Ontogeny of hearing in the plainfin midshipman, *Porichthys notatus*

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2013

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Program Authorized to Offer Degree:

Psychology

University of Washington

Abstract

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The plainfin midshipman, *Porichthys notatus,* has become a model for vocal communication and auditory reception because acoustic communication plays an important role in spawning behavior in this fish species. The auditory system of the teleost fish, *P. notatus*, is an important sensory receiver system used to encode intraspecific social communication signals in adults, but the response properties and function of this receiver system in pre-adult stages are less known. I describe what is known about the ontogeny of auditory sensitivity in juvenile and larval stages as well as other important background information in chapter one. In chapter two, I examine the response properties of auditory evoked potentials from the midshipman saccule, the main organ of hearing in this species, to determine whether the frequency response and auditory threshold of saccular hair cells to behaviorally relevant single tone stimuli change during ontogeny. I address the question of auditory sensitivity during larval development in chapter three and discuss when in the larval stage audition begins using the innate acoustically evoked behavioral response (AEBR) as a hearing assay.

Specifically, I demonstrate that auditory reception develops early in larval development and that auditory sensitivity is similar throughout all larval stages. Additionally, during ontogeny, the saccule undergoes profound growth. I examine the saccular epithelia of larval, juvenile, and adult plainfin midshipman in order to describe the morphological changes that occur in saccular ultrastructure during development. These results are described in greater detail in chapter four. The results from chapters two through four are summarized and placed into broader contexts and I suggest future studies in chapter five.

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GLOSSARY

ABR	auditory brainstem response
AEBR	acoustically evoked behavioral response
AEP	auditory evoked potential
BEF	best evoked frequency
BF	best frequency
CF	characteristic frequency
CNS	central nervous system
GSI	gonad somatic index
IEG	immediate early gene
PBS	phosphate buffered saline
PH3	phosphorylated histone H3
SL	standard length
SPL	sound pressure level
TL	total length

PREFACE

Chapter 2 has previously been published as:

Alderks, PW and Sisneros, JA (2011) Ontogeny of auditory saccular sensitivity in the plainfin midshipman fish, *Porichthys notatus*. J Comp Physiol A 197:387–398.

Chapter 3 is currently in review:

Alderks, PW and Sisneros, JA (2013) Development of the acoustically evoked behavioral response in larval plainfin midshipman fish, *Porichthys notatus. in review*.

Chapter 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.

ACKNOWLEDGMENTS

First and foremost I would like to thank my wife Jenne and my children Willem, Belle, and Elizabeth for the love and support you have given me as well as making collecting trips to the beach more fun and enjoyable. You help me strive to always do my best and help pick me up when I fall short of that goal. I am also grateful for the love and support of my parents David and Kathy, my grandparents Fred and Marge, and my brother David, and my sister Sarah. You helped nurture the seeds of science as I grew and continue to support my efforts and me to this day. I would not be the person I am today without your influence in my life.

I would like to thank the members of the Sisneros Lab, Liz Whitchurch and my fellow graduate students Kam Leon, Ashwin Bhandiwad and Rob Mohr who have assisted with numerous aspects of this dissertation and become friends and colleagues. There have also been many undergraduates who have assisted in various ways including Victoria Nultch, Kiel Shaub, Andrew Acob, Meagan Wong, Jackie Chen, and Christina Jarvis. Thank you for the inviting lab atmosphere and the fun.

A heartfelt thank you goes to my collaborators. Dick Fay and David Zeddies in particular have taught me more about doing research than any other individuals. It has been a treat working with them and brainstorming about science and the future. Thank you also to Raquel Vasconcelos, Allison Coffin, Mike Gray, Peter Rogers, and Andrew Brown who

have also given me opportunities to expand my learning into new areas and patiently worked with me on side projects.

I would like to thank the members of my committee, Michael Beecher, Ellen Covey, Ted Pietsch, and Chris Stecker. You have provided valuable advice and mentoring without which I would not have been able to complete my studies at the University of Washington. Thank you for all that you have done, and for your willingness to serve on my committee.

Thanks to the graduate students in the animal behavior program and my cohort. You have enriched the learning environment and expanded my horizons. I wish you the best in the future.

Finally, I am grateful for my advisor Joe Sisneros. You have been patient with my failings and have always tried to look out for my best interests. I am grateful for the advice you have given and hope to emulate your friendly mentoring style with my own students someday. I am honored to be your first graduate student and hope that we will be able to continue to collaborate in the future. Thanks for all the fish!

