Neural response patterns to social acoustic signals and the role of the sexually dimorphic swim bladder in the plainfin midshipman fish, *Porichthys notatus*

Robert Alex Mohr

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2017

Reading Committee:

Joseph Sisneros, Chair

Eliot Brenowitz

Michael Beecher

Program Authorized to Offer Degree:

Psychology

University of Washington

Abstract

Neural response patterns to social acoustic signals and the role of the sexually dimorphic swim bladder in the plainfin midshipman fish, *Poricthys notatus*

Robert Alex Mohr

Chair of the Supervisory Committee:
Professor Joseph Sisneros
Department of Psychology

The reception and processing of socially relevant auditory signals is crucial for the reproduction and survival of many species including the plainfin midshipman fish, *Porichthys notatus*. Numerous behavioral, physiological and neuroanatomical studies have made *P. notatus* a robust model system for studying vocal acoustic communication. The ways in which fish are able to perceive acoustic information varies across species and I will detail the underlying morphological structures, including the swim bladder, that fish have adapted to enhance underwater hearing. I characterize the morphology of the midshipman swim bladder chapter two, in each of the three sexual phenotypes; females, type I and type II males. Using micro-computerized tomography I measure and quantify the swim bladder's shape and relative distance to the inner ear organs in each midshipman sexual phenotype. In chapter three, using the immediate early gene cFos as a marker for neuronal activation, I quantify the response properties

of gravid female midshipman to both conspecific advertisement calls and heterospecific white seabass calls. Specifically, I demonstrate the brain areas differentially activated during the perception of these socially relevant acoustic signals. To assess the role of the swim bladder in the neural processing of acoustic signals, in chapter four I conducted playback of conspecific advertisement calls to reproductive females with their swim bladders experimentally removed. Finally, in chapter five, I summarize the conclusions of my work as well as the broader implications and suggestions for future studies.

TABLE OF CONTENTS

LIST OF FIGURES

GLOSSARY

AEBR - Auditory evoked behavioral response
AEP- Auditory evoked potential
AT - Anterior tuberal nucleus of the hypothalamus
CP - Central posterior nucleus of the thalamus
CT - Computerized tomography
dB - Decibels
DOri - Rostral intermediate division of the descending octaval nucleus
GSI - Gonadal somatic index
Hz - Hertz
IC - Inferior colliculus
IEG - Immediate early gene
MicroCT - Microcomputerized tomography
SOV - Ventral division of the secondary octaval nucleus
SL - Standard length
TSnc - Nucleus centralis of the torus semicircurlaris

PREFACE

Chapter 2 has previously been published as:

Mohr RA, Whitchurch EA, Anderson RD, Forlano PM, Fay RR, Ketten DR, Cox TC, Sisneros JA (2017) Intra- and Intersexual swim bladder dimorphisms in the plainfin midshipman fish (*Porichthys notatus*): Implications of swim bladder proximity to the inner ear for sound pressure detection. *Journal of Morphology* 278:1458-1468.

Chapter 3 is currently in preparation:

Mohr RA, Chang Y, Bhandiwad AA, Forlano PM, Sisneros JA. Brain activation patterns in response to conspecific and heterospecific social acoustic signals in the plainfin midshipman fish, *Porichthys notatus*. *in prep*.

Chapters 4 has been prepared in publication format.

Please excuse any redundancies in the text that are a result from having prepared these chapters in publication format.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my family for continually supporting me throughout all of my life endeavors. In particular, thank you mom and dad for being extremely instrumental in making me the person I am today. My love for creepy, crawly and slimy critters started at a young age and I am forever grateful for all of the snakes and lizards you threw into my bed as a child, dad – without them, who knows where I would have ended up.

I would like to offer a huge thanks to my advisor Joe Sisneros. Ever since I was a bright-eyed and naïve sophomore in college you have supported and guided me with my best interests in mind. I am extremely grateful for your openness and truly hands-on mentoring. I will always be a proud member of Team Midshipman.

A great thank you to my committee members: Eliot Brenowitz, Michael Beecher and Horacio de la Iglesia. I greatly appreciate your time and thoughtful constructive criticism that have helped me develop into the scientist I am today.

I would like to make a huge thank you to Paul Forlano, for being an amazing mentor to me throughout my undergraduate and graduate career. I am so grateful for your generosity in time and resources and this Ph.D. would not have been possible without you. I would also like to thank the members of the Forlano lab (past and present), in particular Jon Perelmuter, Zack Ghahramani, Miky Timothy, Chris Petersen and Spencer Kim. You all have been extremely helpful in teaching me the techniques of your lab and your hospitality in Brooklyn was greatly appreciated.

To the members of the Sisneros lab (past and present): Peter Alderks, Liz Whitchurch, Ashwin Bhandiwad, Orphal Colleye, Ivy Chang, William Palmer, Nick Lozier, Ruiyu Zeng and Brooke Vetter, I am very thankful to have worked with you all. Both the hard work and fun times we have shared have truly been a highlight of my graduate school career. From trawling, to staying up all-night running experiments, to freezing our hands off in a cryostat, to singing karaoke on Bourbon street – it's been fun.

I wish to thank all of my friends across the globe, I'm thankful to have you all in my life. In particular, I'm grateful to Andre Dayani for being the best friend a guy could ask for.

I would like to acknowledge my Psychology cohort along with the other students in the animal behavior area for being excellent peers and providing a strong environment to work in. A special thank you to the Psychology department staff and in particular Jeanny Mai for guidance with every step of graduate student life.

Lastly, I would like to thank my fiancée Melissa Reilly and angel-puppy, Yuki for being an unwavering support system for me. Your love and belief in me, along with your masterful editing skills and tech-savvy helped me make it to the finish line. Happiness is a warm puppy.

This work was funded by grants from National Science Foundation awarded to the Sisneros and Forlano labs, the National Institutes of Health's training grant in Speech and Hearing Sciences at the University of Washington, along with the Psychology Department Scholars Award.

DEDICATION

This work is dedicated to Jay Ogawa, who inspires me to a better person every day. Kanpai.

Chapter 1. BACKGROUND AND SIGNIFICANCE

1.1 HEARING IN FISHES

Fish represent the most ancient and diverse group of vertebrates on the planet (Popper and Fay 1997, Ladich and Popper 2004). As such, fish have developed numerous specializations related to how they perceive the world. Hearing in fishes has been a topic of interest for over 100 years, and a multitude of unique adaptations have yielded a great diversity in the hearing structures and abilities across fish taxa (Parker 1903, Braun and Grande 2008, Ladich 2016). As diverse as the structures are, so, too, are the hearing ranges of fishes, which vary from low frequencies (<100Hz-400Hz) (Popper et al. 2003) to the extreme case of ultrasonic detection in some clupeids (Mann et al. 1998). Life history and ecology are important factors in determining what acoustic signals are relevant amongst fishes, from auditory scene analysis and predator detection to courtship and navigation (Popper and Fay 1993, Brantley and Bass 1994, Mann et al. 1998, Simpson et al. 2005).

The study of fish hearing is valuable as the anatomical structures and genetic basis of the auditory and vestibular systems are generally conserved across all vertebrates, including mammals (Whitfield et al. 2002, Nicolson 2005). While in general, fishes rely primarily on the detection of particle motion (the near-field component of sound) and mammals detect mostly changes in sound pressure (the far-field component of sound (de Vries 1950)), some fishes have developed morphological adaptations to detect sound pressure as well.

1.2 PROCESSING OF ACOUSTIC INFORMATION

Teleost fishes possess three semicircular canals and three otolithic end organs: the saccule, utricle and lagena (Popper and Fay 1999). In most fish, the saccule is the primary end organ for hearing while the roles of the lagena and utricle in auditory and vestibular functions likely vary amongst species (Popper and Fay 1999, Popper and Schilt 2008, Webb et al. 2008). All fish are able to detect the particle motion component of underwater sound via direct displacement of their otolithic end organs, which act as biological accelerometers (Platt and Popper 1981, Popper and Tavolga 1981, Fay 1984). When sound passes through a fish the highly dense otoliths lag in phase causing a shearing force of the sensory hair cells relative to the otoliths in the inner ear (Dijkgraaf 1960, Fay and Popper 1975). Bending of the stereocilia towards the kinocilium in the hair cell bundles leads to depolarization and an action potential (Fay and Popper 1974, Popper and Fay 1993). Measurement of fish hearing abilities are commonly assessed either with the use of electrophysiological or behavioral measures.

1.2.1 Physiological measures

Single unit, field potential and auditory evoked potential recordings (AEPs, sometimes referred to as auditory evoked behavioral responses AEBR) have been instrumental in determining the hearing thresholds and abilities of many fish species (Enger 1963, Fay and Edds-Walton 1997, Sisneros et al. 2005, Sisneros 2009, Ladich and Fay 2013, Maruska and Sisneros 2016). These recording methods provide fast and precise results, though as with all techniques, they have their limitations. All electrophysiology requires the immobilization of the animals either with physical restraints or with the use of drugs to paralyze. Single unit and field potential recordings require highly invasive surgeries, which may yield stress and unnatural physiological responses. While

AEP recordings are less invasive, the specificity of the recording sites and the output of the recordings are not as straightforward to characterize.

1.2.2 Behavioral measures

Behavioral measures of hearing can be used on fish that are too sensitive to undergo surgery, are of too extreme a size to be used for physiological preparations or are of the subjects of longitudinal studies. Non-invasive behavioral measures can use psychoacoustic techniques to evoke behavioral responses to conditioned stimuli along with involuntary reflexive behaviors in freely swimming subjects (Bhandiwad et al. 2013). While, behavioral measures can provide rapid assessment of hearing abilities generally these measures are less sensitive and precise than physiological hearing measurements.

1.2.3 Immediate early genes

While single unit recordings have been used to assess response properties to acoustic stimuli in nuclei of the central auditory system, this technique is highly invasive and often only yields one or a few units per animal. Recently, the use of the immediate early gene (IEG) response has become a popular technique to assess neural activation in response to various external stimuli (Clayton 2000, Maruska et al. 2013, Petersen et al. 2013). IEGs, including egr-1 and cFos, rapidly encode transcription factors yielding mRNA or protein products, which can be mapped and quantified as a proxy for neural activity (Pinaud and Tremere 2006, Perez-Cadahia et al. 2011). As with all techniques, the use of the IEG response has its limitations. Baseline neural activity varies throughout the brain causing similar changes in baseline IEG levels, which can make analysis difficult. Furthermore, neuronal activation can occur without IEG expression and neurons rapidly firing may not express IEGs (Kovacs 2008). The optimal IEG response time varies across species and when performing acoustic playback, stimuli and analysis often vary greatly between species, which hinders the ability for comparative studies. However, the use of IEGs allows for sampling throughout the entire brain simultaneously, which can provide valuable insights into neural networks and targeting for more fine scale physiological techniques. Studies of central auditory processing in fish focus on nuclei along the ascending auditory pathway.

1.3 THE ASCENDING AUDITORY PATHWAY

Acoustic signals are decoded in the central auditory system in an interconnected suite of nuclei known as the ascending auditory pathway. These nuclei have been identified using both physiological and neuroanatomical techniques and are well characterized (McCormick 1999, Bass et al. 2000). Auditory stimulation of the inner ear hair cells yields action potentials, which are sent to the brain via the VIIIth cranial nerve. Projections first synapse in the hindbrain descending octaval nucleus (DO) (Bass et al. 1994, McCormick 1999, Bass et al. 2000, Tomchik and Lu 2005). The DO has several unique subdivisions related to both auditory and non-auditory processing (Bass et al. 2000). Ascending projections are sent to populations within both the secondary octaval nucleus in the hindbrain and the midbrain torus semicircularis (TS) (McCormick 1999, Bass et al. 2000). Nucleus centralis (TSnc) is the primary auditory center of the TS and is homologous to the mammalian inferior colliculus (Potter 1965, Wilczynski 1981, Echteler 1984, Feng and Lin 1991, Bass et al. 2000, Endepols and Walkowiak 2001, Hoke et al. 2004). TS sends projections to a number of nuclei within the midbrain and forebrain including the periaqueductal grey, the ventral and anterior tuberal nuclei of the hypothalamus and the central posterior nucleus of the auditory thalamus (CP) (McCormick 1999, Bass et al. 2000, Goodson and Bass 2002). CP also shares connections with the ventral telencephalon (McCormick 1999, Bass et al. 2000).

1.4 MORPHOLOGICAL ADAPTATIONS

Many teleost fishes possess swim bladders which function to maintain buoyancy, act as an oxygen reservoir, aid in sound production and amplification and aid in hearing (Harden Jones and Marshall 1963, Blaxter et al. 1979, Parmentier et al. 2007, Braun and Grande 2008, Pelster 2011). Rostral swim bladder extensions are present in several teleost fish families that increase the proximity, or make direct physical contact, to the inner ear or skull (Coombs and Popper 1979, Braun and Grande 2008, Ladich 2016). Comparative physiological experiments in squirrelfishes (family: Holocentridae) revealed that sound pressure detection is greater in species with swim bladder extensions versus those lacking extensions (Coombs and Popper 1979). Behavioral studies in cichlids (family: Cichlidae) have revealed similar findings where swim bladder extensions that come into contact with the inner ear of the Asian cichlid, *Etroplus maculates,* broaden the hearing range to higher frequencies and increase auditory sensitivity compared to species lacking such adaptations (Schulz-Mirbach et al. 2012). Cod (family: Gadidae) have similar swim bladder extensions, and when experimentally deflated physiological hearing sensitivity of the saccule decreases in response to high frequency sounds (Sand and Enger 1973). Together these findings suggest a functional role for swim bladder extensions and their role in sound pressure detection.

In addition to swim bladder extensions, gas filled vesicles such as auditory bullae (which retain connections to the swim bladder), otic gas bladders and suprabranchial chambers (which have no connection to the swim bladder) may also enhance hearing sensitivity and broaden bandwidth. In herring and shad (family: Clupeidae), air bubbles located in the auditory bullae are separated via a membrane from the fluid filled utricular chambers and are attached to the sensory macula via an elastic connection (Blaxter et al 1981, Mann et al 1998). This direct connection between the auditory bullae and inner ear is implicated in the wide bandwidth of hearing in these species, which are known to detect ultrasound (Mann et al 1998, Braun and Grande 2008). These specializations may also provide a system by which these fish can compare both the pressure and particle motion components of sound, which are thought to play an important role in sound source localization (Denton et al 1979, Braun and Grande 2008, Zeddies et al. 2011). In weakly electric elephantfish (family: Mormyridae), otic gas bladders make direct contact with the saccules and when deflated bilaterally, physiological auditory brainstem responses diminish across all frequencies (Yan and Curtsinger 2000). Gouramis (family: Osphronemidae), possess suprabranchial chambers, which are labyrinths of air filled space that come into contact with the cranium and are in close proximity to the inner ear. These fish swallow and store air in these labyrinths allowing them to survive in otherwise unsuitable environments. A membrane between the gas filled labyrinths and the cranium allows for stimulation of inner ear and heightened hearing abilities in this family up to 4500 Hz (Schneider 1941, Liem 1963, Yan 1998). Further evidence for the role of a gas filled chamber in proximity to the inner ear and heightened hearing ability can be found in manipulative studies using flatfish. Flatfish lack swim bladders and other hearing specializations, but when a gas filled condom was placed in close proximity to the head of a dab (family: Pleuronectidae), their hearing sensitivity was instantly heightened (Chapman and Sand 1974). These studies provide great support that a gas filled structure in proximity to the inner ear can lead to greater auditory abilities in fishes.

Physical connections between the swim bladder and inner ear provide perhaps the most extreme adaptation for sound pressure detection in fishes. The Otophysi order of fishes (e.g. minnows, carp, goldfish and catfishes) possess skeletal connections known as Weberian ossicles that provide a direct physical connection from the swim bladder to the inner ear saccules. Displacement of the swim bladder via a sound source causes vibrations of the swim bladder leading to direct stimulation of the inner ear, allowing these fish to detect sound pressure, increase sensitivity and detect higher frequencies than fishes lacking connections. Fish possessing Weberian apparati are known as hearing specialists. Comparative studies measuring the effects of swim bladder deflation in hearing specialists (i.e. goldfish, family: Cyprinidae) versus non-specialists (i.e. toadfish, family: Batrachoididae and gouramis, family: Osphronemidae) have shown that hearing specialists suffer major decreases in hearing sensitivity compared to little to no change in the hearing non-specialists (Yan et al 2000).

1.5 THE PLAINFIN MIDSHIPMAN FISH AS A MODEL

The plainfin midshipman, *Porichthys notatus*, has become a powerful model for studying vocal acoustic communication as this species relies on the production and reception of auditory signals (Brantley and Bass 1994, Bass and McKibben 2003, Bass 2006). During the late spring, nesting or 'type I' males migrate from the depths (>100m) to the shallow intertidal zone in order to set up nesting territories underneath rocks (Hubbs 1920, Greenfield et al. 2008). From there, these males produce a long duration low frequency advertisement call known as the 'hum' to attract receptive females to breed (Greene 1924, MacGinitie 1935, Brantley and Bass 1994). Females must detect and localize calling males in order to successfully spawn. Once in the nest, females deposit their eggs on the underside of the rock while the type I male fertilizes them externally. The female leaves for deeper water and the type I male will be the sole provider of parental care and continue to solicit other females for mating (Sisneros et al. 2009). The male's hum not only attracts females, but also sneaker or 'type II' males which use an alternative mating strategy to steal fertilizations from unknowing type I males. Type II males actively sneak spawn by fanning sperm into the nests of mating type I males. Recent physiological studies have revealed that females and type II males undergo seasonal changes in their hearing abilities to be more suited to hear the type I male advertisement call in the shallow-water nesting environment (Sisneros et al. 2004b, Sisneros 2009, Bhandiwad et al. 2017). Morphological studies have also shown that female midshipman fish have a greater number of saccular hair cells during the breeding season compared with the non-breeding season (Coffin et al. 2012). Interestingly, results from our micro-computerized tomography study have revealed that female and type II males have elongated rostral extensions of their swim bladders which are absent in type I males (Mohr et al. 2017). These extensions increase the proximity of the swim bladder to the inner ear and likely afford females and type II males sound pressure sensitivity (Mohr et al. 2017). Manipulative studies, in which the swim bladder was deflated in female midshipman, revealed that an intact swim bladder is likely necessary for sound source localization (Coffin et al. 2014). These findings support current models of sound source localization, which rely on the detection of both sound pressure and particle motion (Sisneros and Rogers 2016).

1.6 QUESTIONS ADDRESSED IN THIS WORK

The goal of this work is to further understand how fish process complex acoustic signals and how the morphology of the swim bladder alters neural responses to auditory stimuli. I used a variety of techniques, both in the laboratory and in the field, to characterize swim bladder morphology, how the fish brain responds to complex behaviorally relevant acoustic signals and how the presence of the swim bladder alters patterns of neural activation. In my work I address the following questions:

1.6.1 Are there intra- and intersexual differences in swim bladder morphology that may be related to hearing?

I address this question in chapter two by conducting a detailed analysis of the swim bladder morphology of the three sexual phenotypes of the plainfin midshipman fish, *P. notatus.* I also provide detailed measurements between the swim bladder and the three inner ear end organs.

1.6.2 Do neuronal response patterns differ based on exposure to conspecific versus heterospecific vocalizations?

In chapter three I used a semi-naturalistic playback paradigm to expose gravid female *P. notatus* to conspecific advertisement calls, heterospecific white seabass calls or ambient noise in the intertidal zone. The immediate early gene protein product cFos was used to visualize and quantify neural response properties throughout various nuclei along the ascending auditory pathway.

1.6.3 Does the swim bladder alter neuronal response properties to conspecific advertisement calls?

By surgically removing the swim bladder, in chapter four I characterized the neural response properties of reproductive female *P. notatus* exposed to conspecific advertisement calls or no sound trials. Again, the protein product of the immediate early gene cFos was used as a proxy for neuronal activity in auditory regions of the brain.

The following chapters will illustrate empirical findings for one of the first cases of intra- and intersexual dimorphisms in a fish species and the potential implications for sound pressure detection. This work also provides insights into the brain areas necessary for the processing of complex acoustic signals. In the final chapter I will address the conclusions of the findings of chapters two through four and provide possible avenues for future research in the field of fish auditory neuroscience.

Chapter 2. INTRA- AND INTERSEXUAL SWIM BLADDER DIMORPHISMS IN THE PLAINFIN MIDSHIPMAN FISH (*PORICHTHYS NOTATUS*): IMPLICATIONS OF SWIM BLADDER PROXIMITY TO THE INNER EAR FOR SOUND PRESSURE DETECTION

2.1 SUMMARY

The plainfin midshipman fish, *Porichthys notatus*, is a nocturnal marine teleost that uses social acoustic signals for communication during the breeding season. Nesting type I males produce multi-harmonic advertisement calls by contracting their swim bladder sonic muscles to attract females for courtship and spawning while subsequently attracting cuckholding type II males. Here, we report intra- and intersexual dimorphisms of the swim bladder in a vocal teleost fish and detail the swim bladder dimorphisms in the three sexual phenotypes (females, type I and II males) of plainfin midshipman fish. Micro-computerized tomography revealed that females and type II males have prominent, horn-like rostral swim bladder extensions that project toward the inner ear end organs (saccule, lagena and utricle). The rostral swim bladder extensions were longer, and the distance between these swim bladder extensions and each inner-ear end organ type was significantly shorter in both females and type II males compared to that in type I males. Rostral swim bladder extensions are known to increase sound pressure sensitivity and higher frequency detection; swim bladder size is also a known factor implicated in affecting sound pressure sensitivity to higher frequencies in other teleosts. Our results revealed that the normalized swim bladder length of females and type II males was longer than that in type I males while there was no difference in normalized swim bladder width among the three sexual

phenotypes. We predict that these intra- and intersexual differences in swim bladder morphology among midshipman sexual phenotypes will afford greater sound pressure sensitivity and higher frequency detection in females and type II males and facilitate the detection and localization of conspecifics in shallow water environments, like those in which midshipman breed and nest.

2.2 INTRODUCTION

Teleost fishes have swim bladders that serve multiple primary functions including operating as an oxygen reservoir, regulating buoyancy and maintaining hydrostatic position in the water column (Harden Jones and Marshall 1953, Blaxter et al. 1979, Pelster 2011). In some cases, a secondary swim bladder function has evolved that enables these species of fish to produce sound for acoustic communication (Parmentier and Diogo 2006, Kasumyan 2008, Fine and Parmentier 2015). In grunts (family: Haemulidae) and clownfishes (family: Pomacentridae), the swim bladder acts an acoustic amplifier to increase the sound level produced by the stridulation of hard skeletal parts including vibrations of the rib cage driven by jaw snapping (Parmentier et al. 2007, Colleye et al. 2012, Bertucci et al. 2014). In toadfishes and midshipman (family: Batrachoididae), sound is produced predominantly by reproductive males that have enlarged sonic muscles attached to their swim bladders and when contracted vibrate the bladder to yield high sound level acoustic signals for social communication (Fine et al. 1990, Fine et al. 1993, Fine et al. 2001, Bass and McKibben 2003, Fine and Parmentier 2015).

As an acoustic organ, the swim bladder in many species of fish can also aid in the reception of acoustic signals. All fish are thought to be able to detect the particle motion component of sound using their inner-ear otolithic end organs as biological accelerometers that respond directly to the displacement of the fish by particle motion (De Vries 1950, Fay and Popper 1980, Sisneros and Rogers 2016). In some species of more recently derived teleost fishes, the detection of sound pressure has evolved through the use of specialized morphological adaptations that permit pressure-induced vibrations of the swim bladder to be transduced to the inner ear for the detection of sound pressure. These 'pressure sensitive' teleosts have evolved either skeletal adaptations known as Weberian ossicles (e.g. Otophysans) that connect the anterior part of the swim bladder to the inner ear or possess gas-filled vesicles that are in close proximity to the inner ear to enhance sound pressure sensitivity (e.g., herring, Family: Clupeidae and squirrelfishes, family: Holocentridae) (O'Connell 1955, Allen et al. 1976, Coombs and Popper 1979, Popper and Coombs 1980, Blaxter et al. 1981, Braun and Grande 2008). The detection of sound pressure is also thought to be important for sound source localization by fishes. Current models for sound source localization depend on the detection and processing of both sound pressure and acoustic particle motion (Schuijf 1981, Hawkins 1986, Zeddies et al. 2011, Sisneros and Rogers 2016). Recent behavioral studies of the plainfin midshipman fish (*Porichthys notatus*) have suggested that the swim bladder is necessary for both acoustic pressure detection and near-field sound source localization (Coffin et al. 2014).

