particle acceleration sensitivity (dB re: 1 ms^{-2}) from 105 to 1005 Hz (Figure 4.5). Our results confirm previous studies, which showed that the utricle in batrachoid fishes (toadfishes and midshipman) serves an auditory function and is capable of detecting behaviorally-relevant acoustic stimuli (Maruska and Mensinger 2015; Rogers and Sisneros 2020). Further support for the utricle of batrachoids serving an auditory function is the neuroanatomical evidence provided by Highstein et al. (1992) and Sisneros et al. (2002). Highstein et al. (1992) showed that utricular afferents in toadfish project to the rostral “finger” and dorsolateral aspect of the hindbrain descending octaval nucleus (DON), while Sisneros et al. (2002) showed that the midshipman utricle has extensive projections to the intermediate and rostral intermediate auditory zones of the hindbrain DON; note that the rostral “fingerlike” extension described by Highstein et al. (1992) is similar in position and extent to the rostral intermediate zone of the midshipman DON, as described by Bass et al. (2000). Furthermore, Sisneros et al. (2002) showed via transneuronal labeling that the principal cells in the midshipman DON that receive input from utricular afferents subsequently project centrally to terminals in the auditory region of the midbrain torus semicircularis similar to the saccule. Taken together, these physiological and neuroanatomical studies in batrachoid fishes strongly suggest that the utricle serves an auditory function and can detect biologically relevant acoustic stimuli.

Seasonal auditory plasticity of the utricle

We show that female utricular hair cells exhibit seasonal, reproductive state-dependent changes in evoked responses to auditory stimuli (Figure 4.3), such that reproductive females exhibit greater evoked utricular potentials compared to non-reproductive females (Tables S4.1-3; https://figshare.com/s/e17c71d36f441dce06e4). The greatest difference in evoked potential magnitude relative to reproductive state occurred at 105 Hz and a sound pressure level of 130 dB re: 1 μPa (-20.9 dB re: 1 ms^{-2}) such that reproductive females displayed average utricular potentials that were approximately 6.3 times greater than in non-reproductive females. Reproductive state-dependent changes in saccular evoked potential magnitude have previously been examined in reproductive females, which have average evoked potentials approximately 7.4 times greater than in non-reproductive females at 105 Hz and a sound pressure level of 130 dB re: 1 μPa [Sisneros unpublished data; (Sisneros 2009b)]. One hypothesis for these changes in the magnitude of the hair-cell evoked potentials may, in part, be related to seasonal increases in hair cell density. Coffin et al. (2012) showed that reproductive female midshipman exhibit a 13% increase in saccular hair cell density, which was paralleled by a dramatic increase in the magnitude of evoked saccular potentials. However, reproductive females and type I males do not exhibit reproductive state-dependent changes in the hair cell density of the utricle (Coffin et al. 2012; Lozier and Sisneros 2019), yet, reproductive females exhibit seasonal changes in the magnitude of evoked utricular potentials. Indeed, seasonal changes in saccular potential magnitude in reproductive females may still be related to the saccular-specific hair cell addition and may explain, in part, some of the evoked potential differences between the saccule and utricle (i.e., the utricle having ~ a 6.3-fold increase vs the saccule having a ~ 7.4-fold increase). An alternative, but not mutually exclusive, hypothesis for the change in the magnitude of hair cell potentials may be due to reproductive state-dependent changes in ion channel expression and the current kinetics of hair cells in the utricle and saccule [see (Rohmann et al. 2013)]. Future studies that characterize the ion channel current kinetics of hair cells in non-reproductive and reproductive females may provide insight into the mechanism responsible for the reproductive state-dependent changes in the magnitude of evoked potentials in the midshipman utricle and saccule.

Concurrent with the dramatic increase in utricular potential magnitude, we also observed a remarkable increase in the utricular auditory sensitivity of reproductive females when compared to non-reproductive females. The greatest change in utricular auditory sensitivity occurred from 105–505 Hz (Figure 4.5), with reproductive females exhibiting particle acceleration thresholds that were 7-10 dB (re: 1 ms^{-2}) lower than non-reproductive females (an increase in sensitivity equal to approximately 2-3 times) (Figure 4.6a). This reproductive state-dependent increase in female auditory sensitivity corresponds with the dominant frequencies contained within type I male vocalizations, which include grunts, growls, and advertisement calls or “hums” (Figure 4.6b). Grunts are short duration (50-200 ms), broadband acoustic signals that are produced either individually or in a series of “trains” (Figure 4.6b, *bottom*), whereas growls are longer duration (> 1s) broadband signals (Figure 4.6b, *middle*). In general, these vocalizations are produced in an agonistic context to fend off potential rivals/intruders and during nest defense (Sisneros 2009c). In contrast, hums are long duration (up 2 hrs in captive conditions) multiharmonic acoustic courtship signals that have fundamental frequencies ranging from 80 to 102 Hz (Bass et al. 1999; Feng and Bass 2016; Balebail and Sisneros 2022). In comparison to broadband grunts and growls, which have much of their spectral energy at frequencies <600 Hz, hums have prominent harmonics ranging up to ~500 Hz, with additional lower amplitude harmonics ranging up to 1000 Hz (see Figure 4.6b, *top*). Together, our results suggest the utricle of reproductive females is better adapted than in non-reproductive females to detect the dominant spectral energy contained within midshipman social acoustic signals (hums, growls, and grunts), which correspond to frequencies < 600 Hz (Figure 4.6b). Thus, reproductive state-dependent changes in utricular auditory sensitivity may represent an adaptive auditory plasticity that complements the saccular auditory sensitivity of reproductive female midshipman (Figure S.3; https://figshare.com/s/e17c71d36f441dce06e4) and helps facilitate midshipman social and reproductive acoustic communication.

Potential mechanisms for utricular auditory plasticity

The observed changes in utricular auditory sensitivity are likely due to seasonal changes in circulating gonadal steroids (androgens and estrogens), which are related to seasonal changes in midshipman reproductive state (Sisneros et al. 2004b). Saccular afferents in non-reproductive females treated with either testosterone or 17β-estradiol exhibit enhanced frequency sensitivity and phase-locking accuracy to higher frequencies within the midshipman hearing range, which effectively enhances acoustic communication (Sisneros et al. 2004a). Concurrent with reproductive state-dependent changes in gonadal steroid levels are parallel changes in the large-conductance, calcium-activated potassium (BK) channels, which are responsible for the rapid outward currents that contribute to the electrical resonance and low-frequency (<1 kHz) tuning of hair cells in non-mammalian vertebrates (Lewis and Hudspeth 1983; Roberts et al. 1988; Fettiplace and Fuchs 1999). Rohmann et al. (2013) demonstrated that saccular hair cells of reproductive midshipman exhibit increased expression of calcium-activated BK channels, which is correlated with enhanced higher frequency sensitivity (>145 Hz) and that pharmacological inhibition of BK channels results in decreased saccular sensitivity similar to non-reproductive fish. Together, these studies suggest that gonadal steroids may modulate seasonal changes in frequency sensitivity via the regulation of hair cell BK channel expression to effectively enhance auditory sensitivity for social acoustic communication.