Chapter 1. Background and Significance

1.1 Hearing in fishes and important questions for the field

Fish represent the earliest branches of the vertebrate tree and as such have evolved to a greater diversity than any other vertebrate group (Popper and Fay 1997). The auditory system serves a variety of functions in fish and many basic functions of the auditory system first evolved in an early fish common ancestor and are conserved throughout vertebrate groups (Fay and Popper 2000). Some of these functions include communication, sound intensity discrimination, frequency discrimination, sound source localization, predator and prey detection, and auditory scene analysis (Chapman and Johnstone, 1974, Schuijf 1975, Gans 1992, Hauser 1997, Fay 1997, 1998a,b, 2000, Bass et al. 1999). Diversity can also be seen in the structures of the auditory system (Retzius 1881, van Bergeijk 1967). The functional significance of this diversity of auditory structures and its implications for hearing remains unclear.

1.1.1 Important questions

In a seminal series of papers, Popper and Fay (1973, 1993, 2011) reviewed the current state of the literature and proposed important questions in the field of fish hearing. It would be impossible to relate all the questions raised by Popper and Fay in these papers, however some of these important questions relate directly to the research presented in this work. The goal of this work is to gain insight into ontogenetic mechanisms that shape hearing in fishes. One general question brought up by Popper and Fay is, "what are the mechanisms that account for the differences observed in auditory sensitivity

among fishes?" This question relates specifically to chapters two and three in which I investigate auditory sensitivity (saccular sensitivity and acoustically evoked behavioral response (AEBR) sensitivity respectively) during development from larvae to adult. Although these experiments focus primarily on the midshipman rather then using the recommended comparative method, they do provide valuable information about the ontogeny of hearing because the midshipman has become a model for auditory research. Another broad area where further research is needed is to determine the functional significance of the inter-specific variation seen in fish auditory structures. In chapter four I describe structural changes that occur in the inner ear morphology of the saccule during ontogeny that more fully explain the functional results from chapters two and three. Such structure-function relationships will inform future research necessary for a more complete understanding of the ontogenetic mechanisms controlling auditory development in fishes.

1.2 The fish ear and its development

Like other vertebrates, fish have ears that detect acoustic stimuli (Weber 1820, Parker 1903, von Frisch and setter 1932). The fish ear is made up of three otolithic endorgans, the lagena, utricle, and saccule, complete with otoliths and sensory epithelia, as well as three semicircular canals (Retziuz 1881, see figure 1.1). It is believed that all three otolithic endorgans are capable of detecting both inertial stimuli as well as acoustic stimuli, however it is likely that the three endorgans differ in their relative contribution to motion detection and audition (Popper and Fay 1993, Popper et al. 2003). The saccule is the considered the primary auditory endorgan in most teleost fishes (Popper and Schilt 2008, Webb et al. 2008).

1.2.1 The saccule and its role in hearing

The saccule responds to acoustic particle motion much like an accelerometer (Platt and Popper 1981, Popper and Tavolga 1981, Fay 1984). The saccule contains a dense otolith named the sagitta, which is about three times more dense than the the fish's body (de Vries 1950, Popper and Lu 2000). When sound passes through the fish, the sagitta moves at a different phase and amplitude than the saccular epithelium (Dijkgraaf 1960, Fay and Popper 1975). A shearing motion results as the otolith and sensory epithelium move past each other, causing the ciliary hair bundles to bend (Fay and Popper 1974, Popper and Fay 1993). Signal transduction occurs as the hair bundles bend toward the kinocilium and generate a receptor potential (Popper 1983, Fay and Popper 2000). Otolithic organs are most effective at responding to low frequencies (Fay 1988, Popper and Fay 1999).

1.2.2 Development of the fish inner ear

The inner ear develops very early in larval development. In the zebrafish*, Danio rerio,* responses to acoustic stimuli begin five days after hatching (Zeddies and Fay 2005). The inner ear continues to grow throughout a fish's life, and fish continue to grow hair cells. Thus the total number of hair cells increase post-embryonically (Platt 1977, Corwin 1983, Popper and Hoxter 1984). In the oscar, *Astronotus ocellatus*, there is also an increase in the number of afferent neurons innervating the saccule, however this proliferation occurs at a slower rate than hair cell addition (Popper and Hoxter 1984, 1990). The eighth cranial nerve arbors grow to accommodate the increased number of hair cells and the convergence ratio between hair cells and afferent neurons likewise increases (Popper and

Fay 1993). Recordings from the eighth cranial nerve have shown an increase in sensitivity during development as the convergence ratio increases in *Raja clavata, Carassius auratus, and Porichthys notatus* (Corwin 1983, Sento and Furukawa 1987, Sisneros and Bass 2005). The functional significance of hair cell addition and the increase in the number of hair bundles innervated by each afferent neuron is not well understood (Popper and Fay 1993, Lanford et al. 2000, Popper and Fay 2011, but see also Smith et al. 2011).