The plainfin midshipman has become the focus of recent sound localization studies because gravid females exhibit robust phonotaxis and localization of simulated male advertisement calls during the breeding season (McKibben and Bass 1998, Zeddies et al. 2010, Zeddies et al. 2012, Coffin et al. 2014). During the late spring and summer, nesting or type I male plainfin midshipman attract females by producing a multi-harmonic advertisement call known as a 'hum'. Midshipman fish also produce two other vocalizations known as 'grunts' and 'growls' used in agonistic social interactions. As in other batrachoidid fishes, vocal signals are produced by the contraction of the sonic muscles attached to the swim bladder. The hum contains a fundamental frequency (80-102 Hz; varies with temperature) with multiple harmonics that range up to 1 kHz (McKibben and Bass 1998). Females rely on their auditory sense to detect and locate vocalizing mates. Previous studies have shown that females exhibit seasonal changes in physiological auditory sensitivity and morphological changes in saccular hair cell density that enhance their ability to detect and locate males that "sing" in their nests (Sisneros et al. 2004b, Sisneros 2009, Coffin et al. 2012, Rohmann et al. 2013). Once in the nest, females lay their eggs while type I males externally fertilize the eggs and then guard them throughout development. The advertisement call not only attracts females but also type II males or "sneakers" that employ an alternative mating strategy to obtain fertilizations from type I males actively courting females in the nests (Brantley and Bass, 1994). Adult type II males are significantly smaller than nesting type I males and these "sneaker" males do not invest energy in building or guarding nests nor in parental care, but instead invest in the development of their testes, which can be up to 20% of their body mass for increased sperm competition (compared to the testes of type I males which are only 1-3% of their body mass) (Brantley et al. 1993).

While previous gross morphological differences in swim bladder sexual dimorphisms in the plainfin midshipman have been reported earlier by Bass and Marchaterre (1989), we now provide a more detailed examination of swim bladder sexual dimorphisms among the three midshipman sexual phenotypes: females, type I and type II males. We provide evidence for both intra- and intersexual dimorphisms of the swim bladder and differential distance measurements between the swim bladder and inner ear in the plainfin midshipman and interpret our findings as they relate to possible morphological adaptations for the detection of sound pressure and its implications for acoustic communication in this species.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Adult plainfin midshipman, *Porichthys notatus* (Girard 1854), were collected by hand at low tide during the spring breeding season near Marshall in Tomales Bay, CA. Animals used for computerized tomography (CT) were housed in flow-through seawater aquaria at the Marine Resources Center at the Marine Biological Laboratory (MBL) in Woods Hole, MA where they were maintained at 13-14°C on a 14:10 hour light cycle and fed live mysid shrimp 3 times weekly. Five type I males, three females and three type II males were scanned using CT and varied in size from 105-209 mm standard length (measured from the snout to caudal peduncle). Animals used for micro-computerized tomography (microCT) were housed at the University of Washington where they were maintained at 13-14°C on a 14:10 hour light cycle and fed frozen vitamin enriched shrimp 3 times weekly before being euthanized for microCT scanning via overdose of ethyl p-aminobenzoate in a saltwater bath. Twenty-seven adult type I males, type II males and females (nine from each sexual phenotype) of similar standard length were selected for imaging. While adult plainfin midshipman can vary greatly in size within and across morphs, all animals chosen for microCT imaging were between 112-138 mm to ensure an overlap in size between all three sexual phenotypes.

2.3.2 CT Imaging

Initial observations of swim bladder dimorphisms in the plainfin midshipman based on gross dissections (Figure 2.1) led to a pilot group of animals being imaged using a CT scanner in the Computerized Scanning and Imaging Facility (http://csi.whoi.edu) at the Woods Hole Oceanographic Institution.

Whole animal overviews (topograms) were obtained to assess the positioning for the CT sequences. Ultrahigh resolution sectional data were obtained on a Siemens Volume Zoom computerized tomography (CT) unit using a spiral protocol with parameters of 120 kV, 180 mAS, 0.5 mm acquisitions at 0.5 mm table increments. Primary images were formatted in the transaxial plane at 0.1 mm sectioning and a 50 mm field of view (FOV), providing 512 matrix, 100 micron isotropic voxel resolution, in both soft (H40) and ultrahigh bone kernels (H90) with extended scales to enhance soft tissue detail and eliminate image artifacts from hyper-attenuating structures such as the otoliths. Some images were also obtained and reconstructed at 1 mm increments at larger FOVs to document the entire body of the fish and assess sexual differences. Composite three-dimensional reconstructions of multiple regions of interest (ROI), such as the gas-filled areas, swim bladder surface, strictures, and otoliths, were obtained using Siemens proprietary software, with ROIs segmented by Hounsfield values. The DICOM images and raw data were archived onto magneto-optical (MO) disks and additional DICOM copies were transferred via CDs for off-line use with eFilmLite 3.1 (Merge Heathcare) and OsiriX (MacOS) software.

Standard length (SL) and body mass (BM) were recorded and the gonads were dissected and weighed to calculate gonadal somatic index (GSI).

2.3.3 MicroCT Imaging

Specimens were scanned at the Small Animal Tomographic Analysis Facility (SANTA) at Seattle Children's Research Institute where they were imaged using a Skyscan 1076 microCT scanner at 50kV, 170µA with a scan resolution of 35.26 µm. Whole body X-ray images were used to determine a rostral point just anterior to the inner ear otoliths and a caudal point just posterior to the swim bladder for microCT imaging. All data were reconstructed using NRecon (v1.6.9.4) with consistent thresholding parameters. Reconstructions were rendered in 3D using the freeware, Drishti v2.6 (Limaye, 2006). Measurements were made in Dristhi using the 'path' option after landmark placement. For final images, the swim bladders were segmented from the reconstucted data using Drishti Paint v2.5, while otoliths were pseudo-segmented using simple thresholding as their structures represent the highest mineral densities within the fish, with post-rendering clean-up using Drishti. Scalebars were incorporated within Drishti. Images taken in Drishti were then imported into Adobe Photoshop CS5 to optimize contrast and to facilitate appropriate image scaling. After scans were completed, animals were weighed intact before their gonads were dissected and then weighed to calculate GSI and confirm sexual phenotype.

2.3.4 Morphometric Analysis

Measurements represent absolute distance in three-dimensional space in millimeters and calculated from the microCT generated data. Measurements were taken from the rostral-most point of the swim bladder to the caudal most points of the otoliths in each respective end organ (saccule, utricle and lagena, see: Figure 2.2.). Swim bladders were also measured for length and width. The rostral swim bladder extensions or ‘horns’ were defined as the anterior projections of the swim bladder that project beyond the round shape of the bladder at the rostral end (Figure 2.3). This metric was calculated as the difference between the length of swim bladder minus the width or diameter of the round swim bladder, which yields the relative length of the rostral extensions of the swim bladder. All swim bladder to otolith distance measurements were measured bilaterally and averaged within each subject to account for any differences in laterality. In order to account for sexually dimorphic differences in animal size, swim bladder and the swim bladder extension-to-otolith measurements were divided by the fish’s standard length to normalize the measurements, thus creating a normalized distance ratio for all measurements taken. Statistical analyses were conducted using SPSS 19 Software (SPSS, Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA USA) with an α set at 0.05. A multivariate analysis of variance (MANOVA) using Wilk’s Lambda multivariate test compared the swim bladder to otolith distance measurements for all three end organs across the three sexual phenotypes. The effect of sexual phenotype on swim bladder size (length and width) and distance between swim bladder extensions and each inner otolith was determined using a one-way analysis of variance (ANOVA) followed by Tukey post-hoc tests for planned comparisons.

2.4 RESULTS

Initial observations from our gross dissections and preliminary CT scans of the midshipman swim bladders (Figure 2.4) revealed remarkable morphological differences in swim bladder morphology among the three sexual phenotypes. Notably, we observed the presence of horn-like projections on the rostral ends of the swim bladders in both females and type II males. In contrast, type I males lacked the prominent horn-like swim bladder extensions and the rostral swim bladder was observed to be more rounded in shape. In addition, the rostral swim bladder extensions in females and type II males appeared to project closer to the inner-ear end organs (saccule, lagena and utricle) than in type I males (Figure 2.4). However, our initial sample sizes of the CT scanned swim bladders were too few to adequately characterize the morphological differences observed for the three sexual phenotypes. Thus, a more detailed examination of the swim bladders was conducted on a second set of subjects using a microCT scanner.

2.4.1 MicroCT Analysis of Midshipman Swim Bladders

MicroCT analyses of the swim bladders were conducted on 27 plainfin midshipman: nine type I males, nine type II males and nine females. Subjects were selected for microCT analysis based on their SLs in millimeters (mm) to minimize variation between sexual phenotypes. Type I males had a size range of 119-138 mm SL (mean SL = 132 ± 7 mm SD) and 21.4-31.4g BM (mean BM = 28.3 ± 3.1 g SD) and a GSI of 1.7 ± 0.6 SD. Type II males had a size range of 112-121 mm SL (mean SL = 116 ± 3 mm SD) and 15.0-20.5 g BM (mean BM = 17.8 ± 2.1 g SD) and a GSI of 15.6 ± 3.0 SD. Females had a size range of 110-130 mm SL (mean SL = 123 ± 7 mm SD) and 21.7-26.2 g BM (mean BM = 20.9 ± 4.4 g SD) and a GSI of 26 ± 9.0 SD. Type I males were significantly larger (based on SL) than both females ($p< 0.001$) and type II males ($p< 0.05$) while females were larger than type II males ($p< 0.05$) (one-way ANOVA, post hoc Tukey test, $F(2, 24) = 16.59$, $p< 0.001$). Similarly, type I males had a significantly greater BM than both females and type II males ($p< 0.001$), but females and type II males did not differ in BM ($p= 0.13$) (one-way ANOVA, post hoc Tukey test, $F(2, 24) = 23.75$, $p< 0.001$).

The swim bladders of the three sexual phenotypes differed in both length and width. Female swim bladders (mean = 22.68 ± 0.95 mm SD) were significantly longer than the swim bladders of type I males (mean = 20.19 ± 1.57 mm SD, $p < 0.001$) and type II males (mean = 20.85 ± 1.13 mm SD, $p < 0.05$), while type I and type II males did not differ in swim bladder length ($p = 0.52$) (one-way ANOVA, post hoc Tukey test, $F(2, 23) = 8.82$, $p < 0.001$). When normalized for animal size (SL), the swim bladder length to SL ratios did not differ between females (mean = 0.18) and type II males (mean = 0.18) ($p = 0.52$), however both females and type II males had significantly longer swim bladder to SL ratios than type I males (mean = 0.15) (one-way ANOVA, post hoc Tukey test, $F(2, 23) = 33.83$, $p<0.001$). Although the swim bladder width of females (mean = 14.10 ± 1.34 mm SD) and type I males (mean = 14.23 ± 0.97 mm SD) did not differ ($p = 0.96$), the swim bladder widths of females and of type I males were wider than that of type II males (mean = 11.91 ± 0.82 mm SD) (one-way ANOVA, post hoc Tukey test, $F(2, 23) = 13.67$, $p < 0.001$). When normalized for animal size (SL), the swim bladder width to SL ratios also did not differ between females (mean = 0.11) and type I males (mean = 0.11) ($p = 0.21$) nor between type II males (mean = 0.10) and type I males ($p =0.35$), however the female swim bladder width to SL ratio was wider than that for type II males (one-way ANOVA, post hoc Tukey test, $F(2, 23) = 4.87$, $p< 0.05$).

The rostral swim bladder extensions were longer and more prominent in the female and type II male sexual phenotypes (Figure 2.5). Although the lengths of the rostral swim bladder extensions in females (mean = 8.57 ± 0.51 mm SD) and type II males (mean = 8.93 ± 0.67 mm SD) did not differ ($p = 0.55$), the swim bladder extensions in both females and type II males were significantly longer than in type I males (mean = 5.96 ± 0.83 mm SD) (one-way ANOVA, post hoc Tukey test, $F(2, 23) = 49.69$, $p< 0.001$, see Figure 2.5). When normalized for animal size (SL), the rostral swim bladder extension length to SL ratio in type II males (mean = 0.77) was longer than that of females (mean = 0.70) ($p < 0.05$) and type I males (mean = 0.45) ($p < 0.001$) while the swim bladder extension length to SL ratio in females was also longer than that of type I males (one-way ANOVA, post hoc Tukey test, F (2, 23) = 81.08, $p<0.001$).

The distance between the rostral swim bladder extensions and the otoliths in the three inner ear end organs (saccule, lagena, and utricle) differed across sexual phenotype (MANOVA, effect of sexual phenotype,F(6,44) = 11.41, $p< 0.001$) (Figure 2.6). Although there was no difference in the distance between the swim bladder extensions and the saccular otoliths between females (mean = 2.59 ± 0.52 mm SD) and type II males (mean = 2.02 ± 0.25 mm SD) ($p = 0.27$), the distance between the swim bladders extensions and the otoliths in the saccule, which is the primary auditory end organ in the midshipman, was significantly shorter (approximately ½ the distance) in females and type II males compared to type I males (mean = 5.21 ± 1.20 mm SD) (one-way ANOVA, post hoc Tukey test, F (2, 24) = 43.78, $p< 0.001$) (Figure 2.6B). When the swim bladder extension to saccular otolith distance was normalized based on SL, females (mean = 0.021) and type II males (mean = 0.017) had significantly shorter distances between swim bladder extensions to saccular otoliths than type I males (mean = 0.040) with no difference ($p = 0.20$) between females and type II males (one-way ANOVA, post hoc Tukey Test, F (2, 24) = 39.52, $p<0.001$).

The distance between the rostral swim bladder extensions and the otoliths of the lagenae was significantly shorter in females (mean = 2.89 ± 0.16 mm SD) and type II males (mean = 2.29 ± 0.22 mm SD) compared to type I males (mean = 4.71 ± 1.05 mm SD) with no difference ($p= 0.16$) observed between females and type II males (one-way ANOVA, post hoc Tukey Test, F (2,

24) = 30.79, $p < 0.001$) (Figure 2.6C). When normalized for SL, females (mean = 0.024) and type II males (mean = 0.020) had significantly shorter distances between swim bladder extensions to lagenar otoliths than type I males (mean = 0.036) with no difference ($p = 0.14$) between females and type II males (one-way ANOVA, post hoc Tukey Test, F (2, 24) = 23.65, $p < 0.001$).

The distance between the rostral swim bladder extensions and the otoliths of the utricles was also significantly shorter in females (mean = 5.24 ± 0.76 mm SD) and type II males (mean = 5.01 ± 0.50 mm SD) than in type I males (mean = 8.84 ± 1.22 mm SD) with no difference ($p = 0.84$) observed between females and type II males (one-way ANOVA, post hoc Tukey Test, F (2, 24) = 53.55, $p < 0.001$) (Figure 2.6D). When normalized for SL, females (mean = 0.042) and type II males (mean = 0.043) had significantly shorter distances between swim bladder extensions to utricle otoliths than type I males with no difference ($p = 0.76$) between females and type II males (mean = 0.067) (one-way ANOVA, post hoc Tukey Test, F (2, 24) = 45.76, $p < 0.001$).

2.5 DISCUSSION

The goal of this study was to characterize the morphology of the swim bladder and quantify the relative distance between the swim bladder and inner ear organs in the three sexual phenotypes (females, type I and type II males) of the plainfin midshipman fish, *Porichthys notatus*. Our results show that females and type II males have prominent horn-like extensions on the rostral ends of the swim bladders that extend towards the auditory end organs. These rostral swim bladder extensions were longer in both females and type II males compared to that of type I males. In addition, the distance between the rostral swim bladder extensions and each inner-ear end organ (saccule, lagena, and utricle) was significantly shorter in females and type II males compared to that in type I males, and this decreased distance between the swim bladder and auditory end organs likely increases auditory sensitivity to sound pressure and extends the upper range of frequencies that can be detected.

2.5.1 Functional Significance of the Swim Bladder in Female and Type II Male Midshipman

Previous studies have reported sexual dimorphisms in the swim bladders of South Asian torrent minnows (family: Psilorhynchidae) (Conway et al. 2014), Atlantic croakers (family: Sciaenidae) (Hill et al. 1987), oyster toadfish (family: Batrachoididae) (Fine et al. 1990) along with several species of cusk eels (family: Ophidiidae) with implications for sound production (Courtenay 1971, Kéver et al. 2012, Rose 1961,). While Bass and Marchaterre (1989) reported overall size differences in gross dissections of the swim bladders of plainfin midshipman across sexual phenotypes, no study has yet provided evidence for functional differences in swim bladders between the sexes, nor between alternative sexual phenotypes of a given sex as it relates to sound reception. Our results of intrasexual and intersexual dimorphic differences in swim bladder morphology suggest that these differences may be functionally related to differences in sound pressure sensitivity, especially as it relates to social behavior in this species. We show that females and type II males have rostral swim bladder extensions that were 1.4 to 1.5 times longer than those in type I males and the presence of these swim bladder extensions results in a shorter distance between the swim bladder and inner ear otoliths (especially the sagitta of the saccule and the asteriscus of the lagena).

The presence of rostral horn-like swim bladder extensions has been previously observed in other fishes from several teleost families including Holocentridae, Gadidae, Gerreidae, Sciaenidae Chaetodontidae, and more recently Cichlidae (Nelson 1955, Hawkins 1986, Ramcharitar et al.

2006, Braun and Grande 2008, Parmentier et al. 2011, Tricas and Webb 2016, Ladich 2016). These previously reported swim bladder extensions have been posited to enhance sound pressure sensitivity by providing closer proximity of the swim bladder to the auditory end organs (Ramcharitar et al. 2006, Braun and Grande 2008, Parmentier et al. 2011, Fine and Parmentier 2015, Schulz-Mirbach et al. 2012, Schulz-Mirbach et al. 2013). While some fishes such as otophysan fishes (e.g., goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*)) have specialized skeletal adaptations known as Weberian ossicles that directly link the swim bladder to the inner ear for sound pressure sensitivity, other fishes such as holocentrids and gadids (i.e., squirrelfishes and cods, respectively) possess paired rostral swim bladder extensions that approach the skull and provide increasing pressure sensitivity the closer the swim bladder extensions are to the inner-ear end organs (Chapman and Hawkins 1973, Coombs and Popper 1979). The close proximity of the swim bladder to the inner ear afforded by the rostral swim bladder extensions is thought to allow the auditory end organs (e.g., saccule and lagena) to detect the local particle motion produced by pressure-wave induced vibrations of the swim bladder when exposed to sound. This indirect mechanism for sound pressure detection is posited to increase overall auditory sensitivity and extend the upper range of acoustic frequencies that are detected by the fish (Sisneros and Rogers 2016, Tricas and Webb 2016).

The distance between the horn-like swim bladder extensions and the otoliths of the saccule and lagena in the midshipman was less than 3 mm in both females and type II males compared to a distance greater than 4.7 mm for the same measurements in type I males. Similar studies of sciaenids (Ramcharitar et al. 2006), ophidiids (Kéver et al. 2014), and cichlids (Schulz-Mirbach et al. 2012), species with swim bladders less than 3 mm away from the otic capsule, which contains the inner ear end organs, showed enhanced auditory sensitivity to acoustic stimuli over 1500 Hz. In addition, Schulz-Mirbach et al. (2012) showed that in cichlids the size of the swim bladder may also affect hearing such that fish with larger swim bladders could detect higher frequencies while the presence of rostral swim bladder extensions increased overall auditory sensitivity to acoustic stimuli between 500 and 1000 Hz. In our study, we show that the absolute swim bladder length was longer in females than in type I and type II males, however when normalized for standard body length (SL), both females and type II males had significantly larger swim bladder to SL ratios than type I males. Taken together, our findings predict that the relatively long rostral horn-like swim bladder extensions and larger swim bladders in midshipman females and type II males should facilitate enhanced sound pressure sensitivity and increase the upper range of detectable frequencies in these two sexual phenotypes. Future studies that investigate saccular and lagenar auditory physiology of females and type II males that involve experimental deflation or removal of the swim bladder will be informative as to the role of the swim bladder in sound pressure sensitivity in this species.

The predicted enhancement of sound pressure sensitivity and higher frequency detection in females and type II male midshipman would likely increase the detection of social acoustic signals in shallow water environments like those in which midshipman breed and interact during the reproductive season. Plainfin midshipman fish are known to produce three types of vocalizations that include hums, growls and grunts. These vocalizations are produced during social and reproductive behaviors and are generated by the contraction of the sonic muscles attached to the swim bladder. During the breeding season, type I males produce the multi-harmonic hum or advertisement call to attract females for spawning. The hum contains a fundamental frequency that ranges from 80-102 Hz with harmonics that can extend up to 1000 Hz (personal observation, JAS; but also see Ibara et al. 1983, Brantley and Bass 1994, Bass et al. 1999). Growls and grunt trains, which are agonistic calls also produced by type I males, are broadband signals that contain frequency information up to 800 Hz (Bass et al. 1999, Maruska and Sisneros 2015). Previous studies have shown that females and type I and II males exhibit an adaptive form of reproductive-state dependent auditory plasticity that results in increased sensitivity during the reproductive season to a broad range of frequencies including the dominant higher frequencies in type I male vocalizations (Sisneros and Bass 2003, Sisneros 2009, Rohmann and Bass 2011, Bhandiwad et al. 2017). In very shallow waters (< 3-4 m deep), the relatively high frequencies (>180 Hz) contained within midshipman vocalizations (hum, grunts and growls) will propagate farther than the calls' fundamental frequency due to the inverse relationship between water depth and the cutoff frequency of sound transmission (Fine and Lenhardt 1983, Rogers and Cox 1988, Bass and Clark 2003). The mechanism responsible for the seasonal plasticity of auditory sensitivity in females and type I males is known to be due in part to seasonal fluctuations in circulating steroid hormones; i.e. testosterone and 17B-estradiol (Sisneros et al. 2004a, Sisneros et al. 2004b, Rohmann and Bass 2011, Forlano et al. 2015). In addition, females are known also to exhibit seasonal changes in saccular hair cell density that are concurrent with changes in hair cell sensitivity, which may be another contributing factor to the seasonal increase in auditory sensitivity (Coffin et al. 2012). Another morphological feature that may enhance the auditory sensitivity of the saccule is the mass of the saccular otolith. A recent study of midshipman saccular otoliths revealed that females and type II males have sagittae (saccular otoliths) that are larger and of greater mass than those in type I males (Bose et al 2016). Otoliths with greater mass are thought to improve auditory sensitivity especially at low frequencies; as otolith mass becomes heavier, auditory sensitivity increases and the frequency at which otolith amplitude displacement is greatest shifts towards lower frequencies (Lychakov and Rebane 2000). Thus, the sagittae with greater mass in females and type II males may be another potential structure that affects sound pressure detection, given heavier mass loaded otoliths in females and type II males may be more sensitive to the local particle motion produced by the pressure induced oscillations of the swim bladder during sound exposure. Finally, the increased pressure sensitivity afforded by the horn-like swim bladder extensions in females and type II males should also aid in the localization of sound sources; i.e., vocalizing conspecifics. Recent behavioral experiments that investigated the role of pressure reception by females using the swim bladder during sound source localization revealed that sound pressure reception is likely required for the localization of sound sources (based on the results of females performing phonotaxis to a sound source with intact versus deflated swim bladders) (Coffin et al. 2014). In summary, the predicted enhanced sound pressure sensitivity in females and type II males due to the rostral swim bladder extensions may be yet another potential mechanism that enhances the probability of detection and localization of conspecifics during social behaviors in the reproductive season.

2.5.2 Functional Significance of the Swim Bladder in Type I Males

As with the other sexual phenotypes, type I males produce sound by contracting their sonic muscles that are attached to the swim bladder. Although females and type II males are capable of producing grunts, only type I nesting males can produce sustained grunt trains and long duration growls and hums. The hum or advertisement call can be produced by type I males for over an hour in duration and at relatively high sound levels (Bass et al. 1999). The sound levels of type I male hums have been recorded to be as high as 153-161 dB (re 1 mPa) at or near the entrance of the nests from captive calling males maintained in artificial nests at the Friday Harbor Laboratories on San Juan Island, WA (personal observations, JAS). Overexposure to loud sounds for relative long periods of time can have potentially damaging effects on the auditory system and lead to temporary and/or permanent deficits in auditory sensitivity and perception (Le Prell et al. 2012). The swim bladder shape and distance from the saccule and lagena in type I males may be adaptive in terms of reducing sound overexposure of the inner ear in nesting type I males during the breeding season as has been suggested for males of the closely related oyster toadfish (*Opsanus tau*) which lack swim bladder extensions (Barimo and Fine 1998). Furthermore, experimental deflations of male swim bladders in *O. tau* have revealed no differences in hearing thresholds versus animals with intact swim bladders (Yan et al. 2000) supporting the idea that swim bladders are adapted primarily for sound production in males. The distance between the swim bladder and otoliths of the saccule and lagena in type I males was approximately 1.5 times greater than that in females and type II males. In addition, the normalized swim bladder length of type I males was approximately 17% shorter than that of females and type II males while the normalized swim bladder width of type I males did not differ from that of females and type II males. Thus, the increased distance between the swim bladder and the saccular and lagenar end organs coupled with a smaller normalized swim bladder may act to reduce sound pressure sensitivity and higher frequency stimulation of the inner ear in type I males from sounds that are both self-generated and produced from nearby calling males. While it is possible that type I males have swim bladders of similar shape to females and type II males early in development, future studies that investigate how ontogeny affects swim bladder shape and proximate distance to the inner ear end organs in type I males will be needed to determine if there are life history dependent changes in sound pressure sensitivity in this male sexual phenotype.