In addition, reproductive state-dependent changes in dopaminergic efferent projections to the inner ear may also be responsible for the observed seasonal, reproductive state-dependent changes in utricular sensitivity. Previous work by Forlano et al. (2015) showed that dopaminergic innervation of the saccule varied with reproductive state such that reproductive females have a seasonal reduction in dopaminergic input. Furthermore, Perelmuter et al. (2019) showed that dopamine decreases saccular auditory sensitivity via a D2-like receptor and that D2a receptor expression is reduced in the saccule during the midshipman breeding season. Perelmuter et al. (2019) also found that saccular auditory sensitivity is modulated by the dopaminergic efferent system, whereby a release in inhibition effectively mimics the reproductive auditory phenotype and enhances peripheral encoding of social acoustic signals. Furthermore, Perelmuter et al. (2021) recently showed that testosterone treatment mimics the seasonal downregulation of dopamine in the midshipman saccule, which provides evidence that steroid regulation of peripheral auditory sensitivity is mediated, at least in part, by dopamine. Future studies that examine similar reproductive state-dependent, gonadal steroid regulatory mechanisms of hair cell ion channel expression and dopaminergic innervation to the utricle will be instrumental in understanding the neuroendocrine basis of peripheral auditory sensitivity modulation in midshipman fish and other vertebrates, including mammals.

Conclusion

The utricle in mammals primarily serves as a vestibular organ for detecting linear acceleration and sensing translational movements in the horizontal plane. However, in fishes, the utricle is one of three inner ear otolithic end organs (saccule, utricle, and lagena) that act as biological accelerometers and respond to acoustic particle motion and horizontal linear accelerations. While, to some degree, all three otolithic end organs in teleost fishes are posited to serve both an auditory and vestibular function, the teleost utricle is often thought to primarily serve a vestibular function. Here, we show that the utricle in the vocal plainfin midshipman serves an auditory function that is seasonally plastic and modulated by the animal's reproductive state, effectively enhancing the utricle's auditory sensitivity to conspecific acoustic signals. Whether these seasonal-dependent changes extend beyond the auditory system to the vestibular system has yet to be assessed and should be considered in future vestibular research, given the multimodal function of the inner ear end organs.

Conflict of interest: The authors declare no competing financial interests.

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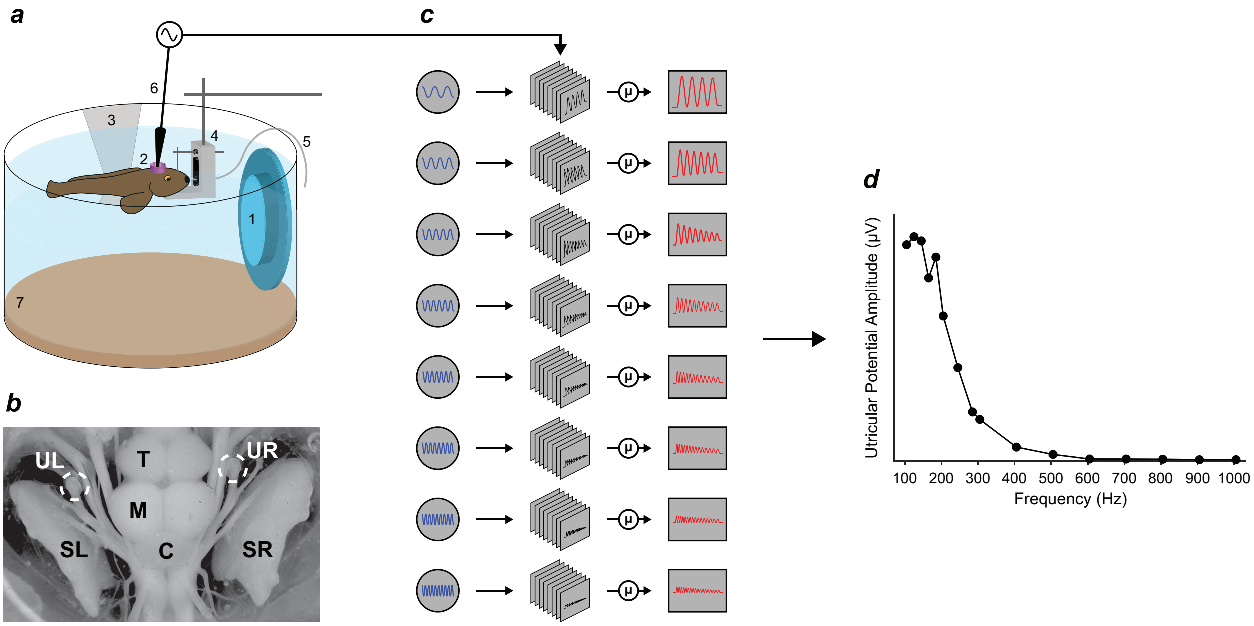

Figure 4.1: Experimental design. (*a*) Schematic of experimental physiology tank. For all physiology experiments, fish were suspended 4 cm below the water's surface and positioned such that the face of the underwater speaker was 10 cm rostral of the otic capsule. Labels are as follows: *1* underwater speaker, *2* hydrophobic water dam, *3* parafilm suspension, *4* head holder, *5* respiration tube, *6* glass microelectrode, and *7* rocky sediment. Physiology tank dimensions: 40 cm diameter and 20 cm water depth. (*b*) Dorsal view of midshipman cranial cavity. The dashed circles indicate the position of the left (UL) and right (UR) inner ear utricle. Abbreviations are as follows: *SL* and *SR* left, and right saccule, respectively, *T* telencephalon, *M* midbrain, *C* cerebellum. (*c*) For all experiments, pure tone acoustic stimuli (right; 500 ms duration; 8 repetitions) were delivered via an underwater speaker and evoked hair cell responses were recorded to each acoustic stimulus presentation. Consecutive utricular hair cell evoked responses (middle) were averaged across stimulus frequency, and frequency-dependent averaged output signals (right) were used to construct (*d*) iso-intensity level response curves.