1.3 Larval fish hearing

1.3.1 Larval stage terminology

The terminology of the life history stages for larval marine fishes is very complex and many different classification systems exist to describe early fish development (Balon 1975, Shardo 1995, Bartsch et al. 1997, Martinez and Bolker 2003, Elliott et al. 2007, Martin et al. 2009,). Most marine fishes reproduce by broadcast spawning where male and female fish simultaneously release gametes into the water (Leis 2002, Montgomery et al. 2006). In this system, embryonic and larval development takes place in a pelagic stage and the developing fish share little resemblance to an adult fish of the same species (Leis et al. 2011). At the end of the pelagic larval stage, the larvae are recruited to suitable habitat where they settle and undergo metamorphosis into a juvenile fish that closely resembles an adult fish of the same species (Leis et al 2003, Leis and Lockett 2005). The majority of classification systems differentiate between small differences in this more general pattern of larval development (Kendall et al. 1984).

Batrachoidid fishes, including midshipman, lay demersal eggs that undergo development in benthic nests without a pelagic larval stage (Greenfield et al. 2008). Additionally larval midshipman closely resemble adult midshipman from the earliest stages of development (MacGinitie 1935, Arora 1948). Because the development pattern observed in plainfin midshipman is markedly different from the more common pelagic larval development, existing classification systems don't adequately define developmental stages in *P. notatus*. In this work I define the midshipman embryonic stage as the developmental period from fertilization of the ova to when the developing embryos hatch. The larval stage is defined as the time period from hatching to when the larval completely absorb their yolk and detach from the nest substrate. The juvenile stage is defined as the time from when the juveniles become free-swimming after detaching from the nest until they reach sexual maturity. This description of larval development and use of larval terminology is in agreement previous studies for Batrachoidid fishes (Gill 1907, Hubbs 1920, Crane 1981, Greenfield et al. 2008).

1.3.2 Auditory sensitivity in larval fishes

Although much is known about the development of auditory structures, there have only been a few studies that investigate auditory sensitivity in larval fish and fewer still that answer the: "question when does auditory responsiveness begin" (Retzius 1881, Titova 1970, Platt 1977, Fay and Popper 1985, Sokolowski and Popper 1987, 1988). The majority of the research in these areas has focused on the hearing abilities of post-pelagic settlement stage larval fish and post-settlement juvenile coral reef fishes. Simpson et al.

(2010) found that settlement stage larvae have similar hearing abilities as juvenile fish in *Pomacentrus amboinensis, P. brachialis, P. moluccensis, and P. nagasakiensis*.

Recently, the auditory evoked potential (AEP) technique has allowed researchers to begin gathering auditory physiology data on larval fishes. Wright et al. (2005, 2008, 2010) investigated auditory sensitivity in larval coral reef fish and also found that larvae have hearing abilities similar to that of post settlement juvenile reef fish. Only one study has investigated the development of auditory sensitivity throughout the larval stage (Wright et al. 2011). Wright et al. (2011) found ontogenetic improvements in hearing in four of the species tested *(Caranx ignobilis*, *Epinephelus coioides, Eleutheronema tetradactylum*, and *Macquaria novemaculeata),* however no clear ontogenetic trend in hearing could be identified in *Epinephelus fuscoguttatus*.

Two studies have investigated the onset of auditory responsiveness in the zebrafish (Zeddies and Fay 2005, Tanimoto et al. 2009). Zeddies and Fay (2005) found that the AEBR begins at day five post-hatch. In contrast, Tanimoto et al. (2009) found that *Danio rerio* begins showing auditory responsiveness as early as 40 hours post fertilization using electrical physiological techniques. Although the significance of this variation in ontogenetic effect during larval development remains unclear, it is clear that the auditory system develops early and therefore must play an important role in larval stages.

1.3.3 Role of audition in larval fishes

One of the major roles of the auditory system in larval fishes is predator avoidance. The ability to detect and localize sound during the larval stage can significantly affect fish mortality (Gagliano et al. 2008). Also recent behavioral studies in broadcast spawning coral reef fishes have provided evidence that sound cues may be an important for navigation and reef recruitment (Leis et al. 2002, Leis et al. 2003, Tolimieri et al. 2004, Simpson et al 2005, Leis and Lockett 2005, Simpson et al. 2008, Simpson et al. 2010, for review see Leis et al. 2011).