2.6 FIGURES

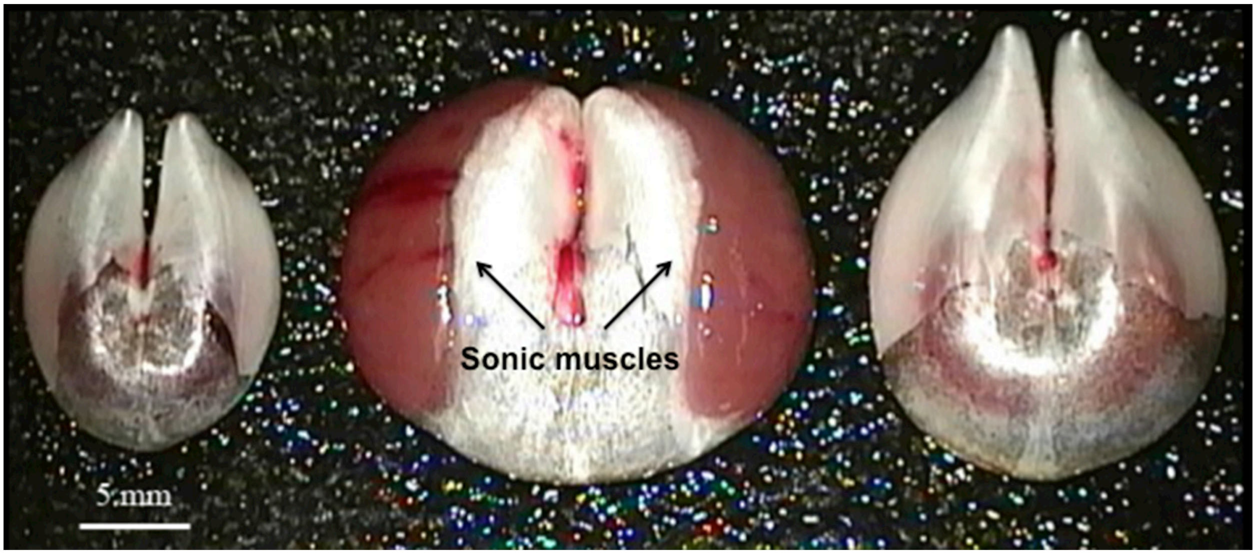

Figure 2.1. **Gross dissections of midshipman swim bladders**. Dorsal view of dissected swim bladders from the three sexual phenotypes of the plainfin midshipman, *Porichthys notatus* of varying standard lengths (SL) (from left to right: type II male (SL = 105mm), type I male (SL = 170mm) and female (SL = 134mm)). Note, type I males have enlarged sonic muscles attached to their swim bladders that are used to produce the seasonal advertisement call while females and type II males have greatly reduced sonic muscles in both size and vasculature.

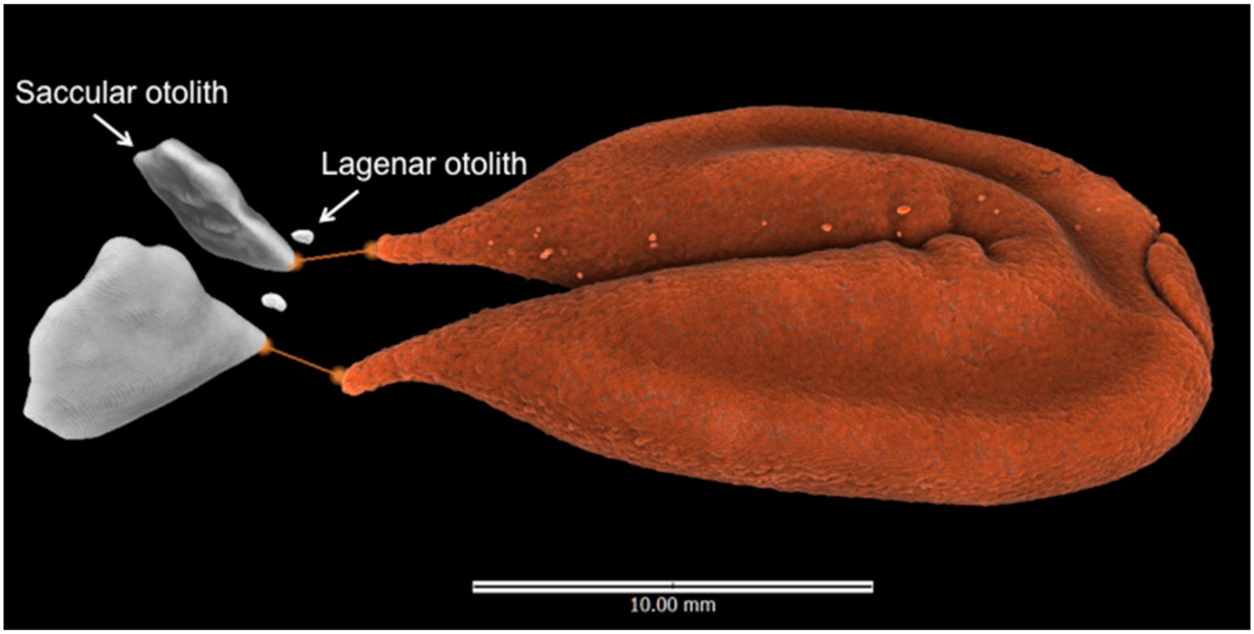

Figure 2.2. **Example of the swim bladder to inner ear distance measurements.** Representative image showing the close proximity of the rostral swim bladder extensions to the inner ear otoliths in the plainfin midshipman. Such images were used to determine the distance between the rostral swim bladder extensions and the otoliths in each of the auditory end organs (only the otoliths of the saccule and lagena are shown in this image while the utricle has been omitted). Note, the lines connecting the swim bladder extensions to the saccular otoliths indicate the calculated distance measurements and are not physical structures.

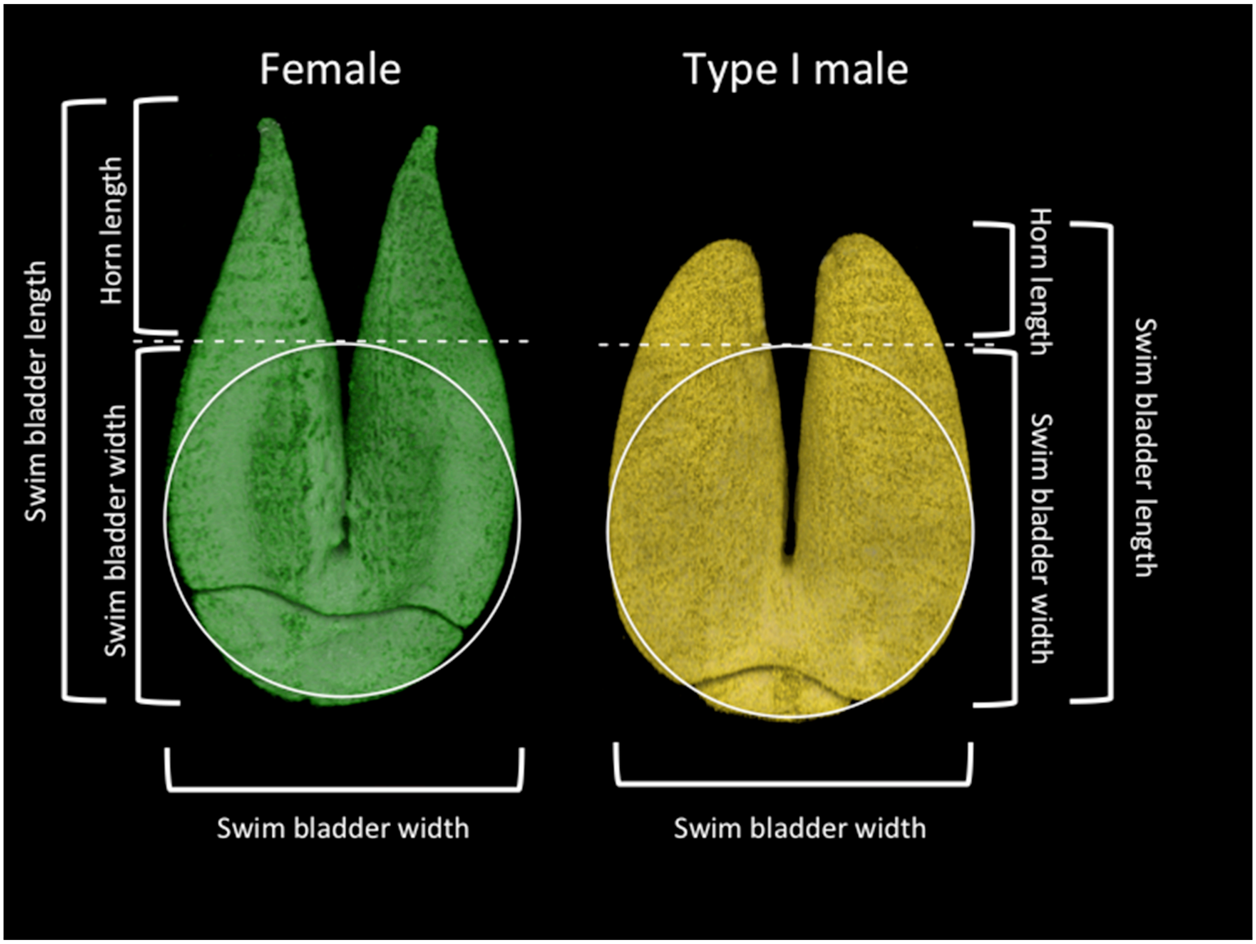

Figure 2.3. **Example of the swim bladder horn length measurements**. Representative images that show the differences in swim bladder morphology between female and type I male plainfin midshipman. Females have horn-like projections on the rostral ends of the swim bladders while type I males lacked such prominent swim bladder extensions. The rostral swim bladder horns were defined as the anterior projections of the swim bladder that projected beyond the primary round shape of the swim bladder and was measured as the difference between the length of the swim bladder minus the width (i.e., diameter of the circle) of the swim bladder. Note that when viewed dorsally, the swim bladder has a primarily round shape as can be seen by the overlaid circles.

Figure 2.4. **Diagram of midshipman swim bladder CT scans**. Representative computerized tomography (CT) scans of female, type I and II male plainfin midshipman that show differences in swim bladder shape and relative proximity of the swim bladder to the inner ear otoliths. The largest otoliths reside in saccule (SO), the main end organ of hearing in this species, while ventro-caudal to the saccular otoliths are the otoliths of lagena (LO), also a putative midshipman auditory end organ, and then medial-anterior to the saccular otoliths are the otoliths of the utricle (UO), a putative vestibular and auditory end organ. These swim bladder CT scans are from animals that varied in size (i.e., standard length (SL)) from 105 to 209 mm (female SL = 134mm, type II SL = 105mm, and type I SL = 209mm).

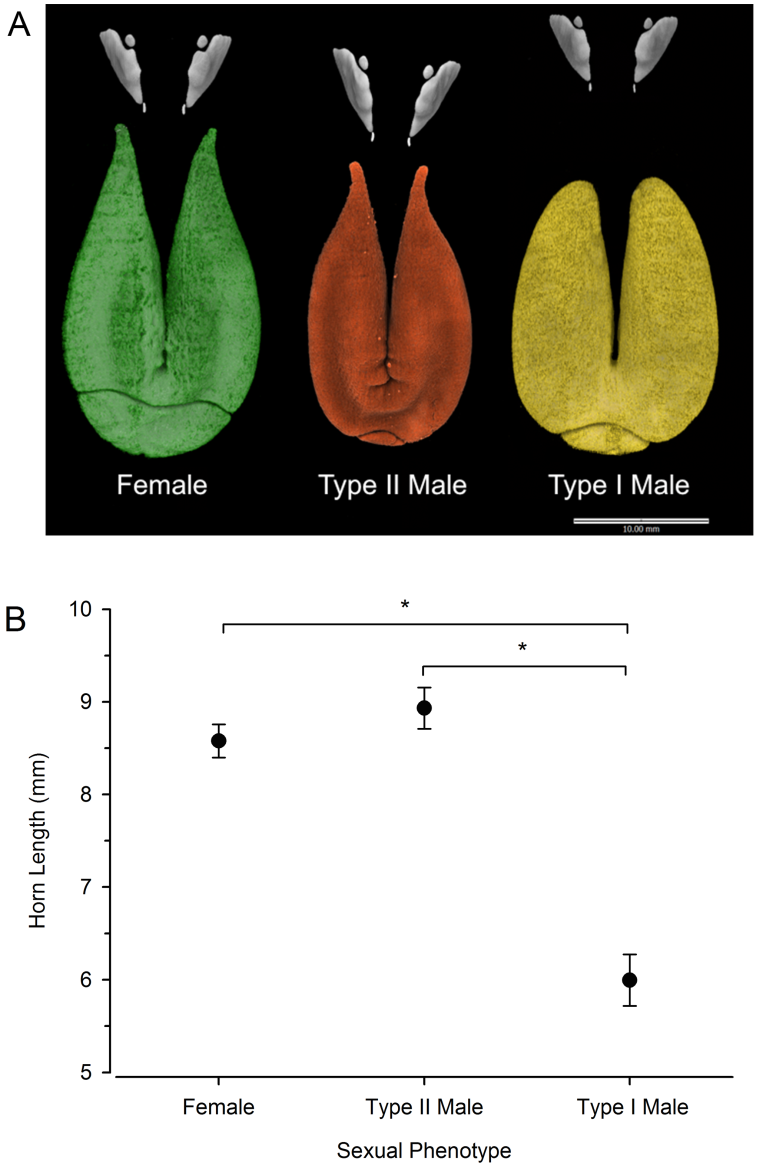

Figure 2.5. **Dorsal microCT scans and horn length measurements**. Micro computerized tomography (microCT) scans and analysis of the swim bladder horn extensions in the sexual phenotypes (females, type I and II males) of the plainfin midshipman. A) Representative microCT scans showing the dorsal view of the swim bladders along with the inner ear otoliths in midshipman females, type I and II males. All three of the scanned sexual phenotypes were similar in size (standard length measurements: female= 119mm, type II male = 118mm and type I male = 125mm). B) Comparison of swim bladder horn length across the three midshipman sexual phenotypes. All data are plotted as mean ± SE, * $p<0.001$.

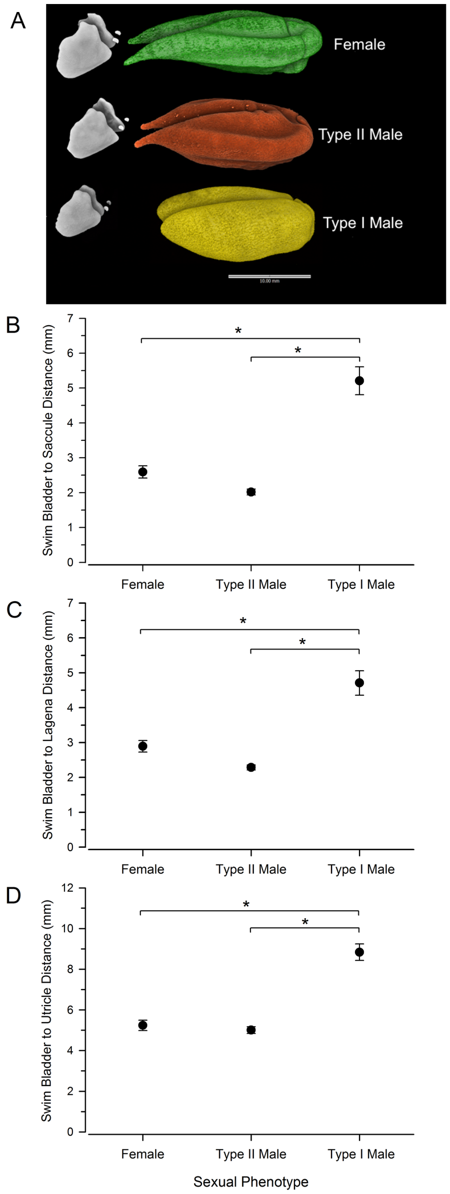

Figure 2.6. **Lateral microCT scans and inner ear distance measurements.** Micro computerized tomography (microCT) scans and analysis of the swim bladder horn extensions and their proximity to the otoliths of the inner end organs (saccule, lagena and utricle) in the plainfin midshipman. A) Representative microCT scans showing a lateral view of the swim bladder and inner ear otoliths. Note that only the otoliths of the saccule and lagena are visible in this lateral view. B-D) Comparisons of the distance between the rostral most points of the swim bladder and the caudal most points of the B) saccular otoliths, C) lagenar otoliths and D) utricular otoliths. Measurements are taken bilaterally and averaged within each animal. All data are plotted as mean ± SE, * $p<0.001$.

Chapter 3. BRAIN ACTIVATION PATTERNS IN RESPONSE TO CONSPECIFIC AND HETEROSPECIFIC SOCIAL ACOUSTIC SIGNALS IN THE PLAINFIN MIDSHIPMAN FISH, *PORICHTHYS NOTATUS*

3.1 SUMMARY

While the peripheral auditory system of fishes has been well studied, less is known about how the fish's brain and central auditory system process complex social acoustic signals. The plainfin midshipman fish, *Porichthys notatus*, has become a good species for investigating the neural basis of acoustic communication because the production and reception of acoustic signals is paramount for this species' reproduction and survival. Nesting males produce long duration advertisement calls that females detect and localize amongst the noise in the intertidal zone to successfully find mates and spawn. How female midshipman are able to discriminate male advertisement calls from environmental noise and other acoustic stimuli is unknown. Using the immediate early gene product cFos as a marker for neural activity, we quantified neural activation of the ascending auditory pathway in female midshipman exposed to conspecific advertisement calls, heterospecific white seabass calls or ambient environment noise. We hypothesized that auditory hindbrain nuclei would be activated by general acoustic stimuli (ambient noise and other biotic acoustic stimuli) whereas auditory neurons in the midbrain and forebrain would be selectively activated by conspecific advertisement calls. We show that neural activation in the rostral intermediate division of the descending octaval nucleus and the ventral division of the secondary octaval nucleus in the auditory hindbrain did not differ via cFos immunoreactive (cFos-ir) activity when exposed to different acoustic stimuli. In contrast, female

midshipman exposed to conspecific advertisement calls showed greater cfos-ir in the midbrain torus semicircularis compared to fish exposed only to ambient noise. No difference in cfos-ir was observed in the torus semicircularis of animals exposed to conspecific versus heterospecific calls. However, cFos-ir was greater in the forebrain central posterior nucleus of the auditory thalamus and in the anterior tuberal hypothalamus of females exposed to conspecific calls versus exposure to either ambient noise or heterospecific calls. Together our findings suggest that neural activation of the midshipman auditory pathway is differentially activated by the exposure to conspecific vs. other stimuli acoustic stimuli. Furthermore, higher order auditory nuclei in the midshipman midbrain torus semicircularis, thalamic central posterior nucleus and in the anterior tuberal hypothalamus may be selectively activated and necessary for the discrimination of complex, species-specific social acoustic signals.

3.2 INTRODUCTION

Acoustic communication is a fundamental component of social behavior across vertebrate taxa. Socially relevant acoustic signals can vary greatly across context- specific behaviors such as during aggression, affiliation and reproduction. The discrimination of biologically relevant acoustic signals from background environmental noise is paramount for appropriate behavioral decision-making. Among vertebrates, fish in general represent perhaps the most ancestral design of the vertebrate auditory receiver system and it is thought that vocal-acoustic communication evolved first in fishes (Bass and McKibben 2003, Bass et al. 2008). Understanding the underlying neural circuitry responsible for the discrimination of behaviorally relevant acoustic signals can potentially provide important insights into the evolution of acoustic communication systems that are conserved across vertebrate taxa.

Among teleosts, the plainfin midshipman fish, *Porichthys notatus*, provides perhaps the best system for studying vocal-acoustic communication because the production and reception of social acoustic signals is necessary for the reproductive success of this species (Bass and McKibben 2003, Bass and Ladich 2008, Forlano et al. 2015). Plainfin midshipman are a nocturnal marine fish found on the west coast of North America that make seasonal migrations from deep off shore sites (>100 m) into the shallow intertidal zone to breed. Type I males excavate nests under rocky shelters from which they contract their sonic swim bladder muscles to produce long duration multiharmonic advertisement calls to attract reproductively receptive females for spawning (Brantley and Bass 1994, Bass and Ladich 2008). Females must be able to detect, discriminate and localize calling type I males amongst the background noise of other soniferous fishes, invertebrates and abiotic factors to successfully locate courting males. The auditory encoding of both conspecific and heterospecific vocalizations likely requires neural mechanisms for signal recognition and discrimination at the level of the midbrain torus semicircularis (TS) and/or higher nuclei in the midshipman auditory pathway, as shown in anurans (Hoke et al. 2004, Hoke et al. 2010), however currently it is unknown how fish discriminate social acoustic signals.

All teleost fishes are thought to be able to detect the particle motion component of underwater sound using their otolithic end organs, which act as biological accelerometers to sense the direct movement of underwater particles relative to the fish. The saccule is thought to be the primary hearing organ in the midshipman as in most other fishes and it is where sound is first transduced and processed before auditory information is sent to hindbrain nuclei in the ascending auditory pathway. Studies in the closely related oyster toadfish, *Opsanus tau*, have shown that neurons in the auditory hindbrain are broadly tuned (Edds-Walton and Fay 2008, Edds-Walton 2016) and that tuning sharpens along the ascending auditory pathway into the midbrain torus semicircularis (Edds-Walton and Fay 2003, Edds-Walton and Fay 2005). Similarly, studies in goldfish and anurans, have revealed that the auditory thalamus is likely involved in the discrimination of complex social acoustic signals and is selectively responsive to species-specific signals (Fuzessey and Feng 1983, Hall and Feng 1987, Mudry and Capranica 1987, Fay and Lu 1995).

The purpose of this study was to characterize neural circuits necessary for the discrimination of complex acoustic signals including conspecific vocalizations. We hypothesized that auditory neurons within major nuclei of the midshipman auditory pathway will be differentially activated by the exposure to ambient noise, conspecific and heterospecific acoustic stimuli. Specifically, we predicted that reproductive females exposed to conspecific advertisement calls would show greater activity of cFos, an immediate early gene product used as a marker for neural activiation, in the midbrain and forebrain auditory nuclei compared to fish exposed to heterospecific vocalizations and ambient noise. Our results support the hypothesis that higher order midshipman auditory nuclei, including the midbrain torus semicircularis, thalamic central posterior nucleus and anterior tuberal hypothalamus, are selectively activated by conspecific vocal signals compared to that of other auditory stimuli (heterospecific vocalizations and ambient noise). Our results are interpreted as they relate to possible auditory mechanisms for conspecific signal recognition and discrimination by the midshipman auditory system during acoustic communication.

3.3 METHODS

3.3.1 Fish collection and housing

Female plainfin midshipman fish, *Porichthys notatus*, were collected by hand during the morning low tides in the intertidal zone at Seal Rock near Brinnon, WA. Fish were housed in aerated 5 gallon buckets with fresh seawater changed every 2-3 hours until experimentation. After dark, fish were transferred to individual buckets with fresh seawater to acclimate for at least 30 minutes prior to testing.