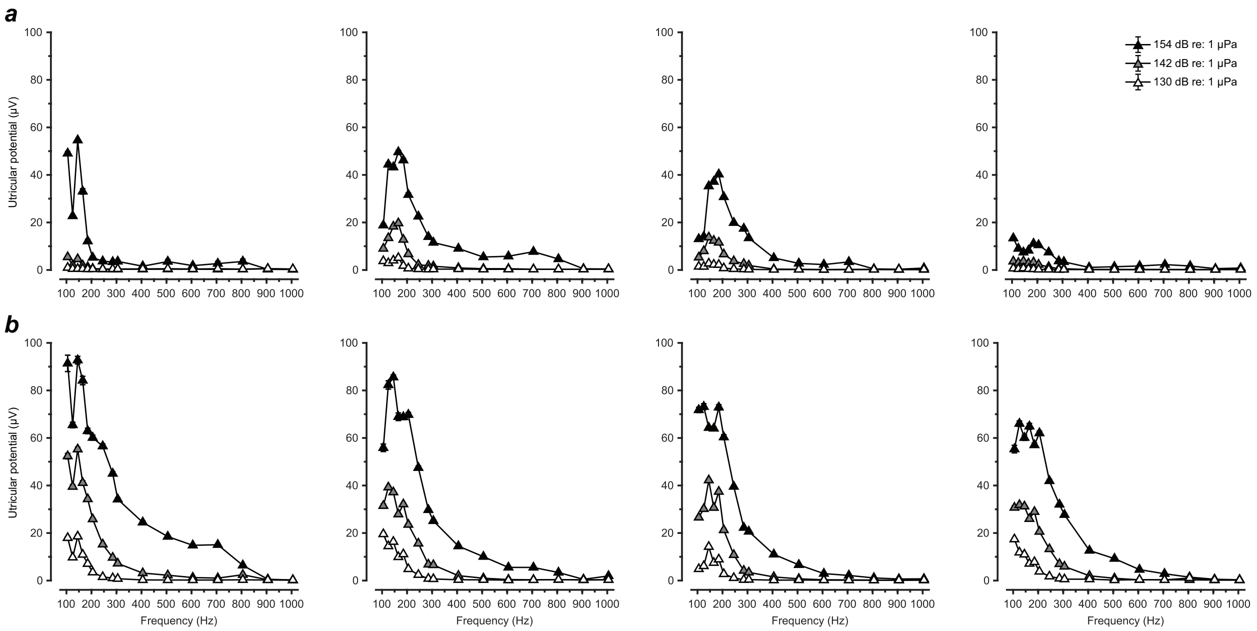

Figure 4.2: Representative examples of iso-intensity level curves recorded from utricular hair cells of (*a*) non-reproductive and (*b*) reproductive female plainfin midshipman. Iso-intensity responses were recorded in response to single tone playbacks at sound pressure levels of 154 (black), 142 (gray), and 130 (white) dB re: 1 µPa. Data are represented as mean ± 1 SD; note that some error bars are minimal, and the symbols may obscure the bars.

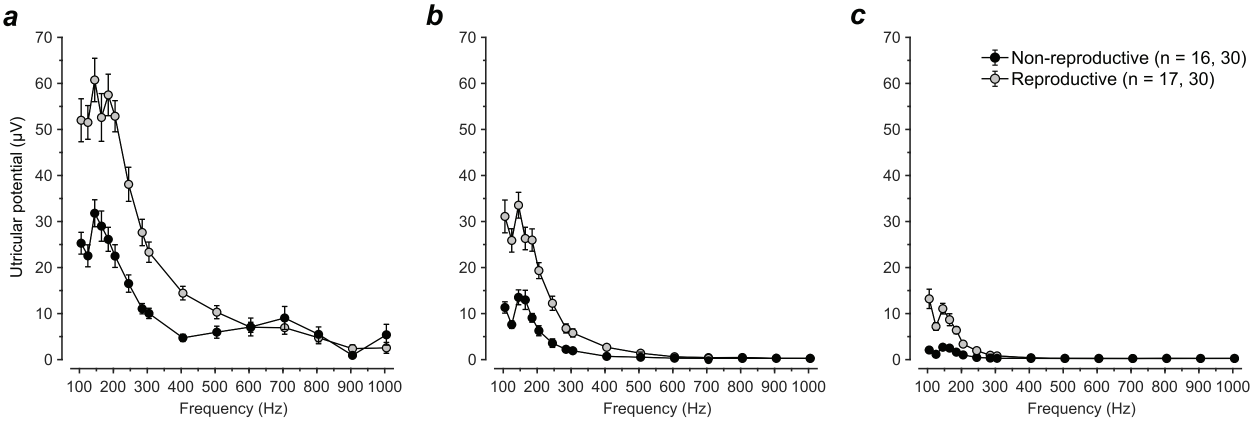

Figure 4.3: Iso-level response curves recorded from utricular hair cells of non-reproductive (black) and reproductive (gray) female plainfin midshipman in response to single tone playbacks at sound pressure levels of (*a*) 154, (*b*) 142, and (*c*) 130 dB re: 1 µPa. Data are represented as mean ± 1 SE; note that some error bars are minimal, and the symbols may obscure the bars. The number of animals and records for each group is indicated in parentheses.

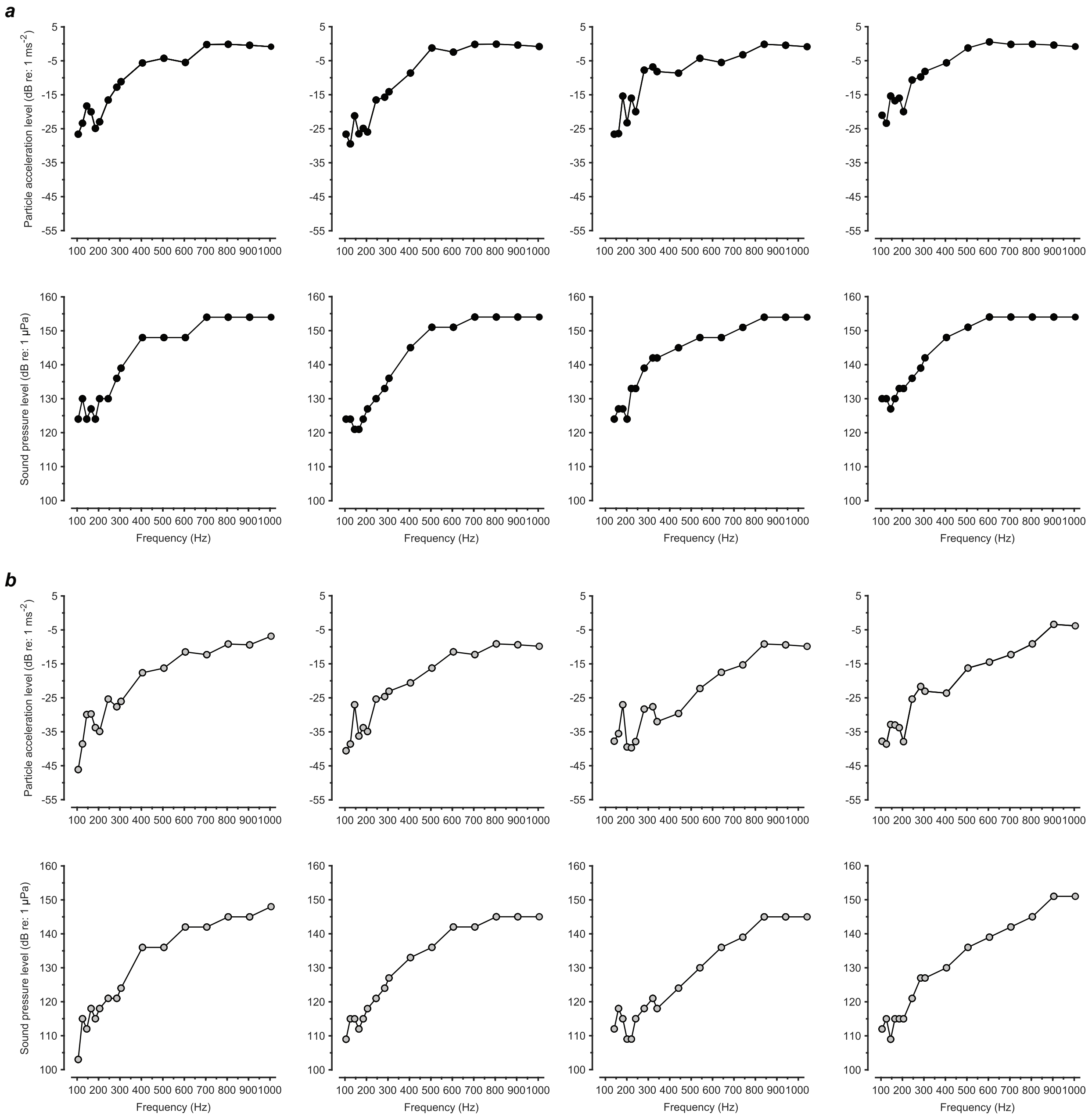

Figure 4.4: Representative (top) particle acceleration (dB re: 1 ms^{-2}) and (bottom) sound pressure (dB re: 1 µPa) level auditory threshold tuning curves recorded from (a) non-reproductive (black) and (b) reproductive (gray) female plainfin midshipman. Tuning curves were constructed using utricular hair cell evoked responses, with thresholds defined as the lowest sound pressure level (dB re: 1 µPa) needed to evoke a utricular potential at least 2 SD above the background electrical noise level.

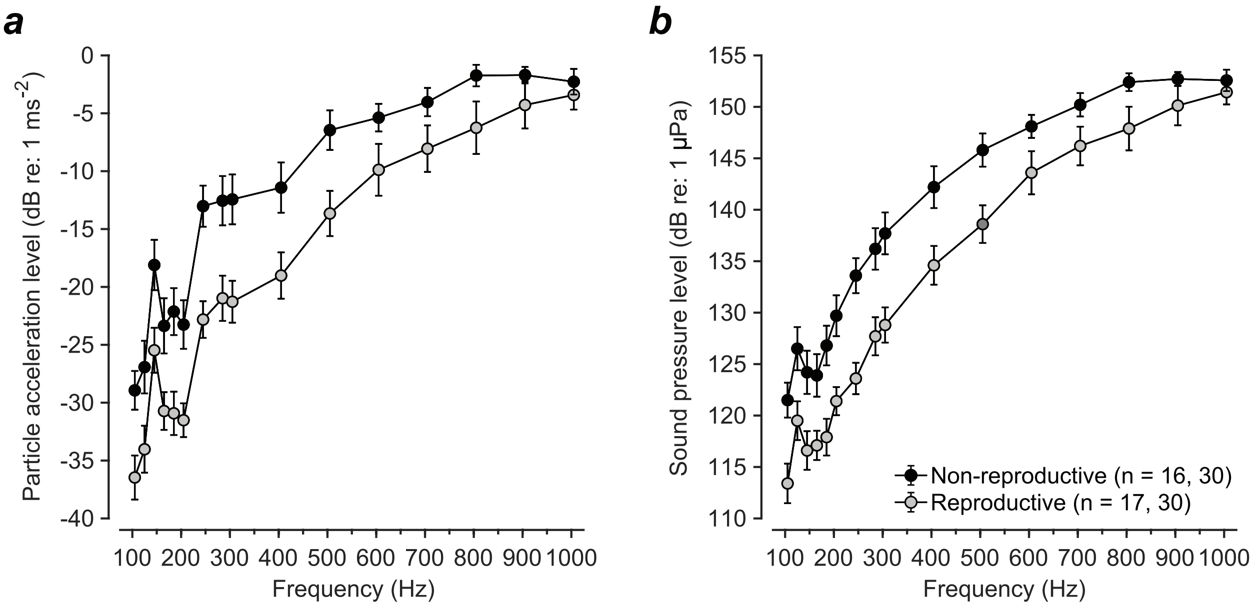

Figure 4.5: (*a*) Particle acceleration (dB re: 1 ms^{-2}) and (*b*) sound pressure (dB re: 1 μPa) level auditory threshold tuning curves recorded from non-reproductive (black) and reproductive (gray) female midshipman utricular hair cells. The auditory thresholds were defined as the lowest auditory stimulus level needed to evoke utricular potentials at least 2 SD above the background electrical noise level. All data are plotted as mean ± 95% confidence interval. The number of animals and records for each group is indicated in parentheses.