3.3.2 Experimental setup

An experimental playback arena was setup in the intertidal zone at Seal Rock (Figure 3.1). At low tide, a UW-30 underwater speaker (Telex Communications, Burnsville, MN, USA) was buried in the substrate ~20 meters from the high tide line and four 1.5 meter rebar were staked into the ground around the speaker for cage support. A removable cage (diameter 40cm, height 120cm) was positioned and secured directly above the speaker. The speaker was powered by an audio amplifier (TOA BG-1120 amplifier), which broadcast audio sound files from a laptop computer. Prior to testing, a field hydrophone (High Tech Inc. HTI-96, Long Beach, MS, USA) and recorder (Zoom H2, Hauppauge, NY, USA) were used to measure ambient noise levels of the testing arena in the natural acoustic environment and calibrate the playback sound levels such that average peak-to-peak amplitude of the acoustic stimuli was adjusted to 130db re: 1µPa at the outer edge of the testing arena. Experiments were then commenced at night after dark when the underwater speaker was submerged 50 cm or greater by the rising or falling tide, and then one female midshipman fish was gently placed into the arena for testing. Water depth at the testing arena was measured at the start and conclusion of each auditory playback experiment, as was water temperature.

3.3.3 Acoustic Stimuli and Playback Procedures

Female midshipman fish were exposed to one of three acoustic stimuli: conspecific advertisement calls, heterospecific calls of white seabass, and ambient environmental noise. The playback of conspecific advertisement calls consisted of a 30 minute looped audio file containing acoustic recordings from 7 male midshipman advertisement calls or “hums” (Brantley and Bass 1994) previously recorded *in situ* from calling type I male midshipman nests at Seal Rock. The audio files were equalized to the same maximum peak-to-peak sound level in MatLab to account for any differences in amplitude between individual male callers (Figure 3.2A). Previous work by Brantley and Bass (1994) showed that the fundamental frequency of male advertisement calls increases with temperature and that female preference is tightly coupled to match the appropriate fundamental frequency across temperatures (McKibben and Bass 1998). To account for the daily fluctuations in water temperature, we linearly shifted the fundamental frequency (along with the harmonics) of the advertisement call stimuli in Matlab to compensate for temperature differences at time of playback. The heterospecific call was a 30 minute looped audio file containing recordings from white seabass (*Atractoscion nobilis*) (Figure 3.2B). The white seabass is a soniferous fish found on the west coast of the United States and is known to be sympatric with the plainfin midshipman (Aalbers and Drawbridge 2008). There is no known predator-prey interaction between these species and theoretically the sounds of the white seabass should represent a familiar and innocuous biotic background sound to plainfin midshipman. The control condition consisted only of the background environment noise present in the intertidal zone during the experiment without the experimental playback of conspecific or heterospecific calls (Figure 3.2C).

After 30 minutes of continuous exposure to one of the three acoustic stimuli, the subjects were removed from the testing cage and were then kept individually in a 5 gallon bucket filled with water from the intertidal for an additional 120 minutes before being sacrificed. The 120 minute post-treatment time before sacrifice was chosen to allow adequate cFos synthesis after sound exposure based on the work by Petersen et al. (2013) and Forlano et al. (2017). Fish were first deeply anesthetized in a 0.025% aminobenzoate bath after which they were weighed and measured for standard length (SL) before being transcardial perfused with ice cold teleost ringers followed by 4% paraformaldehyde in 0.1 M Phosphate buffer (PB; pH 7.2). Brains were harvested and post-fixed for 1 hr before being rinsed 3X in 0.1M PB and stored at 4°C until cryo-sectioned. Prior to sectioning brains were transferred to 30% sucrose in a 0.1M PB solution for 24-48 hrs before being sectioned at 25 μm in two alternating series. Only one series was used for analysis in this experiment..

3.3.4 Immunohistochemistry

Slides were brought to room temperature and then the perimeter of each slide was traced with a hydrophobic pen and soaked 3X for 10 minutes in phosphate buffered saline (PBS; pH 7.2), followed by a 1 hour soak in a blocking solution made of PBS with 0.3% Triton X-100 and 10% normal donkey serum (PBS-DS). Following the blocking procedure, slides were incubated for 16-17 hours at room temperature in PBS-DS containing rabbit anti-cFos (1:2000, Santa Cruz Biotechnology lot# C2510) and mouse anti-Hu (1:2000, Molecular Probes). After incubation, slides were briefly dipped (to remove the majority of the antibody solution) and then rinsed in

PBS + 0.5% normal donkey serum 5X for 10 minutes. The slides were then incubated for 2 hours at room temperature with PBS-DS containing donkey anti-rabbit conjugated to Alexa Fluor 568 (1:200, Life Technologies) and donkey anti-mouse conjugated to Alexa Fluor 488 (1:200, Life Technologies). Finally, slides were dipped and rinsed 4X for 10 minutes in PBS before being cover slipped using ProLong Gold with DAPI and then allowed to cure for 48-72 hours in a dark room. Once dry, the slides were sealed with nail polished and stored at 4°C.

3.3.5 Image Acquisition

Micrographs were obtained on an Olympus BX61 epifluorescence compound microscope using MetaMorph imaging and processing software. All auditory nuclei were identified at low magnification using the GFP and DAPI filters to visualize the neurons and their nuclei before being photomicrographed using a 20X objective. Exposure times and light levels were held constant for each channel across all conditions. Each micrograph was imaged consecutively starting with Texas Red, followed by GFP and DAPI filter sets, respectively. Images were taken in z-series by setting a top and bottom focal plane in the GFP channel with a stack thickness of 1 µm. The stacked photomicrographs were combined into a single projection image using the Z projection maximum intensity feature in ImageJ. cFos immunoreactive (cFos-ir) neurons were quantified manually using a custom ImageJ macro, individual background thresholds were determined for each image and cFos-ir cells were confirmed by comparing across the neuron specific anti-Hu and nucleus specific DAPI channels. To account for the variation in auditory nuclei sizes, similar strategies for analysis were adopted from Petersen et al. (2013). The average number of cFos-ir neurons per section per nucleus was reported for each animal across the sound exposure groups. All cFos-ir quantification was done via experimenters who were blind to the exposure conditions.

3.3.6 Central Acoustic Circuitry

The rostral intermediate division of the descending octaval nucleus (DOri) and ventral division of the secondary octaval nucleus (SOv) receive connections from the saccule and were the two auditory hindbrain nuclei analyzed (Figure 3.3B). While there are several unique subdivisions of the descending octaval nucleus that receive saccular projections (Bass et al. 2000, Sisneros et al. 2002) previous preliminary studies have revealed greatest cFos activation within DOri (RM and PF, personal observations). The caudal extent of DOri was determined by its appearance at the level of the octaval efferent nucleus and sampled rostrally till its disappearance. SOv lies just medial and ventral to DOri throughout most of its extent (slight variations are present due to the angle at which brains were mounted and sectioned). While the secondary octaval nucleus also has a dorsal division, previous preliminary examination revealed less activation in response to auditory stimuli (RM and PF, personal observation). All landmarks were determined by previously published neuroanatomical and physiological studies and the sampling techniques replicated from Petersen et al. (2013). Serial sections of DOri and SOv were sampled unilaterally on the right side throughout their entire extent. No left/right differences in hemispheric brain activation were predicted because fish were allowed to move freely above the underwater playback speaker. On average 8.67 (±2.2 SD) and 7.81 (±1.7 SD) sections were quantified for DOri and SOv, respectively.

In the midbrain, the nucleus centralis of the torus semicircularis (TSnc) was the major auditory region imaged for cFos-ir activity (Figure 3.3C). Landmark and image acquisition for TSnc was held constant as with Petersen et al. (2013) and we sampled every fourth section with two adjacent images to encompass the entire nucleus. Similar to the hindbrain photomicrographs, TSnc images were taken only on the right side of the brain for all treatment groups. On average, 6.32 (± 1.3 SD) sections were analyzed in TS per animal. The TS sends projections to the central posterior nucleus of the thalamus (CP) in the auditory forebrain (Figure 3.3D). The compact division of CP (CPc) is defined by a wing-shaped nucleus adjacent to the midline. While there is a diffuse region of CP (CPd) just lateral and ventral to CPc, only CPc was imaged for analysis due to the lack of clear boundaries in CPd. Photomicrographs of CPc were taken serially on the right side. In CP, 5.1 (±0.9 SD) sections were analyzed on average per animal. Finally, TS also sends projections to the anterior tuberal nucleus of the ventral hypothalamus (AT). Photomicrographs of AT were also taken serially from the right side of the brain. Within AT, cFos-ir activity was quantified in 3.05 (± 0.6 SD) sections on average.

3.3.7 Statistics

Numbers of cFos-ir cells were averaged within each auditory nucleus and compared between playback condition groups using a one-way ANOVA with an alpha set at 0.05. Post-hoc Tukey tests were used to make pair-wise comparisons across groups for each nucleus. Correlations between water temperature and cFos-ir activity were conducted and the Benjamini-Hochberg procedure was used to correct for multiple comparisons with a false discovery rate of 0.25 (Butler and Maruska 2016, Forlano et al. 2017). All statistics were analyzed using IBM SPSS Statistics 19 and GraphPad Prism 5 software.

3.4 RESULTS

A total of 39 female midshipman were used in this study: 15 animals were used in each of the ambient noise and conspecific playback conditions while nine animals were used in the heterospecific playback condition. Fish exposed to ambient noise had a standard length (SL) of

16.54 ± 1.57 cm (mean ± SD), body mass (BM) of 59.48 ± 17.61 g and a gonadosomatic index (GSI) of 22.07 ± 10.54. Fish exposed to conspecific advertisement calls had a SL of 16.43 ± 1.45 cm, BM of 61.95 ± 15.28 g and GSI of 26.53 ± 8.26. Fish exposed to heterospecific vocalizations had a SL of 16.50 ± 0.96 cm, BM of 60.57 ± 14.14 g and GSI of 23.77 ± 5.24. There were no differences between groups for any of the morphometric data analyzed ($p>0.38$ for all cases).

Brain activation of auditory nuclei was examined at the level of the hindbrain (DOri and SOv), midbrain (TS) and diencephalic forebrain (CP and AT) using cFos as a proxy for neural activity. Fluctuations in water temperature during the experiments on different nights ranged from 12-16°C with an average of 14.52 °C (± 1.3 SD). There was no correlation between water temperature at the testing site and cFos-ir activation ($p>0.05$ for all cases).

Neural activation of the hindbrain auditory nuclei did not vary with exposure to the tested acoustic stimuli. The average number of cFos-ir cells in the hindbrain DOri did not vary with acoustic playback stimuli: conspecific advertisement calls (mean = 2.05 ± 1.64 SD), heterospecific vocalizations (mean = 2.22 ± 0.85 SD) and ambient noise (mean = 1.73 ± 1.26 SD), (one-way ANOVA, $F(2, 34) = 0.37$, $p=0.69$) (Figure 3.4). There was also no difference in average cFos-ir neurons between groups for SOv: conspecific (mean = 2.73 ± 1.99 SD), heterospecific (mean = 1.92 ± 0.82 SD) vocalizations and ambient noise (mean = 2.12 ± 1.40 SD) (one-way ANOVA, $F(2, 34) = 0.84$, $p=0.44$; Figure 3.4). There were no differences in the number of sections analyzed within each nucleus between any of the experimental groups ($p>0.05$).

Midbrain neurons were differentially activated by exposure to conspecific advertisement calls versus ambient noise. Specifically, in the midbrain TSnc, there was a significant difference in average cFos number across groups and pairwise comparisons revealed that there were more cFos-ir neurons ($p<0.05$) with conspecific signal exposure (mean = 35.18 ± 7.21 SD) compared to the ambient noise exposure (mean = 25.89 ± 8.14 SD) (one way ANOVA, post hoc Tukey Test, F (2,34) = 4.88, $p=0.013$); however, no differences were found between the ambient noise and heterospecific signal exposure groups ($p=0.90$; mean = 27.47 ± 11.02 SD) or between the conspecific and heterospecific exposure groups ($p=0.096$) (Figure 3.5). There were significant differences in the average number of cFos-ir neurons between playback conditions within the forebrain nucleus CPc and pairwise comparisons revealed there were significantly more cFos-ir neurons in the conspecific signal exposure group (mean = 36.63 ± 8.90 SD) group compared to both the ambient noise (mean = 27.08 ± 9.29 SD, $p<0.05$) and heterospecific signal exposure (mean = 17.89 ± 5.47 SD, $p<0.001$) groups and the ambient noise group had significantly more cFos-ir neurons ($p<0.05$) than the heterospecific signal exposure group (one way ANOVA, post hoc Tukey Test, F (2,35) = 13.90, $p<0.001$) (Figure 3.6).

Forebrain auditory nuclei were differentially activated by exposure to the auditory stimuli presented. In the forebrain nucleus AT, there was a significant difference in average cFos-ir neurons between playback conditions and pairwise comparisons revealed that females exposed to the conspecific advertisement call (mean = 61.09 ± 19.23 SD) had a significantly higher average number of cFos-ir neurons compared to both ambient noise (mean = 45.80 ± 16.21 SD, $p<0.05$) and heterospecific signal exposure (mean = 41.80 ± 12.50 SD, $p<0.05$) groups (one way

ANOVA, post hoc Tukey Test, F (2,36) = 4.80, p=0.014). There was no difference between the average number of cFos-ir cells between the ambient noise and heterospecific signal exposure groups (p=0.84) (Figure 3.6).

3.5 DISCUSSION

The goal of this study was to identify the neural circuits involved in the recognition and discrimination of conspecific social acoustic signals in female midshipman. Using the immediate early gene (IEG) protein product cFos as a marker or proxy for neural activiation, we mapped cFos activity of auditory neurons in the ascending auditory pathway of females held in a semi-naturalistic playback arena while exposed to either conspecific or heterospecific vocalizations or ambient noise. The use of IEG responses, including cFos, have become a powerful tool for mapping neuronal activation patterns as they can be used to assess the entire brain and the IEG response begins within minutes (Luckman et al. 1994, Clayton 2000, Kovacs 2008). Consistent with our hypothesis that exposure to complex acoustic signals would yield differential neural activation across auditory nuclei, our results revealed greater activation in response to conspecific vocalizations compared with ambient noise at the level of the midbrain and greater activation in response to conspecific vocalizations compared with both heterospecific calls and ambient noise in the forebrain. Our results suggest higher order processing is likely necessary for the processing and discrimination of complex social signals in teleosts, consistent with studies in tetrapods.

3.5.1 cFos-ir Response in the Hindbrain

The rostral intermediate division of the descending octaval nucleus (DOri) and the ventral division of the secondary octaval nucleus (SOv) are primary auditory areas of the auditory hindbrain that receive direct innervation from the VIIIth nerve and the auditory end organs (McCormick 1999, Bass et al. 2000, Sisneros et al. 2002, Tomchik and Lu 2005). Neurons within DOri and SOv revealed similar activation levels across the three experimental conditions: ambient noise, conspecific and heterospecific vocalizations. These results are consistent with previous findings in toadfish, *Opsanus tau*, which have revealed nuclei of the auditory hindbrain are broadly tuned and respond to a wide array of acoustic stimuli, including general acoustic stimuli and vocal signals alike (Edds-Walton and Fay 2008, Edds-Walton 2016). Similarly, in the clawed frog, *Xenopus laevis,* first-order hindbrain auditory nuclei have been shown to respond broadly to auditory stimuli (Elliott et al. 2007) and it is hypothesized that the midbrain torus semicircularis (TS) is likely the initial location of auditory filtering for social acoustic signals (Hoke et al. 2004). Midshipman fish receive auditory input from their main end organ of hearing, the saccule, which has been shown to undergo seasonal changes in morphology and auditory sensitivity related to reproductive state modulated by steroid hormones (Sisneros et al. 2004a, Sisneros 2009, Rohmann and Bass 2011, Coffin et al. 2012, Rohmann et al. 2013, Forlano et al. 2015b, Forlano et al. 2016). Specifically, reproductive females, like those used in this study, have increased hearing sensitivity across their entire frequency range to better detect and localize advertising male midshipman fish. Recent studies have also revealed that females have elongated horn-like structures on the rostral ends of their swim bladders, which come into close proximity to the saccule and lagena, which are thought to increase sensitivity to sound pressure and high frequency signals (Mohr et al. 2017). Together these adaptations in female midshipman fish may lead to heightened auditory sensitivity across all frequencies, especially during the reproductive season. Abiotic noise factors along with biotic sounds from other species can cause the intertidal zone to be a very loud and noisy environment (up to 120dB re: 1uPa, personal observation) and such environmental noise levels are well within the hearing range of midshipman fish and are likely a constant source of stimulation to the midshipman auditory system. Furthermore, DOri is also an important part of the vocal-acoustic circuitry in male midshipman fish, as it has direct descending connections to the prepacemaker nucleus of the vocal pattern generator (Bass et al. 1994, Bass et al. 2000, Bass and Ladich 2008). Female midshipman fish are incapable of producing the reproductive advertisement call and the differences in cFos-ir activity in DOri reported by Petersen et al. (2013) in response to conspecific vocalizations versus ambient noise in male midshipman fish, may be a sex-specific difference related to mate call production.

3.5.2 cFos-ir Response in the midbrain and forebrain

The TSnc receives direct innervation from the auditory hindbrain and is the primary auditory center within the midbrain of midshipman and other vertebrates (Bass et al. 2000, Bass et al., 2005). Our results revealed greater cFos-ir activation within TSnc in response to conspecific vocalizations compared to ambient noise but no difference when compared to the heterospecific playback condition. The TS is the anamniote vertebrate homologue to the mammalian inferior colliculus, an area that has been implicated in selectivity to species-specific auditory signals (Echteler 1984, Feng and Lin 1991, Hoke et al. 2004, Klug et al. 2002, Suta et al. 2003, Syka et al. 1997). Subpopulations of auditory neurons within the inferior colliculus have also been theorized to be selectively responsive to a wide array of biologically relevant acoustic stimuli including sounds made by both conspecifics and heterospecifics (Casseday and Covey 1996). Our findings are consistent with the theories of Casseday and Covey (1996) along with results of Petersen et al. (2013), which showed greater cFos-ir activation in male midshipman TSnc in response to conspecific advertisement calls compared to ambient noise in an outdoor laboratory tank. Similarly, our findings also parallel the egr-1 activity quantified in response to auditory playback of conspecific and heterospecific vocalizations in the Tungara frog that showed greater activation to variations of conspecific advertisement calls (whines, chucks and whine-chucks) in TS compared to both silence and heterospecific vocalizations (Hoke et al. 2004). However, unlike the anuran TS which has several distinct toral subdivisions with unique cytoarchitecture and connectivity related to processing of auditory information, the nc is the major auditory center within the midshipman TS (Bass et al. 2000, Potter 1965, Wilczynski 1981, Endepols and Walkowiak 2001, Hoke et al. 2004).

The central posterior nucleus in the auditory thalamus is a major area of higher order processing of auditory information in midshipman and other teleosts (Bass et al., 2000, McCormick, 2011) and receives projections from TS) (Bass et al. 2000, Goodson and Bass 2002, McCormick 1999). Our results from the compact division of CP (CPc) revealed greatest cFos-ir activation in response to conspecific vocalizations compared to both the ambient noise and heterospecific playback conditions. There was also greater cFos-ir activity in the ambient noise condition compared to the heterospecific condition. Previous single unit physiological studies in goldfish, *Carassius auratus*, have suggested that CP may be involved in the processing of complex, wide bandwidth social-acoustic stimuli (Lu and Fay 1995) and along with TS may be a source for the discrimination of conspecific acoustic signals. Earlier work in anurans has also shown characteristics of species-specific selectivity of neurons in the auditory thalamus (Fuzessey and Feng 1983, Hall and Feng 1987, Mudry and Capranica 1987). However, as has been suggested more recently in both teleost fish and anurans, it is likely that CP and other forebrain areas are not solely auditory regions but highly integrative, receiving multisensory (auditory and visual) input with implications for sensorimotor responses (Wilczynski and Endepols 2007, Northcutt

2006, Hoke et al. 2007). Results from Petersen et al. (2013) also showed extreme differences in cFos-ir activity in CP with male midshipman fish exposed to conspecific vocalizations exhibiting much higher levels of cFos compared to males in the ambient noise condition. While the quantitative response properties in CP are similar in both sexes it is possible that the sensorimotor and physiological responses vary greatly between the sexes as they relate to aggression and reproduction, respectively. Future physiological and behavioral studies will be needed to further understand the role of CP In the processing of auditory information and how it relates to sensorimotor responses.

The central posterior nucleus (CP) shares reciprocal connections with the anterior tuberal nucleus (AT) of the hypothalamus, which also receives ascending projections from TSnc (McCormick 1999, Bass et al. 2000, Goodson and Bass 2002). Our results revealed greater cFos-ir activation in AT in response to conspecific compared with both ambient noise and heterospecific acoustic playbacks. Our results are similar to that of Petersen et al. (2013) in which they showed higher levels of cFos in AT in response to conspecific vocalizations compared to ambient noise. AT is not only a part of the ascending auditory system but also part of the descending vocal-motor circuitry (Bass et al. 2000, Goodson and Bass 2000a-b, Goodson and Bass 2002) and the social behavior network (SBN) (Newman 1999, Goodson 2005). The SBN is a group of reciprocally connected nuclei within the midbrain and basal forebrain that are involved in the processing, assessment and action of various social behaviors (Newman 1999, Goodson 2005, Goodson and Kabelik 2009). The anterior tuberal nucleus (AT) along with the ventral tuberal hypothalamus are thought to be homologous, in part, to the ventral medial hypothalamus (VMH) and anterior hypothalamus (AH) in mammals and birds (Goodson 2005, Newman 1999, O'Connell and

Hofmann 2011). Lesion and stimulation studies have revealed that VMH is an important area involved in sexual behavior and female receptivity in rodents and birds (Malsbury et al. 1977, Mathews and Edwards 1977, Pfaff and Sakuma 1979, Meddle et al. 1999, O'Connell and Hofmann 2011). Future studies will be needed to further understand the role of AT in social behavior and central auditory processing in fishes.

3.5.3 Candidate Neuromodulators of the Central Auditory System in Midshipman

Steroid Hormones

Sex steroid hormones have been shown to change the perception or valence of auditory signals and previous findings in the seasonally breeding white-throated sparrows, *Zonotrichia albicollis*, showed that estrogen can modulate the responses of neuronal populations in the SBN, including VMH, in response to conspecific vocalizations (Maney et al. 2008). Similarly, plainfin midshipman fish are seasonal breeders and undergo seasonal changes in reproductive steroid hormones in preparation for reproduction (Sisneros et al. 2004b). Prior to the breeding season, testosterone (T) and estradiol (E) spike in females as their gonads recrudesce in preparation for spawning (Sisneros et al. 2004b). Both male and female midshipman fish have been shown to undergo seasonal changes in their hearing sensitivity at the level of auditory periphery (Bhandiwad et al. 2017, Rohmann and Bass 2011, Sisneros and Bass 2003, Sisneros 2009). Experimental implantation of T and E in non-reproductive and gonadectomized female midshipman have been shown to have similar hearing abilities of reproductive females, supporting the role of steroid hormones in the seasonal plasticity of midshipman audition, at least at the level of the periphery (Sisneros et al. 2004a). Although it is unclear if seasonal changes in steroid hormones directly affect central auditory processing, TS, AT and CP all express androgen and estrogen receptors and the latter two are also replete with aromatase, the enzyme that converts T to E (Forlano et al. 2006, Forlano et al. 2015b, Forlano et al. 2016). Therefore, steroid hormones may modulate higher auditory nuclei to better encode seasonal reproductive-related vocalizations, similar to their effects in the periphery.

Catecholamines

Catecholamines (CA) are well known neuromodulators that can affect attention, motivation and arousal across vertebrate taxa (Berridge 2008, Hurley et al 2004, Riters 2012). Recent studies suggest that seasonal changes in circulating gonadal steroid levels can regulate brain CA levels that act to enhance the valence of conspecific vocal signals (Caras 2013, Maney 2013). Recent studies in midshipman have described robust CA innervation in the central and peripheral auditory system (Forlano et al. 2014, Forlano et al. 2015a, Perelmuter and Forlano, 2017). Immunoreactive tyrosine hydroxylase (TH-ir) fibers were used as a marker for CAs, as TH is the rate limiting enzyme in CA synthesis. TH-ir fibers are present throughout the saccule and nuclei of the central auditory system including SOv, TSnc, CP and AT (Forlano et al. 2014). Interestingly, reproductive female midshipman have increased TH-ir levels in CP which likely receive projections from the dopaminergic periventricular posterior tuberculum (TPp), an area known to have high levels of aromatase, androgen and estrogen receptors (Forlano et al. 2001, Forlano et al. 2005, Forlano et al. 2010). TPp also sends TH-ir projections to the saccule, which could act to modulate sensitivity of the inner ear in response to conspecific vocalizations (Forlano et al. 2014, Forlano et al. 2015a). Together these findings suggest a role for CAs, in particular dopamine, in altering the salience of species-specific acoustic information and appropriate behavioral responses related to reproduction in female midshipman fish. Our results revealed that CP and AT are differentially activated by conspecific versus heterospecific vocalizations, suggesting these brain areas may be a part of a circuit for species-specific recognition. Future physiological studies are needed to further understand the processing and perception of social acoustic signals in CP and AT and how CAs and steroid hormones may influence the salience and recognition of conspecific social signals in plainfin midshipman fish.