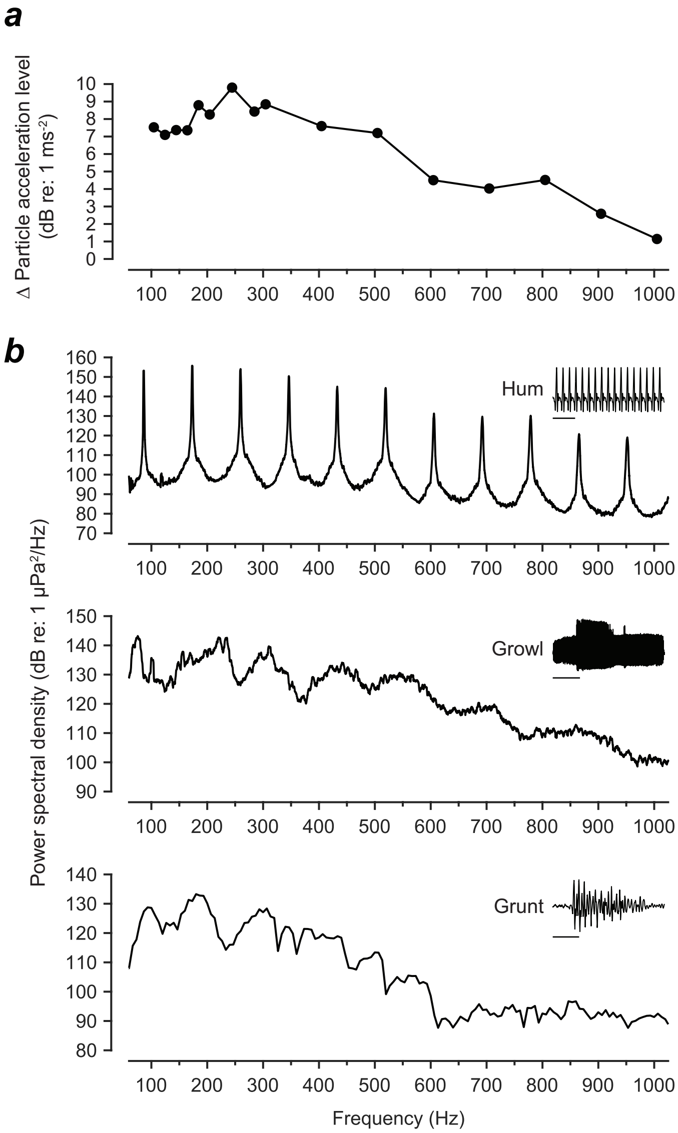

Figure 4.6: Comparison of female midshipman particle motion (dB re: 1 ms^{-2}) sensitivity and male midshipman vocalizations. *a)* Utricular hair cell particle acceleration threshold difference (Δ dB re: 1 ms^{-2}) between reproductive and non-reproductive female midshipman. *(b)* Power spectral density (dB re: 1 μPa^2/Hz) curves of a male midshipman hum (top), growl (middle), and grunt (bottom). *Inset:* Waveform of male midshipman hum, growl, and grunt, respectively. Scale bars represent 5, 1000, and 50 ms, respectively. All vocalizations were recorded from a reproductive type I male midshipman housed in a large, indoor concrete tank (3 m diameter; 14.1 °C) at the University of Washington Friday Harbor Laboratories. Source-level recordings were made using a mini-hydrophone placed directly in front of the entrance of an artificial nest.

Table S4.1: Summary of mean evoked potentials (μV) recorded from utricular hair cells of non-reproductive and reproductive midshipman at 154 dB re. 1 μPa. Non-reproductive and reproductive female evoked potential (μV) responses are displayed as mean ± SE Fold change was calculated as the ratio of the frequency-specific mean evoked response (μV) of reproductive females relative to non-reproductive female midshipman. Non-reproductive and reproductive frequency-specific mean evoked potentials (μV) were compared via *a priori* t-tests, and the *p*-values are shown. *Significant differences between the two groups (*p*<0.05)

	Evoked potential (μV)			
Frequency (Hz)	Non-reproductive	Reproductive	Fold change	*p*-value
105	25.3 ± 2.4	52.0 ± 4.7	2.1	<0.001*
125	22.6 ± 2.4	51.5 ± 3.7	2.3	<0.001*
145	31.8 ± 2.9	60.7 ± 4.7	1.9	<0.001*
165	29.0 ± 3.3	52.6 ± 5.2	1.8	<0.001*
185	26.1 ± 2.6	57.5 ± 4.5	2.2	<0.001*
205	22.5 ± 2.5	52.9 ± 3.4	2.4	<0.001*
245	16.5 ± 1.9	38.1 ± 3.7	2.3	<0.001*
285	11.1 ± 1.1	27.6 ± 2.9	2.5	<0.001*
305	10.0 ± 1.1	23.3 ± 2.2	2.3	<0.001*
405	4.7 ± 0.8	14.4 ± 1.5	3.1	<0.001*
505	6.0 ± 1.3	10.3 ± 1.4	1.7	0.029*
605	7.1 ± 2.0	7.0 ± 1.1	1.0	0.974
705	9.1 ± 2.5	6.9 ± 1.4	0.8	0.462
805	5.5 ± 1.6	4.8 ± 1.3	0.9	0.721
905	0.9 ± 0.2	2.4 ± 0.8	2.6	0.094
1005	5.4 ± 2.3	2.6 ± 1.2	0.5	0.268

Table S4.2: Summary of mean evoked potentials (μV) recorded from utricular hair cells of non-reproductive and reproductive midshipman at 142 dB re. 1 μPa. Non-reproductive and reproductive female evoked potential (μV) responses are displayed as mean ± SE Fold change was calculated as the ratio of the frequency-specific mean evoked response (μV) of reproductive females relative to non-reproductive female midshipman. Non-reproductive and reproductive frequency-specific mean evoked potentials (μV) were compared via *a priori* t-tests and the *p*-values are shown. *Significant differences between the two groups ($p<0.05$).

	Evoked potential (μV)			
Frequency (Hz)	Non-reproductive	Reproductive	Fold change	*p*-value
105	11.3 ± 1.2	31.1 ± 3.6	2.7	<0.001*
125	7.6 ± 0.8	25.9 ± 2.5	3.4	<0.001*
145	13.5 ± 1.6	33.5 ± 2.8	2.5	<0.001*
165	13.0 ± 2.1	26.3 ± 2.4	2.0	<0.001*
185	9.1 ± 0.9	26.0 ± 2.4	2.9	<0.001*
205	6.3 ± 1.1	19.3 ± 1.7	3.1	<0.001*
245	3.6 ± 0.9	12.2 ± 1.5	3.4	<0.001*
285	2.2 ± 0.6	6.8 ± 1.0	3.0	<0.001*
305	1.9 ± 0.6	5.8 ± 0.9	3.0	<0.001*
405	0.7 ± 0.2	2.7 ± 0.5	3.7	<0.001*
505	0.5 ± 0.1	1.4 ± 0.3	2.7	0.004*
605	0.3 ± 0.1	0.6 ± 0.1	2.0	0.008*
705	0.3 ± 0.03	0.4 ± 0.1	1.6	0.022*
805	0.3 ± 0.03	0.5 ± 0.1	1.8	0.042*
905	0.3 ± 0.03	0.3 ± 0.03	1.1	0.606
1005	0.3 ± 0.03	0.3 ± 0.02	1.0	0.786

Table S4.3: Summary of mean evoked potentials (μV) recorded from utricular hair cells of non-reproductive and reproductive midshipman at 130 dB re. 1 μPa. Non-reproductive and reproductive female evoked potential (μV) responses are displayed as mean ± SE Fold change was calculated as the ratio of the frequency-specific mean evoked response (μV) of reproductive females relative to non-reproductive female midshipman. Non-reproductive and reproductive frequency-specific mean evoked potentials (μV) were compared via *a priori* t-tests and the *p*-values are shown. *Significant differences between the two groups ($p<0.05$).

	Evoked potential (μV)			
Frequency (Hz)	Non-reproductive	Reproductive	Fold change	*p*-value
105	2.1 ± 0.3	13.2 ± 2.1	6.3	<0.001*
125	1.2 ± 0.2	7.2 ± 0.9	6.2	<0.001*
145	2.7 ± 0.5	11.1 ± 1.2	4.1	<0.001*
165	2.5 ± 0.6	8.7 ± 1.3	3.5	<0.001*
185	1.6 ± 0.3	6.4 ± 0.8	3.9	<0.001*
205	1.0 ± 0.4	3.4 ± 0.5	3.3	<0.001*
245	0.5 ± 0.1	2.0 ± 0.4	4.1	0.001*
285	0.3 ± 0.1	1.0 ± 0.2	2.8	0.011*
305	0.3 ± 0.04	0.8 ± 0.2	2.7	0.008*
405	0.3 ± 0.03	0.4 ± 0.1	1.6	0.068
505	0.3 ± 0.02	0.3 ± 0.03	1.1	0.426
605	0.2 ± 0.02	0.3 ± 0.02	1.2	0.258
705	0.3 ± 0.02	0.2 ± 0.02	1.0	0.916
805	0.2 ± 0.02	0.3 ± 0.03	1.2	0.232
905	0.3 ± 0.02	0.3 ± 0.01	1.0	0.968
1005	0.3 ± 0.03	0.3 ± 0.02	1.0	0.697

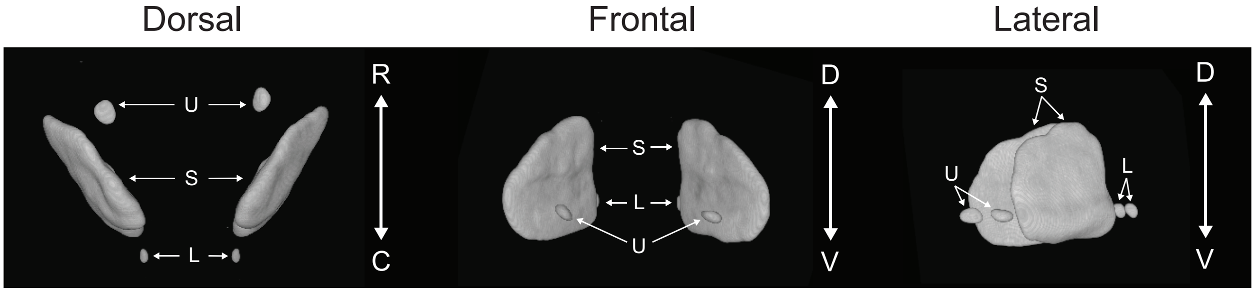

Figure S4.1: Midshipman inner ear morphology. μCT reconstruction of midshipman otolithic end organs, which illustrates variation in otolithic end organ orientation. S, L, and U represent saccule, lagena, and utricle, respectively. μCT data was obtained and adapted from the Virtual Natural History Museum (http://131.220.133.140/VNHM/).

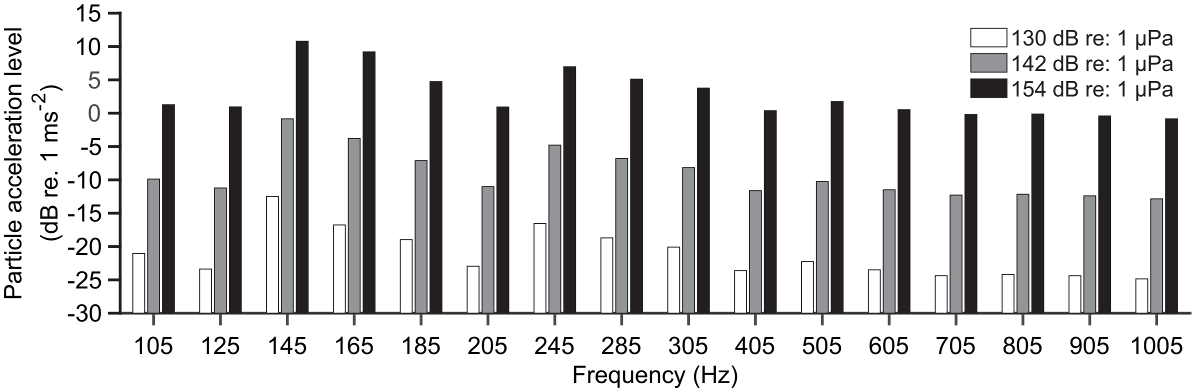

Figure S4.2: Frequency-specific particle acceleration levels (dB re: 1 ms^{-2}) for three representative sound levels [130 (white), 142 (gray), and 154 dB re: 1 μPa (black)]. Particle acceleration level (dB re: 1 ms^{-2}) measurements were measured using a neutrally buoyant waterproofed triaxial accelerometer (Model VW3567A12; Sensitivity at 100 Hz: 10.42 mV/ms^{-2} (*x*-axis), 10.03 mV/ms^{-2} (*y*-axis), 10.37 mV/ms^{-2} (*z*-axis); PCB Piezotronics, Depew, NY, USA) that connected to a signal conditioner (gain = ×100/axis; Model: 482A16; PCB Piezotronics, Depew, NY, USA). The accelerometer was positioned 10 cm perpendicular to the face of the underwater speaker and 4 cm below the water's surface to coincide with the position of the midshipman inner ear during auditory evoked hair cell potential measurements. Particle motion at each tested frequency was acquired using a National Instruments data acquisition system (Model: NI USB-6009, National Instruments, Austin, TX, USA) and visualized using LabVIEW software (National Instruments, Austin, TX, USA). Using a custom LabVIEW (National Instruments, Austin, TX, USA) script, particle motion amplitude measurements (V_{pk-pk}) for each axis (*x*-, *y*-, and *z*-axis) were corrected for the gain (sensitivity) of the accelerometer and then calculated as the combined magnitude vector of particle acceleration in dB scale (Eq. 1).