3.6 FIGURES

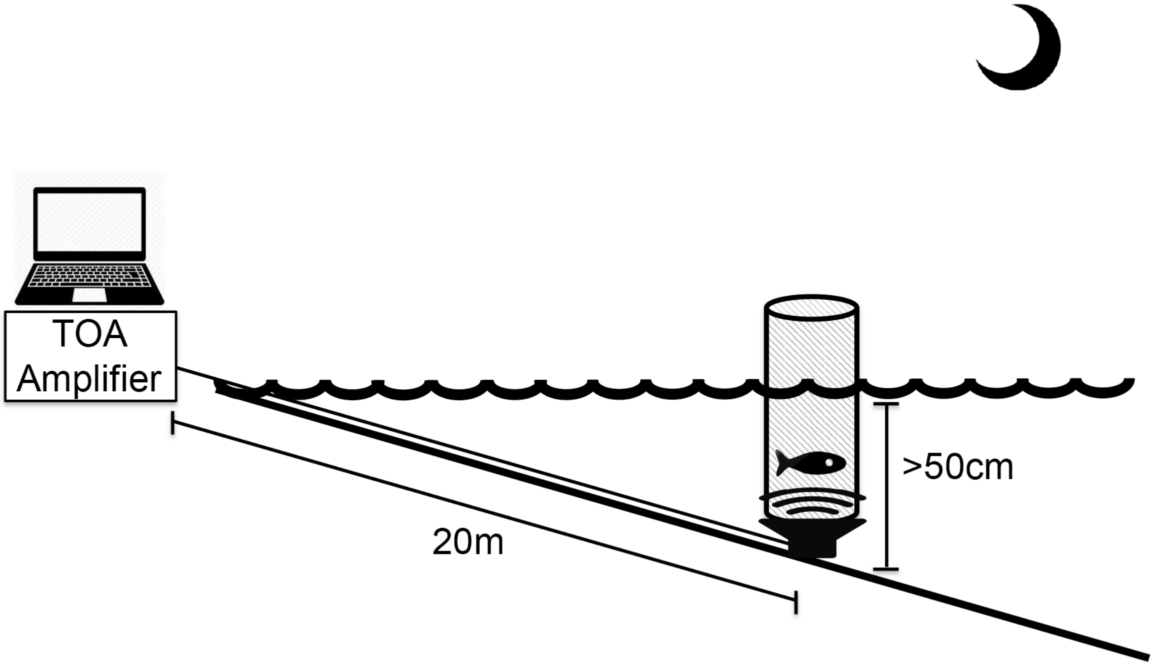

Figure 3.1. **Schematic of the intertidal playback arena**. A schematic representation of the playback paradigm used during auditory exposure. An underwater speaker was buried in the substrate ~20 meters from the shoreline, where it was powered by a TOA amplifier which received acoustic playback files from a laptop computer. Above the speaker, a mesh cage (diameter = 40cm) was suspended in place where the fish was allowed to swim freely during exposure to auditory playback. Experiments were conducted each night after sunset when the cage was covered at least 50cm by the tide. The sound pressure level of the acoustic stimuli was calibrated to 130db re: 1μPa at the perimeter of the cage.

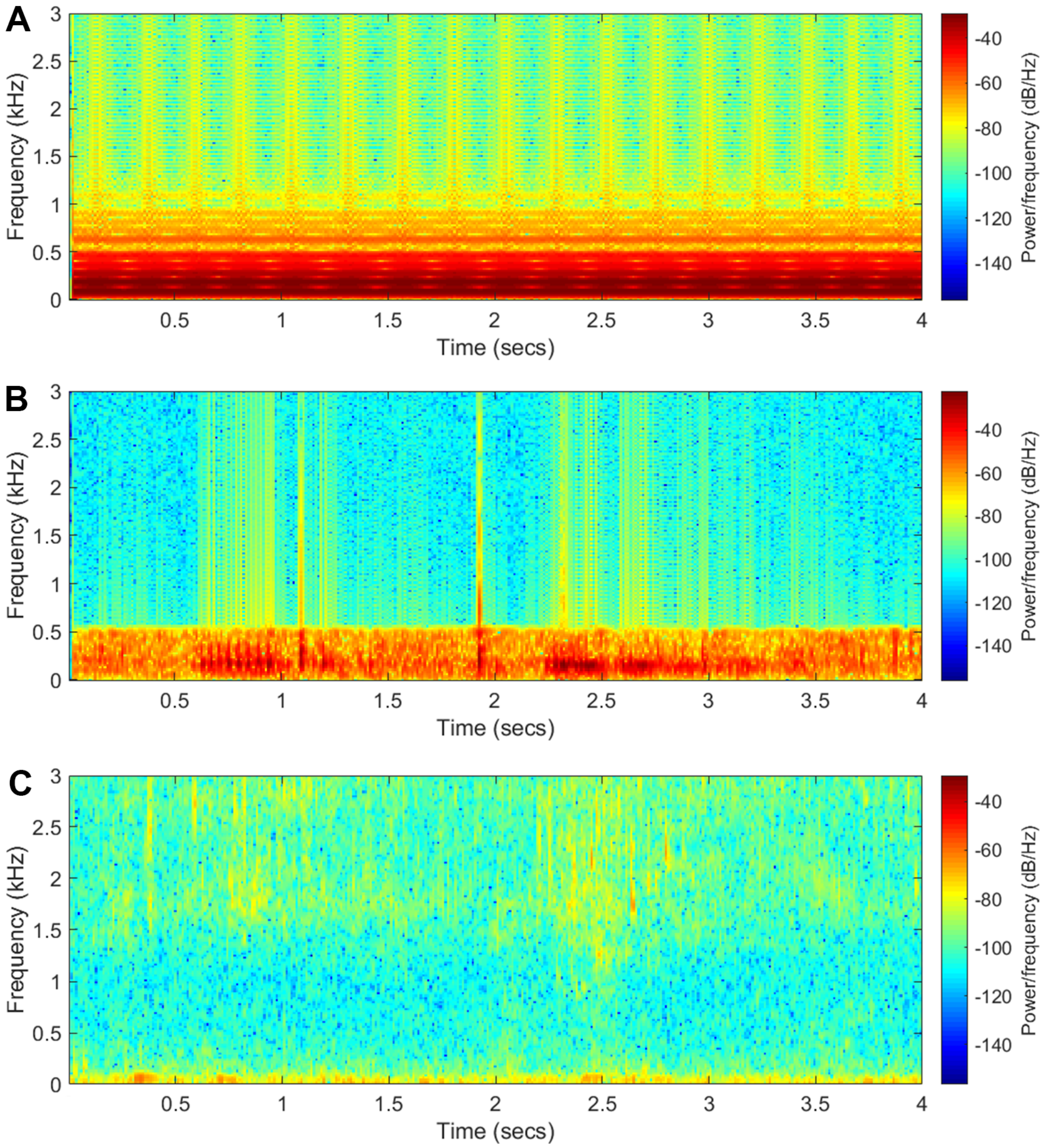

Figure 3.2. **Spectrograms of midshipman and seabass calls**. Representative spectrograms of the sound stimuli used during auditory playback. The advertisement call of the plainfin midshipman (A) is long in duration and primarily sinusoidal while the white seabass calls (B) are short duration pulses produced at random intervals and the ambient noise (C) condition lacks any prominent components.

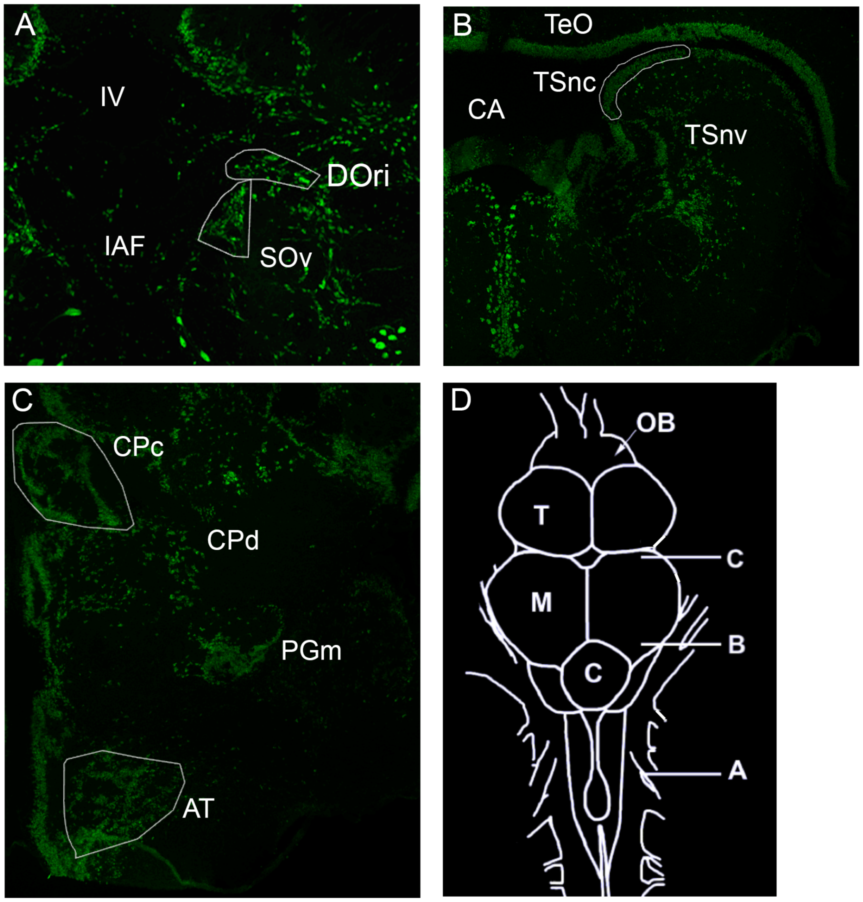

Figure 3.3. **Auditory neuroanatomy of the plainfin midshipman**. Transverse sections with anti-Hu (green) stain showing neuronal cell bodies. Traced areas in white represent auditory nuclei in which cFos-ir neurons were quantified. A) The hindbrain auditory nuclei, rostral intermediate division (DOri) and ventral division of the secondary octaval nucleus (SOv) are shown in. B) The nucleus centralis of the midbrain torus semicircularis (TSnc). C) The compact division of the central posterior nucleus in the auditory and thalamus (CPc) and the anterior tuberal nucleus of the hypothalamus (AT). D) Dorsal view drawing of the midshipman brain with the relative positions of A-C indicated. Abbreviations: Cerebellum (C); Cerebral aqueduct (CA); Diffuse division of the central posterior nucleus (CPd); Internal arcuate fiber tract (IAF); Fourth ventricle (IV); Midbrain (M); Olfactory Bulb (OB); Medial nucleus preglomerulosus

(PGm); Telencephalon (T); Optic tectum (TeO); Torus semicircularis nucleus ventrolateralis (TSnv).

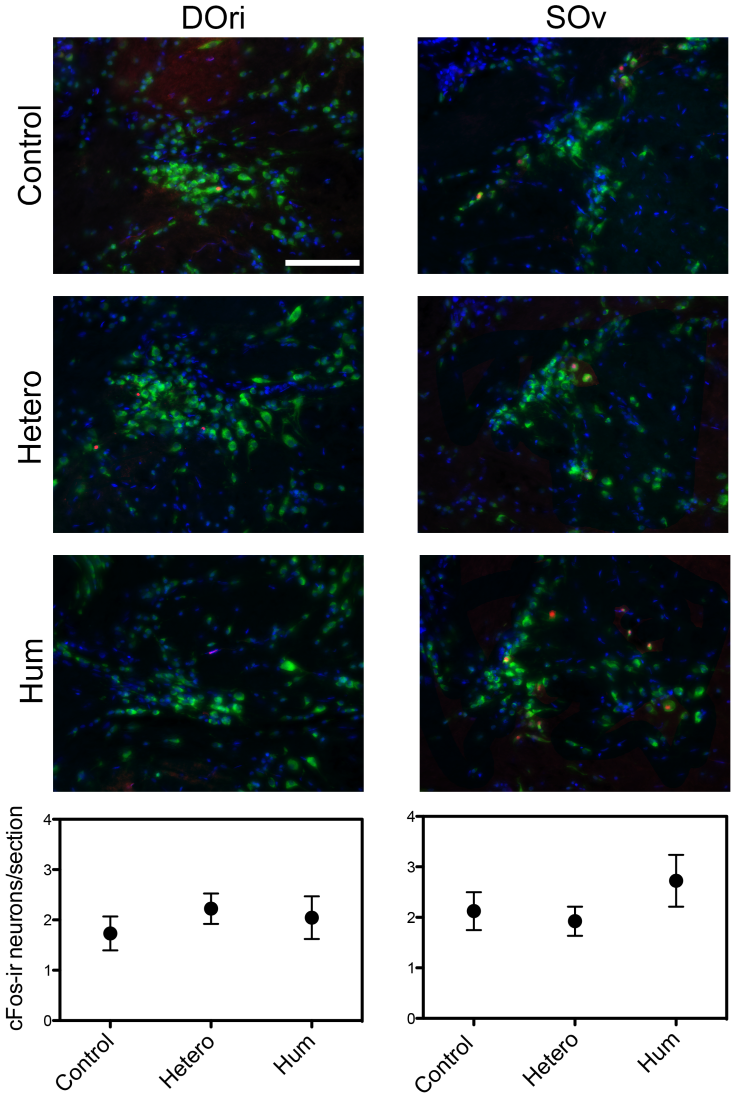

Figure 3.4. **Neuronal activation in the auditory hindbrain**. Representative photomicrographs taken at 20X in the rostral intermediate division of the descending octaval nucleus (DOri) and in the ventral division of the secondary octaval nucleus (SOv). cFos-ir cells (red) were quantified as a marker for neuronal activity. The neuron specific stain anti-Hu (green) and the counterstain DAPI (blue) were used to confirm that the cFos-ir signal was neuron specific. Fish were randomly assigned to exposure of ambient sound (control), white seabass calls (hetero) or

conspecific male advertisement calls (hum). Graphs show the averaged number of cFos-ir neurons per section across the experimental conditions. Scale bar = 100μm.

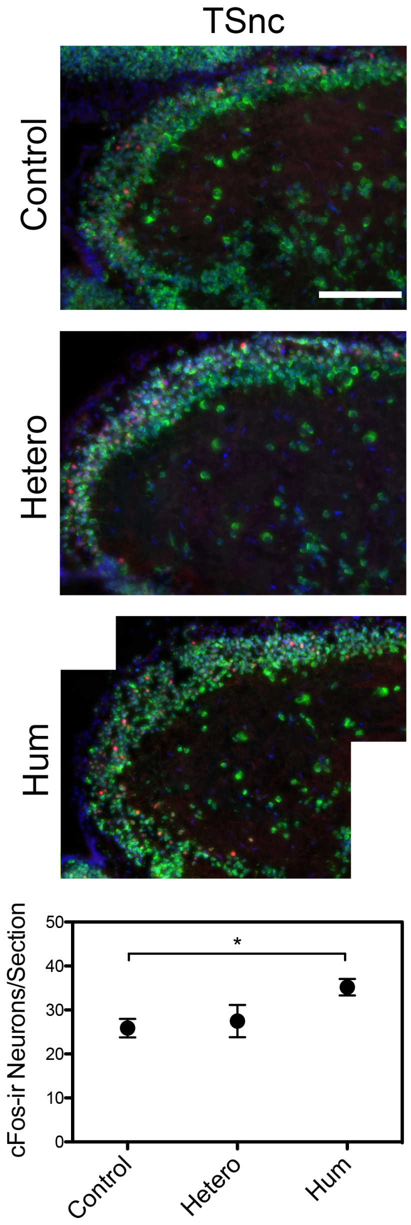

Figure 3.5. **Conspecific playback yields higher activation in the midbrain**. Photomicrographs of the periventricular nucleus centralis of the torus semicircularis (TSnc) in the midbrain. Images were taken at 20X and are representative of the three experimental exposure conditions: ambient sound (control), white seabass calls (hetero) and conspecific advertisement calls (hum). Anti-hu (green) and DAPI (blue) were used to visualize all neurons while only cFos-ir neurons (red) were quantified. Data are represented in the graph as the mean number of cFos-ir neurons per section

in each of the experimental groups. *p<0.05, scale bar = 100µm. Note: two adjacent micrographs were merged to encompass the full area of TSnc in the hum condition.

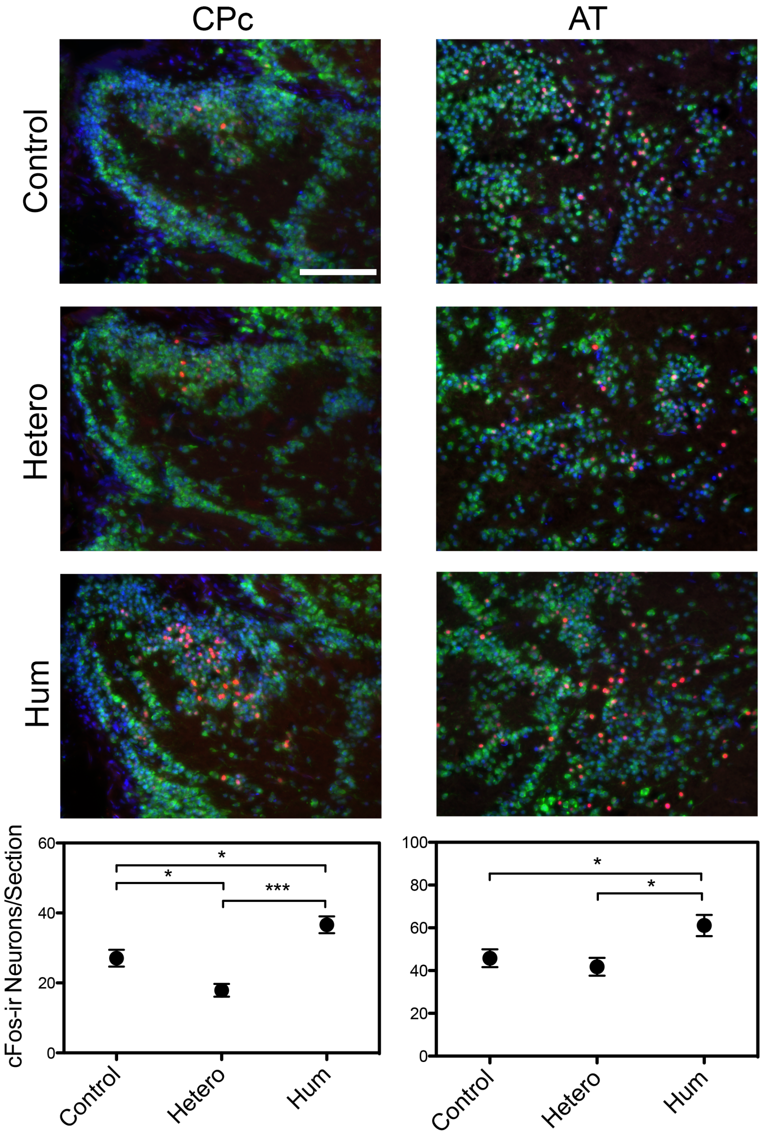

Figure 3.6. **Differential activation in the auditory forebrain**. cFos-ir (red) activation is shown in these representative photomicrographs of the forebrain central posterior nucleus of the thalamus (CP) and the anterior tuberal nucleus of the ventral hypothalamus (AT). Neurons are identified by the presence of both the neuron specific anti-Hu (green) and the nuclear specific counterstain DAPI (blue). Fish were assigned one of three experimental exposure conditions: ambient sound (control), white seabass calls (hetero) or conspecific advertisement calls (hum).

Graphical data are presented at the average number of cFos-ir neurons per section for each of the experimental conditions. $^{*}p<0.05$, $^{***}p<0.001$, scale bar = 100µm.

Chapter 4. USE OF THE SWIM BLADDER FOR THE ENHANCED RECEPTION OF THE MULTI-HARMONIC ADVERTISEMENT CALL IN FEMALE PLAINFIN MIDSHIPMAN FISH, *PORICHTHYS NOTATUS*

4.1 SUMMARY

Teleost swim bladders vary greatly in size and structure across species and in some rare cases sexually dimorphic differences in swim bladders exist within species. Furthermore, morphological specializations, both direct and indirect, between the swim bladder and inner ear are known to enhance hearing abilities of these fishes. While swim bladder adaptations are known to increase hearing abilities at the level of the auditory periphery, the role of the swim bladder in the neural processing of acoustic signals has yet to be examined. The plainfin midshipman fish, *Porichthys notatus*, has become the focus of many recent studies in auditory neuroscience because their life history is reliant on the production and reception of acoustic signals. Advertising male midshipman fish produce a multi-harmonic low frequency call, which must be localized by receptive females in order to spawn. Recently, we showed that there are extreme sexual dimorphisms in the swim bladders of the plainfin midshipman likely related to the detection of sound pressure. Using the immediate early gene product cFos as a marker for neural activity we quantified neural responses of female midshipman to playback of male advertisement calls with intact or experimentally removed swim bladders. Analysis was conducted throughout major nodes of the central auditory pathway in the hindbrain, midbrain and forebrain. Our results revealed no differences in the cFos-immunoreactive response in any of the brain areas analyzed or across all experimental conditions. Here we interpret our results and

propose future studies to further understand the role of the swim bladder in central auditory processing to social acoustic signals.

4.2 INTRODUCTION

Teleost swim bladders serve a variety of functions not limited to respiration, buoyancy control and sound production (Dehadrai 1962, Abdel Magid et al. 1970, Parmentier and Diogo 2006, Kasumyan 2008, Pelster 2011, Fine and Parmentier 2015). Swim bladders are also known to be important in the enhancement of sound reception to higher frequencies and sound pressure stimuli (O'Connell 1955, Allen et al. 1976, Popper and Coombs 1980, Braun and Grande 2008). It is thought that all fish are able to detect the near-field or particle motion component of sound via direct displacement of the otolithic end organs, but that morphological specializations are needed to detect far-field sound pressure stimuli (De Vries 1950, Fay and Popper 1980, Sisneros and Rogers 2016). In some more recently derived teleost fishes direct and indirect connections between the swim bladder and inner ear afford sound pressure detection by transmitting oscillations, caused by the changes in pressure of the gas filled bladder from acoustic stimuli, that stimulate the inner ear by producing a local particle motion field. A common adaptation is the presence of rostral swim bladder extensions and comparative physiological studies between closely related species with and without swim bladder extensions have revealed functional differences related to sound reception, where the presence of swim bladder extensions in close proximity to the inner ear yield more sensitive and extended hearing ranges (Coombs and Popper 1979, Schulz-Mirbach et al. 2012). While the role of the swim bladder in peripheral auditory sensitivity has been well characterized in a number of fish species, the role of the swim bladder in central auditory processing is unknown.

Acoustic information is first transduced in the saccule, the main end organ of hearing in most fish, before being transmitted via the VIIIth nerve to the auditory hindbrain (Bass et al. 1994). Projections ascend to numerous nuclei within the hindbrain, midbrain and forebrain all of which make up the ascending auditory pathway (McCormick 1999, Bass et al 2000). It is thought the neuronal populations within the hindbrain are broadly tuned and respond to general acoustic stimuli whereas neural response properties and tuning sharpen at the level of the midbrain and higher (Fay and Lu 1995, Edds-Walton and Fay 2003, Edds-Walton and Fay 2008, Edds-Walton 2016). Recent studies in female plainfin midshipman fish, *Porichthys notatus*, have revealed that the auditory thalamus and ventral hypothalamus show differential neuronal activation in response to conspecific versus heterospecific vocalizations and may be necessary for species-specific discrimination (Mohr et al. in prep.). How the swim bladder contributes to neuronal activation properties in response to social acoustic signals has yet to be explored.

Among teleosts the plainfin midshipman fish provides perhaps the best species for investigating the role of the swim bladder in central auditory processing because their life history relies on the production and reception of acoustic signals, their neuroanatomy is well characterized and they have sexually dimorphic swim bladders (Brantley and Bass 1994, Bass et al. 2000, Mohr et al. 2017). During the breeding season, nesting or 'type I' males produce a low frequency multi-harmonic advertisement call or 'hum' to attract females to spawn. Females must be able to detect and localize advertising type I males and they likely attend to the higher frequency harmonics which are known to propagate further in the shallow water nesting environment (Fine and Lenhart 1983, Rogers and Cox 1988, Brantley and Bass 1994, Sisneros and Rogers 2016). Morphological studies employing micro computerized tomography have revealed extreme sexual dimorphisms where females have elongated rostral extensions of their swim bladders (absent in type I males), which significantly increase the proximity to the inner ear end organs (Mohr et al. 2017). Recent physiological studies have shown that female midshipman with intact swim bladders have lower hearing thresholds and extended high frequency hearing ranges when compared to both females with surgically removed swim bladders and intact type I males, supporting the role of rostral swim bladder extensions in enhanced hearing high frequency abilities of female midshipman for greater mate call detection (Colleye et al. in prep.).