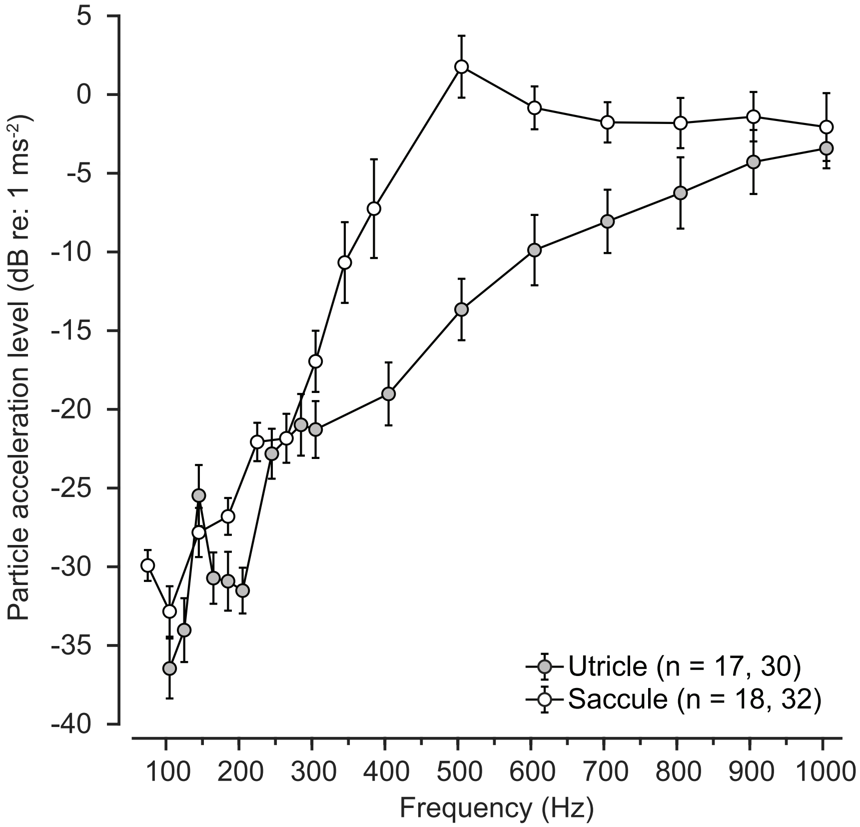

Figure S4.3: Comparison of reproductive female utricular and saccular auditory sensitivity. Particle acceleration level (dB re: 1 ms^{-2}) threshold curve of utricular (gray) and saccular (white) hair cells from summer reproductive female plainfin midshipman. The auditory thresholds were defined as the lowest auditory stimulus level needed to evoke utricular potentials at least 2 SD above the background electrical noise level. All data are plotted as mean ± 95% confidence interval. The number of animals and records for each group is indicated in parentheses. Saccular hair cell data has been adapted from Colleye et al., 2019.

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

This dissertation describes the auditory sensitivity of the midshipman utricular hair cells and adaptations that enhance utricular hair cell auditory sensitivity. I used the well-characterized auditory evoked hair cell potential recording technique and surgical manipulations to conduct this research. The final chapter of this dissertation will briefly summarize each chapter's findings and discusses future research directions for exploring the mechanisms of fish hearing.

Auditory role of the midshipman utricle

In chapter 2, I characterized the evoked potential response of utricular hair cells in nonreproductive type I male midshipman to test the hypothesis that the utricle is sensitive to acoustic stimuli. I showed that the utricle is highly sensitive to a broad range of behaviorally relevant particle motion stimuli along the horizontal plane. In addition, I showed that the utricle is as sensitive as male saccular hair cells to low frequencies (≤305 Hz) and lagenar hair cells at higher frequencies (>605 Hz).

Previous work by Vetter et al. (2019) suggests that the relatively high thresholds of the lagena may be important for the detection of high-intensity sound levels associated with behaviorally relevant acoustic stimuli close to a sound source when the saccule is likely overstimulated. In contrast, the sensitivity of the utricle in nonreproductive type I males was markedly similar to the saccule at frequencies ≤ 305 Hz. The observation that utricular and saccular hair cells of male midshipman exhibit similar auditory sensitivity is rather surprising, given that it has been assumed that the saccule is the main organ for hearing in most fishes.

Sound pressure sensitivity

In chapter 3, I utilized surgical manipulations of the swim bladder to show that removal of the swim bladder results in a 3 – 12 dB re: 1 μPa decrease in auditory sensitivity and decreases the bandwidth of detectable frequencies. Given this adaptive function, we propose, similar to the auditory end organs of female midshipman, that utricular sound pressure sensitivity functions to effectively enhance auditory sensitivity to biologically relevant auditory stimuli, including conspecific acoustic signals important for facilitating social and reproductive interactions. Below I discuss a future study that should be carried out to further explore the mechanisms of sound pressure sensitivity.

Imaging of the inner ear in response to acoustic sound pressure and particle motion

Understanding internal physiology and biomechanics can be challenging; however, the advent of synchrotron x-ray imaging has allowed for studying structure-function relationships in animals [For review, see (Westneat et al. 2008)]. Recently, Schulz-Mirbach and colleagues utilized a standing wave tube-like tank and high-speed (~100 fps) synchrotron x-ray phase contrast imaging to track otolith motion in response to acoustic stimuli (Schulz-Mirbach et al. 2020; Maiditsch et al. 2022). These authors showed that the otolithic end organs exhibit differential motion in response to acoustic sound pressure. However, how otolith motion relates to sound pressure sensitivity is unknown. Development or use of a similar system in female midshipman, which has had the sound pressure sensitivity characterized in all three end organs, would provide novel insight into the structure-function relationship of the inner ear and swim bladder.