The purpose of this study was to investigate the role of the swim bladder in the central processing of conspecific advertisement calls in female plainfin midshipman fish. Using the protein product of the immediate early gene (IEG) cFos as a marker for neural activity we quantified neuronal activation at several nodes of the ascending auditory pathway in female midshipman fish with intact or experimentally removed swim bladders. We hypothesized the presence of the swim bladder would affect neuronal responses and yield differential activation between animals with intact versus experimentally removed swim bladders. Specifically, we predicted that females with intact swim bladders would be capable of processing the higher frequency components of the male advertisement call yielding greater cFos activity at the level of the auditory thalamus and anterior hypothalamus compared to fish with removed swim bladders. Our results revealed no differences in cFos activity in fish with intact or surgically removed swim bladders in response to any playback condition, and we interpret our findings as they relate to the potential role of the swim bladder in central auditory processing.

4.3 METHODS

4.3.1 Fish collection and housing

Reproductive female plainfin midshipman fish, *Porichthys notatus*, were collected by hand during summer low tides at Seal Rock near Brinnon, WA. Fish were then transported in aerated coolers to the University of Washington where they were housed in aquaria and maintained at 14-16°C on a 14:10 hour light cycle and fed vitamin enriched shrimp 3X weekly prior to experimentation.

4.3.2 Experimental setup and auditory playback

Auditory playback was conducted in a 30 cm diameter, 24 cm high Nalgene tank with a UW-30 underwater speaker centered in gravel substrate on the floor of the tank. The tank was housed on a vibration isolation table in an acoustic isolation chamber with the acoustic stimulation equipment and ventilation pump housed externally. The underwater speaker was powered by a TOA BG-1120 Integrated Amplifier, which received auditory input from a laptop computer. The auditory stimulus file was a thirty minute composite created by combining seven unique laboratory recordings from four male plainfin midshipman fish producing multi-harmonic advertisement calls between 87-93 Hz in fundamental frequency (Figure 4.1). The recorded auditory files were equalized to the same maximum peak-to-peak sound level to control for fluctuations in sound intensity between recordings. Prior to acoustic playback, the sound intensity was calibrated to 130 dB (re: 1 μPa) 10cm above the surface of the speaker, at the approximate location of where the fish's heads were positioned. Briefly, the sound intensity was calibrated using a minihydrophone (Bruel and Kjaer model 8103) and single channel amplifier (Bruel and Kjaer Type 2692) connected to an oscilloscope to measure the peak-to-peak voltage.

Following calibration, female plainfin midshipman fish were first anesthetized by immersion in a 0.025% ethyl p-aminobenzoate saltwater bath before intramuscular injection of 0.25% of Bupivacaine (approximately 1 mg/kg) and 0.35-0.5 ml cisatracurium besylate (approximately 0.5 mg/kg) for analgesia and paralysis, respectively. Fish were weighed and measured and then underwent a swim bladder removal surgery in which a ~2 cm incision was cut on the ventral surface of the skin just rostral to the anal pore. The fish was then gonadectomized, to gain access to the swim bladder, by cauterizing the blood vessels on the rostral and caudal ends of the ovaries. The swim bladder was then removed via cauterization of the blood vessels and connective tissue moving caudal to rostral until the entire swim bladder could be easily lifted from the body cavity. The body cavity was then sealed using antimicrobial monofilament sutures. The control groups underwent sham surgeries in which the fish were gonadectomized but the swim bladder remained intact.

After surgery, fish were acclimated by being positioned in the tank 10cm above the speaker with 15°C (+/- 1°C) water perfused constantly over their gills for at least 30 minutes. Once acclimation was complete, the water flow was turned off and the fish were exposed to the acoustic playback stimulus or ambient sound for 30 minutes. After the auditory stimulation was complete, the water flow was turned back on and the fish were maintained in the tank for an additional 120 minutes prior to transcardial perfusion of ice-cold teleost ringer followed by 4% paraformaldehyde in 0.1M Phosphate buffer (PB: pH 7.2). Brains were then harvested and post-fixed for 60 minutes before being rinsed 3X in PB and stored at 4°C until cryo-sectioning. Prior to sectioning, brains were transferred to 30% sucrose in PB for 24-48 hours before embedding in cryogel and sectioning in two alternate series at 25 µm. Only one series was analyzed in this study and brain slides were stored at 4°C until immunohistochemistry.

4.3.3 Immunohistochemistry

Slides were first brought to room temperature before being traced with a hydrophobic pen. Slides were then rehydrated for 2X ten minute soaks in phosphate buffered saline (PBS: pH 7.2) before being transferred to a blocking solution of PBS with 10% normal donkey serum and 0.3% Triton-X (PBS-DS) for 1 hour. After blocking was complete, slides were incubated for 16-17 hours at room temperature with PBS-DS containing rabbit anti-cFos (1:2000, Santa Cruz Biotechnology lot# C2510) and mouse anti-Hu (1:2000). After the incubation of primary antibodies, slides were dipped before being rinsed in PBS in 0.5% normal donkey serum 5X for 10 minutes. Slides were then incubated for an additional 2 hours at room temperature with PBS-DS containing donkey anti-rabbit conjugated to Alexa fluor 568 (1:200) and donkey anti-mouse conjugated to Alexa fluor 488 (1:200). Finally, slides were dipped and rinsed in PBS 4X for 10 minutes and cover slipped with Prolong Gold with DAPI and allowed to cure at room temperature in darkness for 48-72 hours before being sealed with nail polish and stored at 4°C until imaging.

4.3.4 Image Acquisition

Photomicrographs were imaged on an Olympus BX61 epifluorescence compound microscope using MetaMorph imaging and processing software. Low magnification lenses (4X, followed by 10X) were used to identify the rostral and caudal boundaries of all auditory nuclei in the GFP channel to visualize neuronal cell bodies. Photomicrographs were obtained at 20X magnification with exposure times held constant for each channel. Images were taken in z-series by setting a top and bottom focal plane with 1 μm stack thickness. Photomicrographs were imaged consecutively for each channel in the following order: Texas Red, GFP and DAPI. The z-series micrographs were combined into a single projection for each channel using the Z project maximum function in ImageJ and then overlaid. cFos immunoreactive (cFos-ir) neurons were quantified manually using a custom written ImageJ macro by a researcher who was blind to the experimental condition. Background thresholds were determined for each individual photomicrograph and cFos-ir neurons were confirmed by identifying the appropriate size, shape and illumination of the signal across all channels. Numbers reported represent the average number of cFos-ir neurons per section per nucleus for each fish across experimental conditions.

4.3.5 Central Acoustic Circuitry

The saccule, the primary end organ of hearing in midshipman, sends ascending projections to rostral intermediate division of the descending octaval nucleus (DOri) and the ventral division of the secondary octaval nucleus (SOv) which were the two hindbrain nuclei analyzed in this study. The caudal extent of DOri was determined by its appearance at the level of the octaval efferent nucleus and sampled rostrally till its disappearance. SOv lies just medial and ventral to DOri throughout most of its extent though slight variations are present due to the angle at which brains were mounted and sectioned. All landmarks were determined by previously published neuroanatomical and physiological studies and sampling techniques replicated Petersen et al. (2013). Both DOri and SOv were sampled serially on the right side of the brain in their entirety. No hemispheric differences were predicted due to the central location of the fish directly above the speaker. On average 6.8 (± 1.9 SD) and 5.9 (± 1.6 SD) sections were quantified for DOri and SOv, respectively.

Projections from DOri and SOv ascend to the periventricular nucleus centralis of torus semicircularis (TSnc), the major auditory center in the midbrain. TSnc was imaged unilaterally on the right side of the brain with two adjacent micrographs needed to encompass the entirety of the large nucleus. Every fourth section was imaged throughout the extent of the nucleus with landmarks clearly determined from previously published descriptions. On average, 5.9 (± 0.9 SD) sections were imaged and analyzed per animal in TSnc. TSnc sends ascending projections to the central posterior nucleus (CP) of the auditory thalamus in the forebrain. The compact division of CP (CPc) was analyzed for cFos-ir activity and was identified by the clear wing-shaped group of cells along the midline of the brain. Sections were imaged serially on the right side of the brain with an average of 4.9 (± 0.8 SD) sections per animal. TSnc also sends projections to the anterior tuberal nucleus (AT) of the ventral hypothalamus in the forebrain. AT is defined by a triangular grouping of cells adjacent to the midline on the ventral edge of the brain. Photomicrographs were taken serially on the right side of the brain with an average of 2.8 (± 0.5 SD) sections per animal.

4.3.6 Statistics

Average number of cFos-ir neurons were calculated within each auditory nucleus and compared between experimental and playback condition groups using a one-way ANOVA with an alpha set at 0.05. Post-hoc Tukey tests were used to make pair-wise comparisons across groups for each nucleus. All statistics were analyzed using IBM SPSS Statistics 19 and GraphPad Prism 5 software.

4.4 RESULTS

A total of twenty-four reproductive female plainfin midshipman fish were used in this experiment, eight in each experimental group. Fish exposed to ambient sound that underwent sham surgeries had a standard length (SL) of 16.88 ± 1.90 cm (mean ± SD), body mass (BM) or 60.75 ± 18.83 g and gonadosomatic index (GSI) of 21.82 ± 11.04 cm. Fish exposed to auditory playback that underwent sham surgeries had a SL of 16.44 ± 1.32 cm BM of 53.85 ± 12.67 g and GSI of 19.96 ± 10.72. Fish exposed to auditory playback that underwent swim bladder removal surgery had a SL of 17.14 ± 1.65 cm, BM of 61.75 ± 23.76 g and GSI of 17.28 ± 12.88. There were no differences between any of the experimental groups in any of the morphometric data analyzed determined by one-way ANOVA ($p>0.69$ for all cases).

4.4.1 Auditory Brain Activation

Brain activation was quantified using the protein product of the immediate early gene cFos. cFos-ir activity was analyzed in nuclei along the ascending auditory pathway in the previously discussed areas of the hindbrain, midbrain and forebrain. Temperature during auditory exposure ranged from 14-15°C and was maintained until sacrifice. There were no differences in cFos-ir activity between any of the experimental groups in any of the auditory nuclei sampled. In the hindbrain DOri, sham surgery fish exposed to ambient sound had 3.14 ± 1.83 (mean ± SD), sham surgery fish exposed to auditory playback had 1.77 ± 0.42 and swim bladder removal fish exposed to auditory playback had 2.38 ± 1.97 cFos-ir neurons, respectively (one-way ANOVA, $F(2, 19) = 1.42$, $p=0.27$)(Figure 4.2). cFos-ir counts in SOv were 1.92 ± 2.10 in the sham surgery fish exposed to ambient sound, 1.32 ± 0.84 in the sham surgery fish exposed to auditory playback and 1.21 ± 1.18 in the swim bladder removal fish exposed to auditory playback (one-way ANOVA, F (2, 19) = 0.48, p=0.63)(Figure 4.2). In TSnc, the number of cFos-ir neurons in the sham surgery fish exposed to ambient sound was 25.67 ± 22.43, 15.30 ± 9.88 in the sham surgery fish exposed to auditory playback and 21.30 ± 11.77 in the swim bladder removal fish exposed to auditory playback (one-way ANOVA, F (2,19) = 0.77, p=0.47)(Figure 4.3). In CP in the sham surgery ambient sound exposed fish there were 31.58 ± 26.73 cFos-ir neurons, in the sham surgery fish exposed to auditory playback 14.73 ± 7.95 cFos-ir neurons and in the swim bladder removal fish exposed to auditory playback 16.18 ± 12.61 cFos-ir neurons (one-way ANOVA, F (2, 20) = 2.15, p=0.14)(Figure 4.4). Finally, in AT the number of cFos-ir neurons in the sham surgery fish exposed to ambient sound was 100.40 ± 36.20, 86.48 ± 34.26 in the sham surgery fish exposed to auditory playback and 85.62 ± 35.23 in the swim bladder removal fish exposed to auditory playback (one-way ANOVA, F (2, 19) = 0.42, p=0.66)(Figure 4.4).

4.5 DISCUSSION

The goal of this study was to determine the role of the plainfin midshipman swim bladder in the central processing of the multi-harmonic advertisement call. We characterized brain activation by quantifying the protein product of the immediate early gene cFos as a proxy for neural activity in fish with intact and experimentally removed swim bladders. cFos, and other IEGS, are widely used for assessing neuronal activation as the IEG response begins within minutes after exposure and can be quantified throughout the entire brain (Luckman et al. 1994, Kovacs 2008). Our results revealed no differences in brain activation along the ascending auditory pathway in any of the experimental or playback conditions and we discuss and interpret our findings as they relate to previously published literature.

4.5.1 Swim bladders and hearing

A number of previous studies have aimed to characterize the role of the swim bladder and how it affects hearing abilities. Comparative studies between closely related species of squirrelfishes (family: Holocentridae) have shown that species possessing swim bladder extensions that come into close proximity with the inner ear are more sensitive across all frequencies tested and have extended high frequency hearing ranges compared to species lacking swim bladder extensions (Coombs and Popper 1979). More recently, similar results were found when comparing the physiological hearing abilities of several species of cichlid fish (Family: Cichlidae) where the presence of swim bladder extensions and increased swim bladder size yielded greater hearing abilities as measured by auditory evoked potentials Schultz-Mirbach et al. 2012). In a classic study by Chapman and Sand (1974), the presence of a gas filled balloon underneath the head of a dab (a fish species which lacks a swim bladder) increased the hearing sensitivity across all frequencies tested. In the oyster toadfish (Family: Batrachoididae), a fish lacking swim bladder extensions, experimental deflation of the swim bladder had no effect on hearing sensitivity, further supporting that the proximity of a gas filled structure to the inner ear is the essential factor in affecting hearing sensitivity (Yan et al. 2000). In the closely related plainfin midshipman fish, *Porichthys notatus*, we have previously shown that there are sexual dimorphisms in the presence of rostral swim bladder extensions, found in females, which greatly increase the proximity of the swim bladder to the inner ear (Mohr et al. 2017). Physiological saccular potential recordings from female midshipman with intact and surgically removed swim bladders have revealed drastic changes in hearing sensitivity, where the lack of the swim bladder significantly desensitizes hearing thresholds across all thresholds and lowers the high frequency hearing range (Colleye et al. in prep.). Interestingly, our results revealed no differences in brain activation patterns in five nodes of the ascending auditory pathway in female plainfin midshipman fish analyzed in this study. We used an auditory stimulus composed of several male advertisement calls, which contain most of their energy within the fundamental frequency and first few harmonics (~90-400 Hz) and is well within the hearing range of midshipman with intact or removed swim bladders (Brantley and Bass 1994, Sisneros 2007, Sisneros 2009, Colleye et al. in prep.). The stimuli were presented at a constant 130 dB (re: 1 µPa), which has also been shown to be well within the audible and biologically relevant hearing range for midshipman fish, as that is the approximate sound intensity just outside of a midshipman nest (Brantley and Bass 1994, Sisneros 2007). It is possible that pure tone stimuli, especially higher frequencies (above 500 Hz), would have elicited differences in brain activation patterns between fish with intact versus removed swim bladders (Colleye et al. in prep.). However the biological salience of pure tone signals could alter how they are processed in the higher order brain areas which are hypothesized to be selectively responsive to complex signals, including species-specific vocalizations (Mohr et al. in prep.).

4.5.2 The ascending auditory pathway

The primary end organ for hearing in the plainfin midshipman fish is the saccule which sends ascending projections into auditory nuclei within the hindbrain (Bass et al. 2000). The rostral intermediate division of the descending octaval nucleus (DOri) is the initial site for central decoding of information from the auditory periphery, along with the ventral division of the secondary octaval nucleus (SOv) (McCormick 1999, Bass et al. 2000, Tomchik and Lu 2005). Neurons within the hindbrain are known to have high levels of baseline activity and have been shown to respond generally to acoustic stimuli in the clawed frog, *Xenopus laevis* (Feng and Schellart 1999, Elliott et al. 2007). Results from a recent study in plainfin midshipman fish revealed that there were no differences in cFos-ir activity between female midshipman exposed to conspecific playback, heterospecific white seabass calls or ambient noise in a semi-naturalistic playback paradigm in DOri or SOv (Mohr et al. 2017). We predicted there would be no difference between fish with either intact or experimentally removed swim bladders exposed to playback of conspecific advertisement calls, however we also predicted both groups to have greater cFos-ir levels than the control group exposed to ambient sound with intact swim bladders. Our results revealed no differences in cFos-ir activity in DOri or SOv across the experimental conditions and playbacks. It is possible that the use of a sound isolation chamber in these experiments presented an unnatural ambient sound condition yielding supernormal baseline neuronal activity in the hindbrain. Experimentation in a natural or semi-natural environment will be necessary to corroborate the baseline activity levels measured in our study.

DOri and SOv send ascending projections to the major auditory center of the midbrain, nucleus centralis of torus semicircularis (TSnc). TSnc is the anamniote vertebrate homolog to the mammalian inferior colliculus and is likely the primary location for filtering of acoustic stimuli (Echteler 1984, Feng and Lin 1991, Bass et al. 2000, Hoke et al. 2004). Similar findings have been made in the tungara frog, where the IEG, egr-1, showed greater expression in response to conspecific playback compared to ambient sound (Hoke et al. 2004). Our results revealed no differences within TSnc for any of our experimental conditions. While the amphibian TS has several unique toral subdivisions related to auditory processing, there are no known subdivisions of TSnc (Potter 1965, Wilczynski 1981, Bass et al. 2000, Endepols and Walkowiak 2001, Hoke et al. 2004). Previous studies done with free-swimming male and female midshipman fish in either large outdoor tanks or smaller cages within the inter tidal zone have revealed greater cFos-ir activity between fish exposed to conspecific playback and ambient sound (Petersen et al. 2013, Mohr et al. 2017). It is possible that our small testing tank and the use of paralyzed fish does not allow for complete stimulation of the inner ear during auditory playback, as the auditory signal is always presented from directly below the fish. The acoustics of the experimental tank could also present unknown artifacts to the auditory playback that alter the salience of the biologically relevant components of the advertisement stimuli.

The central posterior nucleus of the auditory thalamus (CP) receives projections from TS (McCormick 1999, Bass et al. 2000, Goodson and Bass 2002). While CP is a node in the ascending auditory pathway, CP is likely also important in the processing of multisensory information both auditory and visual (Northcutt 2006, Hoke et al. 2007, Wilczynski and Endepols 2007). Previous work in goldfish, *Carassius auratus*, has implicated CP in the processing of complex social acoustic signals (Lu and Fay 1995). Similarly, earlier work in anurans has shown species-specific characteristics of neurons with the auditory thalamus (Fuzessery and Feng 1983, Hall and Feng 1987, Mudry and Capranica 1987). Plainfin midshipman fish of both sexes have been shown to have significantly greater cFos-ir activity in CP in fish exposed to conspecific advertisement calls compared with ambient sound (Petersen et al. 2013, Mohr et al. 2017). Once again, our results revealed no differences within CP for any of our experimental conditions. CP, as a multisensory area, may be receiving strong input from the visual system, which could be confounding our results. Midshipman fish are nocturnal and previous acoustic playback studies have been conducted at night, while this study was conducted during the day. Circadian rhythm has been shown to be important in various aspects of fish behavior along with gene expression and could be affecting our predicted IEG response (Idda et al. 2012).

The anterior nucleus of the ventral hypothalamus (AT) is reciprocally connected with CP and also receives projections from TS (McCormick 1999, Bass et al. 2000, Goodson and Bass 2002). AT is highly integrative and is a node of the ascending auditory pathway, descending vocal-motor circuitry and social behavior network (SBN) (Newman 1999, Bass et al. 2000, Goodson and Bass 2000a-b, Goodson and Bass 2002, Goodson 2005). SBN is a group of interconnected nuclei in the midbrain and forebrain, which include receptors for steroid hormones implicated in various aspects of social behavior (Newman 1999, Goodson 2005, Goodson and Kabelik 2009). Our results revealed no differences in AT across all experimental and playback conditions. Previous work in midshipman fish have shown strong differences in AT between fish exposed to conspecific playback and ambient sound (Petersen et al. 2013, Mohr et al. 2017). Along with the previously discussed possibilities for the lack of differences we see in cFos-ir activity across all brain areas analyzed in this study, responses in AT may have been modulated by the presence or absence of steroid hormones. Midshipman fish undergo seasonal changes in steroid hormones levels related to their breeding season (Sisneros et al. 2004a). AT is known to possess high numbers of steroid receptors for androgens and have a high presence of aromatase, the enzyme responsible for converting testosterone into estradiol (Forlano et al. 2001, Forlano et al. 2010, Forlano and Bass 2011). Sex steroids are known to alter the perception or valence of acoustic signals in birds, and it is possible that a similar mechanism is present in fish (Maney et al. 2008). Differences in steroid hormone levels between individuals could act as a confounding factor in affecting the cFos-ir response.

4.5.3 Conclusions and future directions

Swim bladder removal in female plainfin midshipman fish did not alter the cFos-ir response in response to conspecific playback of male advertisement calls. Previous studies have shown robust differences in neural activity between midshipman fish exposed to conspecific playback and ambient sound at the level of the midbrain and higher suggesting the experimental setup could be responsible for the lack of differences observed in this study (Petersen et al. 2013, Mohr et al. in prep). Midshipman fish are nocturnally active and conducting these studies during the day could have changed the circadian rhythm negatively affecting normal neuronal response properties. Furthermore, experimental playback was done less than one hour after surgery and it is possible that recovery time, trauma or lasting effects of the drugs used were too severe to accurately represent natural neuronal responses. While playback of midshipman recordings should represent the most biologically relevant acoustic stimuli, it is possible that the small testing tank could yield unforeseen artifacts that alter the salience of the call features, including the high frequency harmonics. Future studies using synthetically altered calls which experimentally manipulate the relative energy in the high frequency harmonics along with playback of broadband pure tone stimuli will be necessary to further understand the role of the swim bladder in auditory processing. Furthermore, to account for potential confounds due to the tank acoustics and laboratory conditions these experiments should be replicated in a much larger tank, free-field or natural environment (see: Petersen et al. 2013, Mohr et al. in prep). Future studies in fish with intact and removed swim bladders should also employ signal unit recording techniques to greater characterize neuronal response properties to pure tone and complex auditory stimuli. These studies and more will be needed to fully understand the role of the swim bladder in central auditory processing to social acoustic signals.

4.6 FIGURES

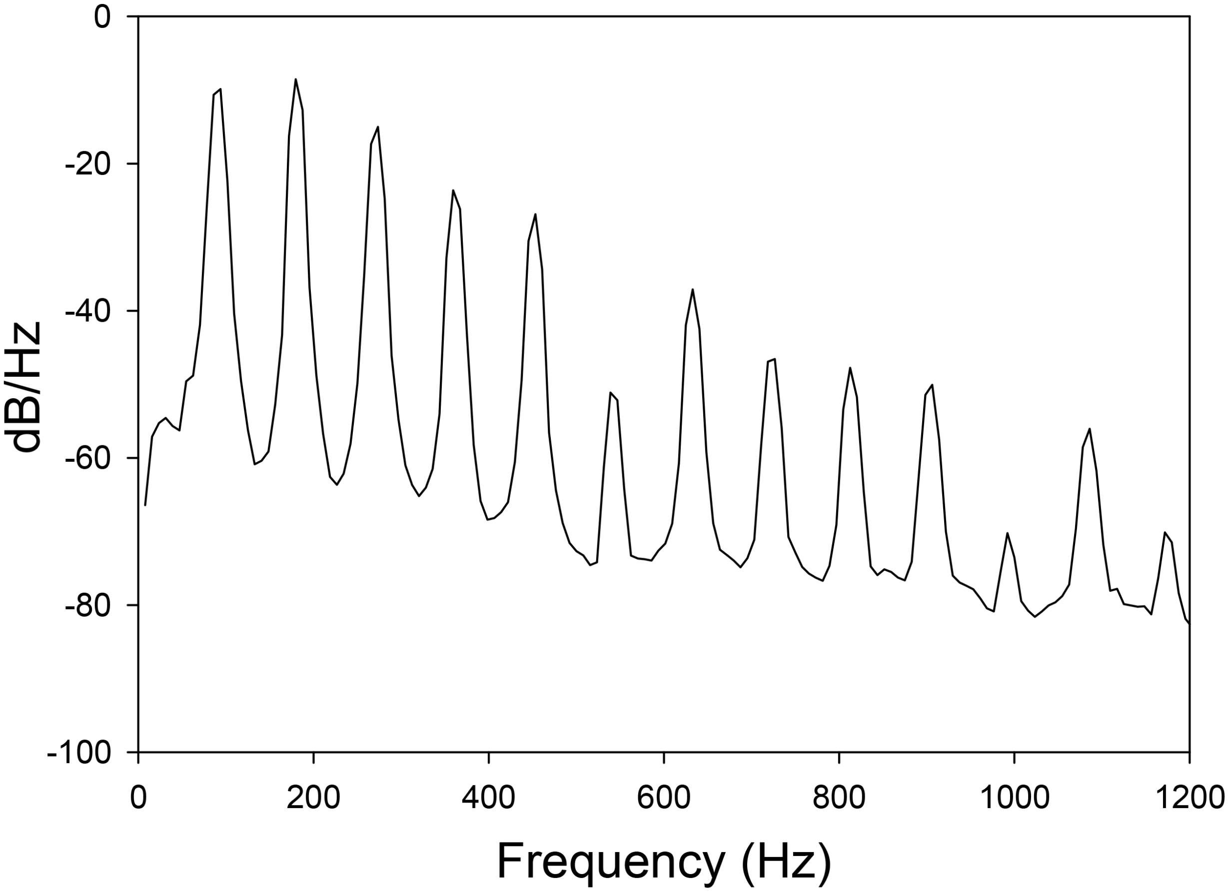

Figure 4.1**. Power spectrum of the midshipman hum**. Power spectrum analysis of the multi-harmonic plainfin midshipman advertisement call showing the relative energy in the fundamental frequency and higher frequency harmonics.

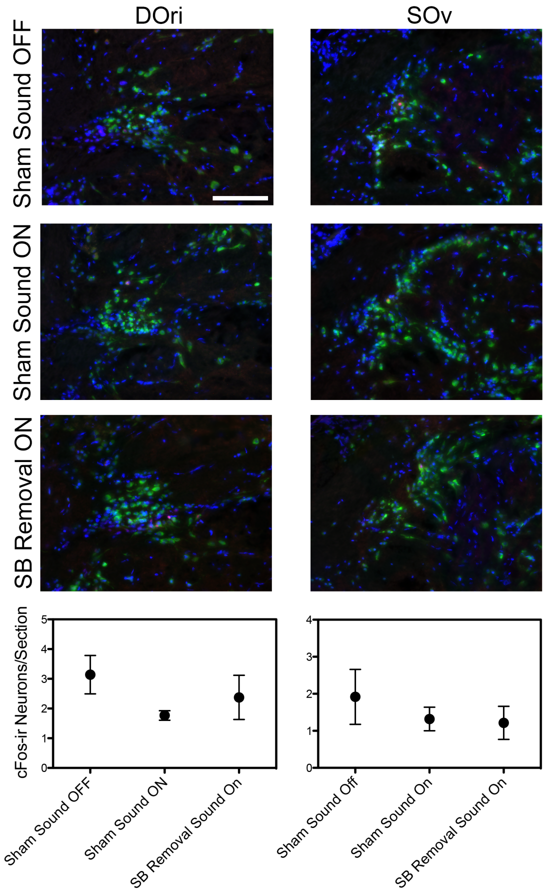

Figure 4.2. **Hindbrain activation with intact and removed swim bladders**. Representative 20X photomicrographs of the rostral intermediate division of the descending octaval nucleus (DOri) and the ventral division of the secondary octaval nucleus (SOv). cFos-immunoreactive (cFos-ir) neurons (red) were used as a proxy for neural activity and quantified. The neuron specific anti-Hu stain (green) and the nuclear counterstain DAPI (blue) were used confirm cFos activity. Fish were assigned randomly to one of three experimental groups. The sham surgery group underwent gonadectomies but their swim bladders remained intact. The swim bladder (SB) removal group had both their gonads and their swim bladders removed. The presence of the sound stimulus (ON) was either the playback of conspecific advertisement calls or the ambient sound of the attenuation booth in which the experiments took place (OFF). Graphs depict the average number of cFos-ir cells per section within each experimental group. Error bars = S.E.M, scale bar = 100 μm.

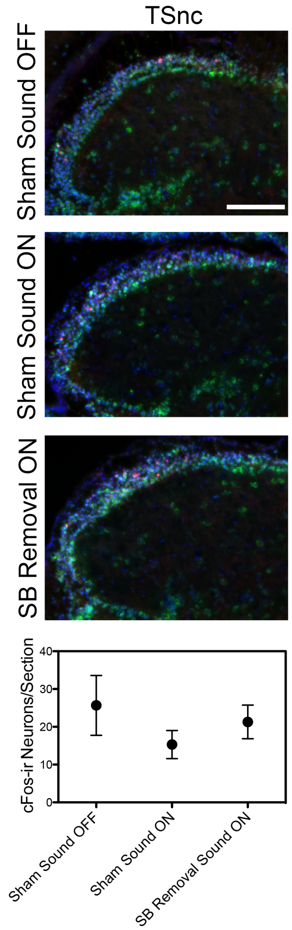

Figure 4.3. **Activation in the midbrain between fish with intact and removed swim bladders**. Photomicrographs of nucleus centralis of the torus semicircularis (TSnc) taken at 20X. Images show cFos-ir neurons (red) within the neuronal cell bodies stained by anti-Hu (green). The nuclear counterstain DAPI (blue) was used to confirm the location of cFos within neurons. Female midshipman were assigned to one of three experimental groups including: the sham sound off group where fish underwent gonad removal surgery and were exposed to ambient noise, the sham sound group where fish underwent the same gonad removal surgery but were exposed to thirty minutes of conspecific advertisement call playback or the swim bladder removal sound on group in which fish underwent surgeries to remove their gonads and swim bladders prior to exposure to thirty minutes of conspecific advertisement calls. The graph shows the mean number of cFos-ir neurons per section for the three experimental groups. Error bars = S.E.M, scale bar = 100 µm.

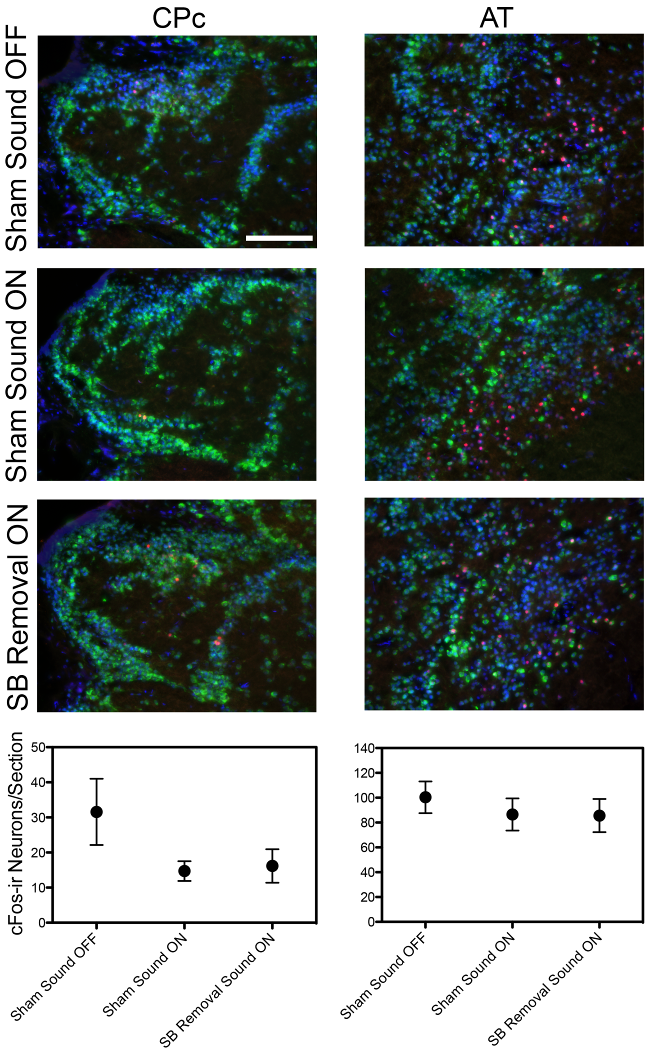

Figure 4.4. **Forebrain activation in fish with intact and removed swim bladders**. Representative photomicrographs taken at 20X of the central posterior nucleus of the thalamus (CP) and the anterior tuberal hypothalamus (AT). Neuron cell bodies are stained with anti-Hu (green) and those that are positive for cFos protein appear red. The nuclear counter stain DAPI (blue) was used to confirm the localization of the cFos protein to neuronal nuclei. Three experimental conditions were tested: sham surgery fish which had their gonads removed which were exposed to either ambient sound or conspecific playback and swim bladder removal fish which had their gonads and swim bladders removed before exposure to playback of conspecific advertisement calls. Graphs show the average number of cFos-ir neurons per brain section for each experimental group. Error bars = S.E.M, scale bar = 100 μm.

Chapter 5. SUMMARY AND FUTURE DIRECTIONS

This dissertation explored how socially relevant acoustic signals are processed in the brain and how the sexually dimorphic swim bladder impacts neural processing in the plainfin midshipman fish. In chapters two through four, I characterized the morphology of the swim bladder and the patterns of neuronal responses to complex social signals and how the presence of the swim bladder alters neuronal properties. In this final chapter, I will summarize my findings in greater detail and provide potential avenues of future research in auditory neuroscience for the midshipman and other fish species.

5.1 SEXUAL DIMORPHISMS IN SWIM BLADDER MORPHOLOGY

In Chapter 2, I detail the sexually dimorphic differences in swim bladder shape and distance to the inner ear organs of the three sexual phenotypes (female, type I and type II male) of the plainfin midshipman fish. Using microCT, I showed that female and type II males have elongated swim bladder horns which are greatly reduced in type I males. These swim bladder extensions greatly increase the proximity of the swim bladder to all three inner ear end organs (the saccule, utricle and lagena).

Having the swim bladder (or other gas filled structure) in close proximity to the auditory end organs has been shown to provide increased hearing abilities, especially to higher frequencies and sound pressure stimuli (Ramcharitar et al. 2006, Braun and Grande 2008, Parmentier et al. 2011, Schulz-Mirbach et al. 2012, Schulz-Mirbach et al. 2013, Fine and Parmentier 2015). Our findings from Chapter 2 suggest that female and type II midshipman may have enhanced sound pressure detection abilities which likely aid in the reception of conspecific advertisement calls during courtship. We recently conducted a study that aimed to determine the role of the swim bladder in peripheral hearing in the midshipman fish, using saccular potential recordings in female midshipman with intact or experimentally removed swim bladders. We found that fish with intact swim bladders were more sensitive to all stimuli presented and had extended high frequency hearing ranges when compared to fish with removed swim bladders (Colleye et al. in prep.) Together, these results inspired the experiments conducted in Chapter 4 in which I experimentally removed the swim bladder and exposed female midshipman to conspecific advertisement before quantifying neuronal activation. Future studies using type I and type II males will be needed to further understand the role of the swim bladder in hearing as well as its potential role in sound source localization. Single unit recordings in auditory nuclei of the brain will also help to determine the role of the swim bladder in central auditory processing.

5.2 NEURONAL RESPONSE PATTERNS TO CONSPECIFIC AND HETEROSPECIFIC SIGNALS

In Chapter 3, I characterized neural response patterns to social acoustic signals in reproductive female midshipman. I showed that exposure to conspecific advertisement calls, white seabass calls and ambient noise yielded no differences in activation patterns, as quantified by cFos, in the hindbrain DOri or SOv. Interestingly, I also showed that fish exposed to conspecific advertisement calls elicited greater neuronal activation in the midbrain TSnc and the forebrain CPc and AT when compared to fish exposed to heterospecific white seabass calls or ambient sound. These findings suggest that auditory nuclei of the hindbrain may respond to general acoustic stimuli and that higher order nuclei are involved in the discrimination and filtering of complex acoustic signals, including species-specific calls.

Our results provide potential target sites for future physiological studies, which are needed to determine how neuronal populations in auditory brain nuclei respond to complex acoustic signals. Future work is also needed to understand the involvement of neural networks in processing of complex auditory signals and whether there are populations that are selectively responsive to species-specific signals. It is also important to gain further understanding into the role of excitatory and inhibitory neurons in the activation patterns we describe. Plainfin midshipman fish are seasonal breeders and have seasonal fluctuations in steroid hormones that are known to alter neuronal substrates (Sisneros et al. 2004a, Forlano et al. 2015). The role of seasonality and steroid hormones on neural processing provides a potential avenue of research, which could greatly enhance our understanding of auditory processing in midshipman and other fishes.

5.3 THE ROLE OF THE SWIM BLADDER IN THE PROCESSING OF CONSPECIFIC ADVERTISEMENT CALLS

In Chapter 4, I quantified neuronal response properties of reproductive female midshipman to conspecific advertisement calls in fish with intact and experimentally removed swim bladders. I showed that neuronal activation, as measured by cFos, was no different between fish with intact or removed swim bladders in response to either playback of conspecific advertisement calls or ambient sound. We measured cFos-ir levels in auditory nuclei of the hindbrain (DOri and SOv), midbrain (TSnc) and forebrain (CPc and AT).

While the presence of the swim bladder did not influence neuronal response properties in my experiments, future physiological studies employing single unit recordings in higher order auditory brain nuclei are needed to further understand the role of the swim bladder in the central processing of acoustic signals. Less invasive techniques, such as filling the swim bladder with liquid via injection, may allow for immediate behavioral and physiological testing without the potentially irreversible side effects of traumatic surgery. Comparisons between midshipman sexual phenotypes may also yield interesting differences in central auditory processing as type I males are not thought to encode sound pressure due to their lack of swim bladder horns as described in Chapter 2.

5.4 FUTURE DIRECTIONS

Together, these studies characterize the sexually dimorphic midshipman swim bladder, provide insights into the neural circuits that may be involved in the discrimination of complex social signals and the role of the swim bladder in central processing of complex acoustic signals. The previous sections focused on future questions raised by my empirical findings. In this section, I will discuss broader avenues of potential future research.

5.4.1 What are the salient features of the midshipman advertisement call?

The midshipman advertisement call is primarily sinusoidal with a low fundamental frequency (~80-100Hz, dependent on temperature) and several harmonics nearing 1kHz (Brantley and Bass 1994). Often, the second and third harmonics contain more energy that the fundamental frequency and are known to travel further in the shallow water nesting environment. Neuronal activation studies in midshipman have relied on either auditory playback of these conspecific advertisement calls or pure tone stimuli near the fundamental frequency of the advertisement call. The salient features of the advertisement call that female midshipman attend to are unknown, and may be important in assessing male quality. Our playback stimuli contain recordings from at least six individuals, however it is unknown how the quality of these specific males may have affected female responsiveness. Future behavioral and physiological studies that manipulate the relative power in the fundamental frequency and harmonics of the advertisement call would provide insights into the most salient components of the male call for optimization of future playback experiments.

5.4.2 Catecholamines in central auditory processing

Catecholaminergic innervation is well described in the midshipman peripheral and central auditory system and is known to influence auditory function and behavioral responses (Forlano et al. 2014, Forlano et al. 2017, Perelmuter and Forlano 2017). However, how monoamines affect central auditory processing of social acoustic signals in female midshipman is unknown. Combining pharmacological manipulations of dopamine and serotonin and physiological recording techniques during acoustic playback of complex social signals could provide insights into the role of catecholamines in midshipman auditory processing.

BIBLOGRAPHY

Aalbers SA, Drawbridge MA (2008) White seabass spawning behavior and sound production. *Trans Am Fish Soc* 137:542-550.

Abdel Magid AM, Vokac Z, Ahmed NED (1970) Respiratory function of the swim-bladders of the primitive fish *Polypterus senegalus*. *J Exp Biol* 52:27-37.

Allen JM, Blaxter JHS, Denton EJ (1976) The functional anatomy and development of the swimbladder-inner ear-lateral line system in herring and sprat. *J Mar Biol Ass UK* 56:471-486.

Barimo JF, Fine ML (1998) Relationship of swim-bladder shape to the directionality pattern of underwater sound in the oyster toadfish. *Can J Zool* 76:134-143.

Bass AH, Marchaterre MA (1989) Sound-generating (sonic) motor system in a teleost fish (*Porichthys notatus*): Sexual polymorphism in the ultrastructure of myofibrils. *J Comp Neurol* 286:141-153.

Bass AH, Marchaterre MA, Baker R (1994) Vocal-acoustic pathways in a teleost fish. *J Neurosci* 14:4025-4039.

Bass AH, Bodnar DA, Marchaterre MA (1999) Complementary explanations for existing phenotypes in an acoustic communication system. In: Konishi M, Hauser M (eds) *Neural Mechanisms of Communication*. MIT Pres, Cambridge. pp 494-514.

Bass AH, Bodnar DA, Marchaterre MA (2000) Midbrain acoustic circuitry in a vocalizing fish. *J Comp Neurol* 419:505-531.

Bass AH, Clark CW (2003) The physical acoustics of underwater sound communication. In: Simmons AM, Fay RR, Popper A (eds.) *Spring Handbook of Auditory Research: Acoustic Communication*. Springer, New York. pp 15-64.

Bass AH, McKibben JR (2003) Neural mechanisms and behaviors for acoustic communication in teleost fish. *Prog Neurobiol* 69:1-26.

Bass AH (2006) Neural mechanisms of vocal communication: Interfacing with neuroendocrine mechanisms. In: Kanwal J, Ehret G (eds.) *Behavior and Neurodynamics for Auditory Communication*. Cambridge University Press, Cambridge England. pp 123-131.

Bass AH, Gilland EH, Baker R (2008) Evolutionary origins for social vocalization in a vertebrate hindbrain-spinal compartment. *Science* 321:417-421.

Bass AH, Ladich F (2008) Vocal-acoustic communication: From neurons to behavior. In: Webb JF, Fay RR, Popper AN (eds.) *Fish Bioacoustics*. Springer, New York. pp 253-278.

Berridge CW (2008) Noradrenergic modulation of arousal. *Brain Res Rev* 58:1-17.

Bertucci, F, Ruppe, L, Van Wassenbergh S, Compere P, Parmentier E (2014) New insights into the role of the pharyngeal jaw apparatus in the sound-producing mechanism of *Haemulon flavolineatum* (Haemulidae). *J Exp Biol* 217:3862-3869.

Bhandiwad AA, Zeddies DG, Raible DW, Rubel EW and JA Sisneros (2013) Auditory sensitivity of larval zebrafish (*Danio rerio*) measured using a behavioral prepulse inhibition assay. *J Exp Biol* 216:3504-3513.

Bhandiwad AA, Whitchurch EA, Colleye O, Zeddies DG, Sisneros JA (2017) Seasonal plasticity of auditory saccular sensitivity in "sneaker" type II male plainfin midshipman fish, *Porichthys notatus*. *J Comp Physiol A* 203:211-222.

Blaxter JHS, Denton EJ, Gray JAB (1979) The herring swimbladder as a gas reservoir for the acoustico-lateralis system. *J Mar Biol Assoc UK* 59:1-10.

Blaxter JHS, Denton EJ, Gray JAB (1981) Acousticolateralis system in clupeid fishes. In: Tavolga WN, Popper AN, Fay RR (eds.) *Hearing and Sound Communication in Fishes*. Springer, New York. pp 39-59.

Bose, APH, Adragna JB, Balshine S (2016) Otolith morphology varies between populations, sexes and male alternative reproductive tactics in a vocal toadfish *Porichthys notatus*. *J Fish Biol* 90:311-325.

Brantley RK, Wingfield JC, Bass AH (1993) Sex steroid levels in *Porichthys notatus*, a fish with alternative reproductive tactics, and a review of the hormonal bases for male dimorphism among teleost fishes. *Horm Behav* 27:332-347.

Brantley RK, Bass AH (1994) Alternative spawning tactics and acoustic signals in the plainfin midshipman fish, *Porichthys notatus* Girard (teleosti, batrachoididae). *Ethology* 96:213-232.

Braun CB, Grande T (2008) Evolution of peripheral mechanisms for the enhancement of sound reception. In Popper AN, Fay RR, Webb JL (eds.) *Springer Handbook of Auditory Research: Fish Bioacoustics*. Springer, New York. pp 99-144.

Butler JM, Maruska KP (2016) The mechanosensory lateral line system mediates activation of socially relevant brain regions during territorial interactions. *Front Behav Neurosci* 10:93.

Caras ML (2013) Estrogenic modulation of auditory processing: A vertebrate comparison. *Front Neuroendocrinol* 34:285-299.

Casseday JH, Covey E (1996) A neuroethological theory of the operation of the inferior colliculus. *Brain Behav Evol* 47:323-336.

Chapman CJ, Hawkins AD (1973) A field study of hearing in the cod, *Gadus morhua* L. *J Comp Physiol A* 85:147-167.

Chapman CJ, Sand O (1974) Field studies of hearing in two species of flatfish *Pleuronectes platessa* (L.) and *Limanda limanda* (L.) (family Pleuronectidae). *Comp Biochem Physiol A* 47:371-385.

Clayton DF (2000) The genomic action potential. *Neurobiol Learn Mem* 74:185-216.

Coffin AB, Mohr RA, Sisneros JA (2012) Saccular-specific hair cell addition correlates with reproductive-state dependent changes in the auditory saccular sensitivity of a vocal fish. *J Neurosci* 32:1366-1376.

Coffin AB, Zeddies DG, Fay RR, Brown AD, Alderks PW, Bhandiwad AA, Mohr RA, Gray MD, Rogers PH, Sisneros JA (2014) Use of the swim bladder and lateral line in near-field sound source localization by fish. *J Exp Biol* 217:2078-2088.

Colleye O, Nakamura M, Frédérich B, Parmentier E (2012) Further insight into the sound-producing mechanism of clownfishes: What structure is involved in sound radiation? *J Exp Biol* 215: 2192-2202.

Colleye O, Mohr RA, Sisneros JA. Sexually dimorphic swim bladder extensions enhance sound pressure sensitivity in female plainfin midshipman, *Porichthys notatus. in preparation*.

Conway KW, Britz R, Siegel DS (2014) Different on the inside: Extreme swimbladder sexual dimorphism in the South Asian torrent minnows. *Biol Lett* 10:20140348.

Coombs S, Popper AN (1979) Hearing differences among Hawaiian squirrelfish (family Holocentridae) related to differences in the peripheral auditory system. *J Comp Physiol A* 132:203-207.

Courtenay WR (1971) Sexual dimorphism of the sound producing mechanism of the striped cusk-eel, *Rissola marginata* (Pisces: Ophidiidae). *Copeia* 1971:259-268.

Dehadrai PV (1962) Respiratory function of the swimbladder of Notopterus (Lacepede). *J Zoo Biol* 139:341-357.

Denton EJ, Gray JAB, Blaxter JHS (1979) The mechanics of the clupeid acousticolateralis system: Frequency responses. *J Mar Biol Assoc UK*:59:27-47.

De Vries HL (1950) The mechanics of the labyrinth otoliths. *Acta Otolaryngol* 38:262-273.

Dijkgraaf, S (1960) Hearing in bony fishes. *Proc. Roy. Soc. London Ser. B* 152:51- 54.

Echteler SE (1984) Connections of the auditory midbrain in a teleost fish, *Cyprinus carpio. J Comp Neurol* 230:536-551.

Edds-Walton PL, Fay RR (2003) Directional selectivity and frequency tuning of midbrain cells in the oyster toadfish, *Opsanus tau*. *J Comp Physiol A* 189:527-543.

Edds-Walton PL, Fay RR (2005) Sharpening of directional responses along the auditory pathway of the oyster toadfish, *Opsanus tau*. *J Comp Physiol A* 19:1079-1086.

Edds-Walton PL, Fay RR (2008) Directional and frequency response characteristics in the descending octaval nucleus of the toadfish (*Opsanus tau*). *J Comp Physiol A* 194:1013-1029.

Edds-Walton PL (2016) What the Toadfish Ear Tells the Toadfish Brain About Sound. In: Sisneros JA (ed.) *Fish Hearing and Bioacoustics: An anthology in honor of Arthur N. Popper and Richard R. Fay. Advances in Experimental Medicine and Biology*, Springer Science + Business Media, New York. pp 197-226.