Sexually dimorphic differences in sound pressure sensitivity

Reproductive males have a swim bladder that is positioned approximately 1.5 – 2 times the distance from the otic capsule when compared to females and type II males (Mohr et al. 2017). Whether the auditory end organs (i.e., saccule, utricle, and lagena) of reproductive male midshipman exhibit sound pressure sensitivity has yet to be considered but would provide valuable insight into the potential adaptations of the midshipman swim bladder as an acoustic organ for sound production and reception.

Reproductive state-dependent modulation of auditory sensitivity

In chapter 4, I characterized evoked utricular hair cell potentials in nonreproductive and reproductive female midshipman to determine if utricular auditory sensitivity is seasonally dependent. I showed that reproductive state modulates female utricular hair cell auditory sensitivity. Below, I discuss potential mechanisms that may contribute to these observed changes in hair cell auditory sensitivity and future questions that should be asked next.

What role do gonadal steroids play in modulating utricular auditory sensitivity?

The observed changes in utricular auditory sensitivity are likely due to seasonal fluctuations in circulating gonadal steroids (androgens and estrogens), which are related to seasonal changes in midshipman reproductive state (Sisneros et al. 2004b). Previously, the saccular afferents of non-reproductive female midshipman when treated with either testosterone or 17β-estradiol, have exhibited enhanced frequency sensitivity and phase-locking accuracy to higher frequencies within the midshipman hearing range, which is thought to effectively enhances acoustic communication (Sisneros et al. 2004a). However, hormone-dependent utricular auditory sensitivity has yet to be determined in the midshipman. If hormone-dependent modulation is observed, then underlying mechanisms, such as those outlined below, should be explored to determine if hormones act on the saccule and utricle in a similar manner.

What are the cellular mechanisms underlying state-dependent auditory sensitivity?

Previously, Rohmann et al. (2013) showed that large-conductance, calcium-activated potassium (BK) channels, which are responsible for the rapid outward currents that contribute to the electrical resonance and low-frequency (<1 kHz) tuning of hair cells, exhibit increased expression in saccular hair cells of reproductive midshipman. Concurrently, seasonal increases in BK channel expression is correlated with enhanced higher frequency sensitivity (>145 Hz). Taken together, these results suggest that gonadal steroids may modulate seasonal changes in frequency sensitivity via the regulation of hair cell BK channel expression to effectively enhance auditory sensitivity for social acoustic communication. In chapter 4, we showed that reproductive females exhibited enhanced frequency sensitivity; however, whether this is due seasonal changes in BK channel expression has not been determined.

Does the auditory efferent system modulate utricular auditory sensitivity?

Previous work by Forlano et al. (2015) showed that dopaminergic innervation of the saccule varied with reproductive state such that reproductive females have a seasonal reduction in dopaminergic input. Later, Perelmuter et al. (2019) showed that dopamine decreases saccular auditory sensitivity via D2-like receptors and that D2a receptor expression is reduced in the saccule during the midshipman breeding season. Additionally, it was noted that saccular auditory sensitivity is modulated by the dopaminergic efferent system, whereby pharmacological manipulations that release inhibition effectively mimic the auditory sensitivity of reproductive midshipman (Perelmuter et al. 2019). More recently, Perelmuter et al. (2021) have shown that androgen treatment mimics seasonal downregulation in dopamine expression in the midshipman, which provides evidence that steroid regulation of peripheral auditory sensitivity is mediated, at least in part, by dopamine. Future studies that examine similar reproductive state-dependent, gonadal steroid regulatory mechanisms of dopaminergic expression at the utricle will be instrumental in understanding the neuroendocrine basis of peripheral auditory sensitivity modulation in midshipman fish.

Future directions

In summary, the results of this dissertation describe the auditory sensitivity of the utricle and adaptations that effectively enhance auditory sensitivity. These changes to inner ear sensitivity likely aid in detecting and responding to biologically relevant auditory cues and signals. In the previous sections, I suggest incremental next steps specific to the questions addressed in this dissertation. In this section, I will discuss future directions in a broader context.

Comparative functions of the teleost auditory inner ear

The teleost auditory system is composed of three paired otolithic end organs (i.e., saccule, utricle, and lagena). Of the more than 34,000 extant fish species, only a handful of fishes have had each of the auditory sensitivity of all three otolithic end organs characterized. Of this subset includes goldfish, sleeper goby, and, most recently, plainfin midshipman. Each of these different model systems has been shown to have developed drastically different auditory adaptations and detection strategies, which influence auditory and frequency sensitivities. Most importantly, we see that the comparative auditory sensitivity of the three auditory end organs within species differs, with goldfish and sleeper gobies exhibiting sensitivity that follows previously posited size-based sensitivity, whereby larger otolithic end organs exhibit greater sensitivity. However, in the plainfin midshipman, we see that this does not hold true. What about other animals?

Hearing in the natural environment

Aquatic organisms are continuously exposed to ambient sound, which is made up of biotic, abiotic, and anthropogenic sound sources, within their marine environment. Complex acoustic scenes, or soundscapes, have the potential to interfere with or mask the detection of biologically relevant acoustic signals (Putland et al. 2018b; Popper and Hawkins 2019). To understand the extent to which complex underwater soundscapes influence the detection of auditory signals, researchers have historically utilized physiological techniques that measure the auditory responses of aquatic organisms using electrodes positioned on the head, known as 'auditory evoked potentials'. A confound in this physiological approach is that they are conducted in laboratory settings, which have their own complex environment [For review, see (Parvulescu 1964; Rogers et al. 2016)], are void of other acoustic cues that may be present in a fishes acoustic environment. Therefore, the development of equipment to measure fish auditory responses in their natural environment would greatly improve our understanding of how fish respond to acoustic stimuli.

Along similar lines, previous investigations into the underlying behavioral and physiological mechanisms of sound source localization have historically been investigated in fishes separately. Some of the most extensively studied species of fish are batrachoid fishes (i.e., toadfish and midshipman), which utilize acoustic communication to facilitate heterospecific and conspecific interactions. Prior behavioral studies in the midshipman have shown that they are capable of localizing underwater sound sources by encoding the local particle motion vectors (Zeddies et al. 2010, 2012). Additionally, physiological studies on the midshipman inner ear saccule and utricle, which are oriented in planes approximately orthogonal to each other (saccule: vertical; utricle: horizontal), have shown that both end organs act as primary auditory end organs (Sisneros 2009b; Rogers and Sisneros 2020). However, these physiological studies were conducted in stationary fish due to technical challenges associated with recording from freely moving fish. Future experiments that attempt to record auditory neural activity in fishes during auditory-driven behaviors, such as sound source localization, would provide novel insight into the function of the teleost inner ear.

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