Elliott TM, Christensen-Dalsgaard J, Kelley, DB (2007) Tone and call responses of units in the auditory nerve and dorsal medullary nucleus of *Xenopus laevis*. *J Comp Physiol A* 193:1243-1257.

Endepols H, Walkowiak W (2001) Integration of ascending and descending inputs in the auditory midbrain of anurans. *J Comp Physiol A* 186:1119-1133.

Enger PS (1963) Single unit activity in peripheral auditory system of a teleost fish. *Acta Physiologica Scandinavica*, *59*, 9.

Fay RR, Popper AN (1974) Acoustic stimulation of the ear of the goldfish (*Carassius auratus*). *J Exp Biol* 61:243-260.

Fay RR, Popper AN (1975) Modes of stimulation of the teleost ear. *J Exp Biol* 62:379-387.

Fay RR, Popper AN (1980) Structure and function in teleost auditory system: In: Popper AN, Fay RR (eds.) *Comparative Studies of Hearing in Vertebrates*. Springer-Verlag, New York. pp 1-42.

Fay RR (1984) The goldfish ear codes the axis of acoustic particle motion in three dimensions. *Science* 225:951-954.

Fay RR, Edds-Walton PL (1997) Diversity in frequency response properties of saccular afferents of the toadfish, *Opsanus tau. Hear Res* 113: 235-246.

Feng AS, Lin W (1991) Differential innervation patterns of three divisions of frog auditory midbrain (torus semicircularis). *J Comp Neurol* 306:613-630.

Feng AS, Schellart NAM (1999) Central auditory processing in fish and amphibians. In: Fay RR, Popper AN (eds.) *Comparative Hearing: Fish and Amphibians*. Springer, New York. pp 218-268.

Fine ML, Lenhardt ML (1983) Shallow-water propagation of the toadfish mating call. *Comp Biochem Physiol A* 76:225-231.

Fine ML, Burns NM, Harris TM (1990) Ontogeny and sexual dimorphism of the sonic muscle in the oyster toadfish. *Can J Zool* 68:1374-1381.

Fine ML, Bernard B, Harris TM (1993) Functional morphology of toadfish sonic muscle fibers: Relationship to possible fiber division. *Can J Zool* 71:2262-2274.

Fine ML, Malloy KL, King C, Mitchell SL, Cameron TM (2001) Movement and sound generation by the toadfish swimbladder. *J Comp Physiol A* 187:371–379.

Fine ML, Parmentier E. (2015) Mechanisms of fish sound production. In: Ladich F (ed.) *Sound Communication in Fishes*. Springer Verlag, New York. pp 77-126.

Forlano PM, Deitcher DL, Myers DA, Bass AH (2001) Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: Aromatase enzyme and mRNA expression identify glia as source. *J Neurosci* 21:8943-8955.

Forlano PM, Deitcher DL, Bass AH (2005) Distribution of estrogen receptor alpha mRNA in the brain and inner ear of a vocal fish with comparisons to sites of aromatase expression. *J Comp Neurol* 483:91-113.

Forlano PM, BA Schlinger, Bass AH (2006) Brain aromatase: New lessons from non-mammalian model systems. *Front Neuroendocrinol* 27:247-274.

Forlano PM, Marchaterre M, Deitcher DL, Bass AH (2010) Neuroanatomical distribution of androgen receptor mRNA in vocal, auditory and neuroendocrine circuits in a teleost Fish. *J Comp Neurol* 518:493-512.

Forlano PM, Bass AH (2011) Neural and hormonal mechanisms of reproductive-related arousal in fishes. *Horm Behav* 59:616-629.

Forlano PM, Kim SD, Krzyminska Z, Sisneros JA (2014) Catecholaminergic connectivity to the inner ear, central auditory and vocal motor circuitry in the plainfin midshipman fish, *Porichthys notatus*. *J Comp Neurol* 522:2887-2927.

Forlano PM, Ghahramani ZN, Monestime C, Kurochkin P, Chernenko A, Milkis D (2015a) Catecholaminergic innervation of central and peripheral auditory circuitry varies with reproductive state in female midshipman fish, *Porichthys notatus*. *PLoS ONE* 10(4): e0121914.

Forlano PM, Sisneros JA, Rohmann KN, Bass AH (2015b) Neuroendocrine control of seasonal plasticity in the auditory and vocal systems of fish. *Front Neuroendocrinol* 37:129-145.

Forlano PM, Maruska KM, Sisneros JA, Bass AH (2016) Hormone-dependent plasticity of auditory systems in fishes. In Bass AH, Sisneros JA, Fay RR, Popper AN (eds.) *Hearing and Hormones.* Springer Handbook of Auditory Research, Vol. 57, Springer Science+Business Media, LLC, New York.

Forlano PM, Licorish RR, Ghahramani ZN, Timothy M, Ferrari M, Palmer WC, Sisneros JA (2017) Attention and motivated response to simulated male advertisement call activates forebrain dopaminergic and social decision-making network nuclei in female midshipman Fish. Int Comp Biol (in press).

Fuzessery ZM, Feng AS (1983) Mating call selectivity in the thalamus and midbrain of the leopard frog (*Rana p. pipiens*): Single and multiunit responses. *J Comp Physiol* 150:333-344.

Goodson JL, Bass AH (2000a) Forebrain peptides modulate sexually polymorphic vocal circuitry. *Nature* 403:769-772.

Goodson JL, Bass AH (2000b) Rhythmic midbrain-evoked vocalization is inhibited by vasoactive intestinal polypeptide in the teleost *Porichthys notatus*. *Brain Res* 865:107-111.

Goodson JL, Bass AH (2002) Vocal-acoustic circuitry and descending vocal pathways in teleost fish: Convergence with terrestrial vertebrates reveals conserved traits. *J Comp Neurol* 448:298-322.

Goodson JL (2005) The vertebrate social behavior network: Evolutionary themes and variations. *Horm Behav* 48:11-22.

Goodson JL, Kabelik D (2009) Dynamic limbic networks and social diversity in vertebrates: From neural context to neuromodulatory patterning. *Front Neuroendocrinol* 30:429-441.

Greene CW (1924) Physiological reactions and structure of the vocal apparatus of the California singing fish, *Porichtys notatus. Amer J Physiol* 70:496-499.

Greenfield DW, Winterbottom R, Collette BB (2008) Review of the toadfish genera (teleostei: batrachoididae). *Proc Cal Acad Sci* 59:665-710.

Hall JC, Feng AS (1987) Evidence for parallel processing in the frog's auditory thalamus. *J Comp Neurol* 258:407-419.

Harden Jones FR, Marshall NB (1953) The structure and functions of the teleostean swim bladder. *Biol Rev* 28:16-23.

Hawkins AD (1986) Underwater sound and fish behavior. In: Pitcher TJ (ed.) *The Behaviour of Teleost Fishes*, Croom Helm, London; Sydney, NSW. pp 114-151.

Hill GL, Fine ML, Musick JA (1987) Ontogeny of the sexually dimorphic sonic muscle in three Sciaenid species. *Copeia* 1987:708-713.

Hoke KL, Burmeister SS, Fernald RD, Stanley Rand A, Ryan, MJ, Wilczynski W (2004) Functional mapping of the auditory midbrain during mate call reception. *J Neurosci* 24:11264-11272.

Hoke KL, Ryan MJ, Wilczynski W (2007) Integration of sensory and motor processing underlying social behavior in tungara frogs. *Proc Roy Soc B* 274:641-649.

Hoke KL, Ryan MJ, Wilczynski W (2010) Sexually dimorphic sensory gating drives behavioral differences in tungara frogs. *J Exp Biol* 213:3463-3472.

Hubbs, CL (1920) The binomics of *Porichthys notatus* Girard. *Amer Nat* 54:380-384.

Hurley LM, Devilbiss DM, Waterhouse BD (2004) A matter of focus: monoaminergic modulation of stimulus coding within mammalian sensory networks. *Curr Opin Neurobiol* 14:488-495.

Ibara RM, Penny LT, Ebeling AW, van Dykhuizen G, Cailliet G (1983) The mating call of the plainfin midshipman fish, *Porichthys notatus*. In: Noakes DLG, Lindquist DG, Helfman GS, Ward JA (eds.) *Predators and Prey in Fishes*, The Hague: Junk Publishers. pp 205-212.

Idda ML, Bertolucci C, Vallone D, Gothilf Y, Sanchez-Vazquez FJ, Foulkes NS (2012) Circadian clocks: Lessons from fish. In: Kalsbeek A, Merrow M, Roenneberg T, Foster RG (eds.) *Progress in brain research: The Neurobiology of Circadian Timing*. Elsevier, Amsterdam, the Netherlands. pp 41-57.

Kasumyan AO (2008) Sounds and sound production in fishes. *J Ichthyol* 48:981-1030.

Kéver L, Boyle KS, Dragičević B, Dulčić J, Casadevall M, Parmentier E (2012) Sexual dimorphism of sonic apparatus and extreme intersexual variation of sounds in *Ophidion rochei* (Ophidiidae): First evidence of a tight relationship between morphology and sound characteristics in Ophidiidae. *Front Zool* 9:34.

Kéver L, Boyle KS, Bolen G, Dragičević B, Dulčić J, Parmentier E (2014) Modifications in call characteristics and sonic apparatus morphology during puberty in *Ophidion rochei* (actinopterygii: Ophidiidae). *J Morphol* 275:650-660.

Klug A, Bauer EE, Hanson JT, Hurley L, Meitzen J, Pollak GD (2002) Response selectivity for species-specific calls in the inferior colliculus of Mexican free-tailed bats is generated by inhibition. *J Neurophysiol* 88:1941-1954.

Kovacs KJ (2008) Measurement of immediate-early gene activation – c-fos and beyond. *J Neuroendocrinol* 20:665-672.

Ladich F (2016) Peripheral hearing structures in fishes: Diversity and sensitivity of catfishes and cichlids In: Sisneros JA (ed.) *Fish Hearing and Bioacoustics: An anthology in honor of Arthur N. Popper and Richard R. Fay. Advances in Experimental Medicine and Biology.* Springer Science + Business Media, New York. pp 321-340.

Ladich F, Fay RR (2013) Auditory evoked potential audiometry in fish. *Rev fish biol fish* 23:317-364.

Ladich F, Popper AN (2004) Parallel evolution in fish hearing organs. In: Manley GA, Fay RR, (eds.) *Evolution of the Vertebrate Auditory System*. Springer Verlag New York. pp 95-127.

Le Prell CG, Henderson D, Fay RR, Popper AN (eds.) (2012) Noise-Induced Hearing Loss. *Scientific Advances, Springer Handbook of Auditory Research*, Springer Science+Business Media, New York.

Liem KF (1963) The Comparative Osteology and Phylogeny of the Anabantoidei (Teleostei, Pisces). Urbana, IL: University of Illinois Pres.

Lu Z, Fay RR (1995) Acoustic response properties of single neurons in the central posterior nucleus of the thalamus of the goldfish, *Carassius auratus*. *J Comp Physiol A* 176:747-760.

Luckman SM, Dyball RE, Leng G (1994) Induction of c-fos expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity. *J Neurosci* 14:4825-4830.

Lychakov DV, Rebane YT (2000) Otolith regularities. *Hear Res* 143:83-102.

MacGinittie GE (1935) Ecological aspects of California marine estuary. *Amer Midl Nat* 16:629-765.

Malsbury CW, Kow LM, Pfaff DW (1977) Effects of medial hypothalamic lesions on the lordosis response and other behaviors in female golden hamsters. *Physiol Behav* 19:223-237.

Maney DL, Goode CT, Lange HS, Sanford SE, Solomon BL (2008) Estradiol modulates neural responses to song in a seasonal songbird. *J Comp Neurol* 511:173-186.

Maney DL, Pinaud R (2011) Estradiol-dependent modulation of auditory processing and selectivity in songbirds. *Front Neuroendocrinol* 32:287-302.

Mann DA, Lu Z, Hastings MC, Popper AN (1998) Detection of ultrasonic tones and simulated dolphin echolocation clicks by a teleost fish, the American shad (*Alosa sapidissima*). *J Acoust Soc Am* 104:562-568.

Maruska KP, Sisneros JA (2015) Sex steroid-dependent modulation of acoustic communication systems in fishes. In: Ladich F, (ed.) *Sound Communication in Fishes*. Spring-Verlag Berlin-Heidelberg-New York. pp 207-233.

Maruska KP, Sisneros JA (2016) Comparison of electrophysiological auditory measures in fishes. In: Sisneros JA (ed.) *Fish Hearing and Bioacoustics: An anthology in honor of Arthur N. Popper and Richard R. Fay. Advances in Experimental Medicine and Biology*. Springer Science + Business Media, New York. pp 121-155.

Mathews D, Edwards DA (1977) Involvement of the ventromedial and anterior hypothalamic nuclei in the hormonal induction of receptivity in the female rat. *Physiol Behav* 19:319-326.

McCormick CA (1999) Anatomy of the central auditory pathways of fish and amphibians. In: Fay RR, Popper AN (eds.) *Comparative Hearing: Fish and Amphibians*. Springer, New York. pp 155-217.

McCormick CA (2011) Auditory/lateral line CNS: Anatomy. In: Farrell AP (ed.) *Encyclopedia of fish physiology: From genome to environment*. Academic Press, San Diego, CA. pp. 283-291.

McKibben JR, Bass AH (1998) Behavioral assessment of acoustic parameters relevant to signal recognition and preference in a vocal fish. *J Acoust Soc Am* 104:3520-3533.

Meddle SL, Foidart A, Wingfield JC, Ramenofskyand M, Balthazart J (1999) Effects of sexual interactions with a male on fos-like immunoreactivity in the female quail brain. *J Neuroendocrinol* 11:771-784.

Mohr RA, Whitchurch EA, Anderson RD, Forlano PM, Fay RR, Ketten DR, Cox TC, Sisneros JA (2017) Intra- and Intersexual swim bladder dimorphisms in the plainfin midshipman fish (*Porichthys notatus*): Implications of swim bladder proximity to the inner ear for sound pressure detection. *J Morph* 278:1458-1468.

Mohr RA, Chang Y, Bhandiwad AA, Forlano PM, Sisneros JA. Brain activation patterns in response to conspecific and heterospecific social acoustic signals in the plainfin midshipman fish, *Porichthys notatus. in preparation.*

Mudry KM, Capranica RR (1987) Correlation between auditory evoked responses in the thalamus and species-specific call characteristics. I. *Rana catesbeiana* (Anura: Ranidae). *J Comp Physiol A* 160:477-489.

Nelson EM (1955) The morphology of the swim bladder auditory bulla in the Holocentridae. *Field Zool* 37:121-130.

Newman SW (1999) The medial extended amygdala in male reproductive behavior: A node in the mammalian social behavior network. *Ann NY Acad Sci* 877:242-257.

Nicolson T (2005) The genetics of hearing and balance in zebrafish. *Ann Rev Genet* 39:9-22.

Northcutt RG (2006) Connections of the lateral and medial divisions of the goldfish telencephalic pallium. *J Comp Neurol* 494:903-943.

O'Connell CP (1955) The gas bladder and its relation to the inner ear in *Sardinops caerulae* and *Engraulis mordax*. *Fish Bull* 104:505-533.

Parker GH (1903) The sense of hearing in fishes. *Am Nat* 37:185-204.

Parmentier E, Colleye O, Fine ML, Frédérich B, Vandewalle P, Herrel A (2007) Sound production in the clownfish *Amphiprion clarki*. *Science* 316:1006.

Parmentier E, Diogo R (2006) Evolutionary trends of swimbladder sound mechanisms in some teleost fishes. In: Ladich F, Collin SP, Moller P, Kapoor BG (eds.) *Fish Communication*. Science Publisher Inc, USA, pp 43-68.

Parmentier E, Mann K, Mann D (2011) Hearing and morphological specializations of the mojarra (*Eucinostomus argenteus)*. *J Exp Biol* 214:2697-2701.

Pelster B (2011) Buoyancy, locomotion, and movement in fishes: swimbladder function and buoyancy control in fishes. In: Farrell AP (ed.) *Encyclopedia of Fish Physiology: From Genome to Environment*. Academic Press, San Diego CA. pp 526-534.

Perelmuter JT, Forlano PM (2017) Connectivity and ultrastructure of dopaminergic innervation of the inner ear and auditory efferent system of a vocal fish. *J Comp Neurol* 525:2090-2108.

Perez-Cadahia B, Drobic B, Davie JR (2011) Activation and function of immediate-early genes in the nervous system. *Biochem Cell Biol* 89:61-74.

Petersen CL, Timothy M, Kim DS, Bhandiwad AA, Mohr RA, Sisneros JA, Forlano PM (2013) Exposure to advertisement calls of reproductive competitors activates vocal-acoustic and catecholaminergic neurons in the plainfin midshipman fish, *Porichthys notatus*. *PLoS ONE* e70474.

Pfaff DW, Sakuma Y (1979) Deficit in the lordosis reflex of female rats caused by lesions in the ventromedial nucleus of the hypothalamus. *J Physiol* 288:203-210.

Platt C, Popper AN (1981) Structure and function in the ear. In: Tavolga WN, Popper AN, Fay RR (eds.), *Hearing and Sound Communication in Fishes*. Springer, New York, pp 3- 38.

Popper AN, Coombs S (1980) Auditory mechanisms in teleost fishes: Significant variation in both hearing capabilities and auditory structures are found among species of bony fishes. *Am Sci* 68:429-440.

Popper AN, Fay RR (1993) Sound detection and processing by fish: critical review and major research questions. *Brain Behav Evol* 41:14-38.

Popper AN, Fay RR (1997) Evolution of the ear and hearing: Issues and questions. *Brain Behav Evol* 50:213-221.

Popper AN, Fay RR (1999) The auditory periphery in fishes. In Fay, RR and Popper, AN (eds.) *Comparative Hearing: Fish and Amphibians.* Springer-Verlag New York. pp 43-100.

Popper AN, Schilt CR (2008) Hearing and acoustic behavior (basic and applied). In: Webb, JF, Fay, RR and Popper, AN (eds.) *Fish Bioacoustics*. Springer Science+Business Media, LLC, New York. pp 17-48.

Popper AN, Tavolga WN (1981) Structure and function of the ear of the marine catfish, *Arius felis*. *J Comp Physiol* 144:27-34.

Potter HD (1965) Mesencephalic auditory region of the bullfrog. *J Neurophysiol* 28:1132-1154.

Ramcharitar JU, Higgs DM, Popper AN (2006) Audition in sciaenid fishes with different swim bladder-inner ear configurations. *J Acoust Soc Am* 119:439-443.

Riters LV (2012) The role of motivation and reward neural systems in vocal communication in songbirds. *Front Neuroendocrinol* 33:194-209.

Rogers PH, Cox M (1988) Underwater sound as a biological stimulus. In: Fay RR, Popper AN, Tavolga WN (eds.) *Sensory Biology of Aquatic Animals*. Springer-Verlag, New York. pp 131-149.

Rohmann KN, Bass AH (2011) Seasonal plasticity of auditory hair cell frequency sensitivity correlates with plasma steroid levels in a vocal fish. *J Exp Biol* 214:1931-1942.

Rohmann KN, Fergus DJ, Bass AH (2013) Plasticity in ion channel expression underlies variation in hearing during reproductive cycles. *Curr Biol* 23:678-683.

Rose JA (1961) Anatomy and sexual dimorphism of the swim bladder and vertebral column in *Ophidion holbrooki* (Pisces: Ophidiidae). *Bull Mar Sci Gulf Carib* 11:280-308.

Schneider H (1941) Die Bedeutung der Atemhöhle der Labyrinthfische fürihr Hörvermögen. *Z Vergl Physiol* 29:171-194.

Schuijf A (1981) Models of acoustic localization. In: Tavolga WN, Popper AN, Fay RR (eds.) *Hearing and Sound Communication in Fishes*. Springer, New York. pp 267-310.

Schulz-Mirbach T, Metscher, BD, Ladich, F (2012) Relationship between swim bladder morphology and hearing abilities - A case study on Asian and African Cichlids. *PLoS ONE* 7(8), e42292.

Schulz-Mirbach T, Martin H, Metscher BD, Ladich F (2013) A unique swim bladder-inner ear connection in a teleost fish revealed by a combined high-resolution microtomographic and three-dimensional histological study. *BMC Biol* 11:75.

Simpson SD, Meekan M, Montgomery J, McCauley R, Jeffs A (2005) Homeward sound. *Science* 308:221.

Sisneros JA, Marchaterre MA, Bass AH (2002) Otolithic endorgan projections of the inner ear in a vocal fish. *Bioacoustics* 12:137-139.

Sisneros JA, Bass AH (2003) Seasonal plasticity of peripheral auditory frequency sensitivity. *J Neuro* 23:1049-1058.

Sisneros JA, Forlano PM, Knapp R, Bass AH (2004a) Seasonal variation of steroid hormone levels in an intertidal-nesting fish, the vocal plainfin midshipman. *Gen Comp Endocrinol* 136:101-116.

Sisneros JA, Forlano PM, Deitcher DL, Bass AH (2004b) Steroid-dependent auditory plasticity leads to adaptive coupling of sender and receiver. *Science* 305:404-407.

Sisneros JA (2007) Saccular potentials of the vocal plainfin midshipman fish, *Porichthys notatus*. *J Comp Physiol A* 193:413-424.

Sisneros JA (2009) Seasonal plasticity of auditory saccular sensitivity in the vocal plainfin midshipman fish, *Porichthys notatus*. *J Neurophysiol* 102:1121-1131.

Sisneros JA, Rogers PH (2016) Directional hearing and sound source localization in fishes. In: Sisneros JA (ed.) *Fish Hearing and Bioacoustics: An anthology in honor of Arthur N. Popper and Richard R. Fay. Advances in Experimental Medicine and Biology*. Springer Science + Business Media, New York. pp 121-155.

Suta D, Kvasnak E, Popelar J, Syka J (2003) Representation of species-specific vocalizations in the inferior colliculus of the guinea pig. *J Neurophysiol* 90:3794-3808.

Syka J, Popelář J, Kvašňák E, Šuta J, Jilek M (1997) Processing of species-specific vocalizations in the inferior colliculus and medial geniculate body of the guinea pig. In: Syka J (ed.) *Acoustical Signal Processing in the Central Auditory System*. Springer, Boston, MA, pp 431-441.

Tomchik SM, Lu Z (2005) Octavolateral projections and organization in the medulla of a teleost fish, the sleeper goby (*Dormitator latifrons*). *J Comp Neurol* 481:96-117.

Tricas TC, Webb JF (2016) Acoustic communication in butterflyfishes: Anatomical novelties, physiology, and behavior. In: Sisneros JA (ed.) *Fish Hearing and Bioacoustics: An anthology in honor of Arthur N. Popper and Richard R. Fay. Advances in Experimental Medicine and Biology.* Springer Science + Business Media, New York. pp 57-92.

Webb JF, Fay RR, Popper, AN (eds.) (2008) *Fish Bioacoustics*. Springer Science+Business Media, LLC, New York.

Wilczynski W (1981) Afferents to midbrain auditory center in the bullfrog *Rana catesbeiana*. *J Comp Neurol* 198:421-433.

Wilczynski W, Endepols H (2007) Central auditory pathways in anuran amphibians: The anatomical basis of hearing and sound communication. In: Popper AN, Feng AS, Narins PN (eds.) *Hearing and Sound Communication in Amphibians*. Springer, Berlin, Germany. pp 221-249.

Whitfield TT, Riley BB, Chiang MY, Phillips B (2002) Development of the zebrafish inner ear. *Dev Dyn* 223, 427–58.

Yan HY (1998) Auditory role of the suprabranchial chamber in gourami fish. *J Comp Physiol A* 183:325-333.

Yan HY, Curtsinger WS (2000) The otic gasgladder as an ancillary auditory structure in a mormyrid fish. *J Comp Physiol A* 186:595-602.

Yan HY, Fine ML, Horn NS, Colon WE (2000) Variability in the role of the gasbladder in fish audition. *J Comp Physiol A* 187:371-379.

Zeddies, DG, Fay RR, Alderks PW, Shaub, KS, Sisneros JA (2010) Sound source localization by the plainfin midshipman fish, *Porichthys notatus*. *J Acoust Soc Am* 127:3104-3113.

Zeddies DG, Fay RR, JA Sisneros (2011) Sound Source Localization and Directional Hearing in Fishes. In: Farrell AP (ed.) *Encyclopedia of Fish Physiology: From Genome to Environment*. Academic Press, San Diego, CA. pp 298-303.

Zeddies DG, Fay RR, Gray MD, Alderks, PW, Acob A, Sisneros JA (2012) Local acoustic particle motion guides sound-source localization behavior in the plainfin midshipman fish, *Porichthys notatus*. *J Exp Biol* 215:152-160.