1.4 Ontogeny of auditory sensitivity in fishes

A variety of behavioral and physiological techniques have been used to study the ontogeny of hearing in fishes. Several studies using classical behavioral conditioning techniques found increases in hearing sensitivity around the characteristic frequency (CF), the frequency with the greatest sensitivity in an audiogram, and a broadening of the audiogram with increased age/size in pomacentrids and *Pagrus major* (Kenyon 1996, Iwashita et al. 1999). Corwin (1983) also reported an increase in hearing sensitivity in the skate (*Raja Clavata*) using extracellular single unit recordings from nerves innervating the macula neglecta. However, Popper (1971) reported that hearing sensitivity did not change with size in the goldfish, *Carassius auratus.*

Studies using the auditory evoked potential (AEP) technique have reported ontogenetic increases in hearing sensitivity in test frequencies near the adult stages CF in *Trichopsis vittata, Lophiobagrus cyclurus, Synodontis schoutedeni*, and *Halobatrachus didactylus* (Wysocki and Ladich 2001, Lechner et al. 2010, Lechner et al. 2011, Vasconcelos and

Ladich 2008). However, other studies also using the AEP technique have reported no change in hearing sensitivity during ontogeny in zebra fish, *Danio rerio,* American shad, *Alosa sapidissima* and spotfin butterflyfish, *Chaetodon ocellatus* (Higgs et al. 2001, Higgs et al. 2003, Higgs et al. 2004, Webb et al. 2012). One study even found a decrease in auditory sensitivity in the range of best hearing with an increase in size in sergeant major damselfish, *Abudefduf saxatilis* (Egner and Mann 2005).

In the plainfin midshipman, *Porichthys notatus*, Sisneros and Bass (2005) found an increase in resting discharge rate and auditory sensitivity at best frequency (BF), the frequency with the lowest threshold in an iso-intensity response profile, with increasing size when analyzing extracellular single unit recordings of saccular afferents in juvenile fish. It is likely that the diversity observed in the auditory structures of adult fish also represent a diversity of ontogenetic mechanisms at work that may affect auditory sensitivity during development. It is also possible that ontogenetic changes at one level of the auditory system are not also observed at subsequent levels of the auditory system (i.e. just because the eighth nerve has an ontogenetic increase in sensitivity, does not mean that you will see ontogenetic increases in auditory sensitivity at the level of the central nervous system, or that the animal will show an increase in behavioral auditory sensitivity).

1.5 The plainfin midshipman as a model

The plainfin midshipman, *P. notatus*, has become a good neuroethological model for studying communication and auditory reception in fishes (Bass and McKibben 2003,

Bass 2006, Sisneros 2009a,b). The survival of the species depends on acoustic communication and as a result auditory reception is very important in midshipman (Sisneros et al. 2009). During late spring and early summer male midshipman migrate to the shallow intertidal region and excavate a breeding nest under suitable rocks (Hubbs 1920, Greenfield et al. 2008). The males then begin advertising to potential mates via an acoustic hum (Greene 1924, MacGinitie 1935). Presumably female midshipman use features of the males' advertisement call to select a mate (Sisneros 2009a,b). Spawning takes place during high tides and the females retreat to deeper water leaving the male to guard the developing embryos and seek additional mating opportunities.

The embryos develop, hatch out, and progress through larval stages all while attached to the roof of the breeding nest (Hart 1973, see figure 1.2). When the larval fish have reached a sufficient size and have absorbed their yolk supply, they detach from their natal rock and adopt a free-swimming juvenile lifestyle (Arora 1948, Crane 1981). It is believed that juvenile midshipman remain in shallow water eel grass beds for a year or more until they are large enough to migrate to deeper water and enter the adult population (personal observations).

Several factors make the plainfin midshipman an ideal model for investigating auditory reception and the ontogeny of hearing. Firstly, midshipman are able to survive and develop in the very dynamic intertidal region where conditions (temperature, salinity, water turbidity, and even a lack of water at low tide) can change rapidly and extremes are common. This inherent "toughness" allows for physiology experiments that have been

all but impossible in more "delicate" fish. Also developing midshipman embryos and larvae can easily be collected attached to rocks in the intertidal region during the breeding season and reared in the lab until they reach the size of interest for the particular experiment. Typically rocks have several broods attached, so the researcher isn't constrained to a small window of one or two weeks during the breeding season to complete all their planned experiments. Lastly, the auditory system is very important for the plainfin midshipman. The fact that the saccule is almost as large as the brain (see figure 1.1) attests to the importance of hearing in this species. The midshipman must complete several difficult auditory tasks (sound source localization, acoustic feature detection, sound intensity discrimination, and frequency discrimination) in order to localize and select an appropriate mate in the shallow water intertidal environment. These three factors make the plainfin midshipman an ideal model for auditory research.

1.6 Questions addressed this work

As previously discussed, the goal of this work is to gain insight into ontogenetic mechanisms that shape hearing in fishes. I used an ethological perspective and a variety of electrical physiological, behavioral, and morphological techniques to investigate auditory sensitivity and changes in inner ear structures in larval and juvenile fish. I specifically asked the following questions:

1.6.1 Do auditory threshold or frequency response properties of saccular hair cells change during ontogeny?

I address this question in chapter two by characterizing the auditory-evoked potentials from the saccule of *P. notatus* and compare the saccular hair-cell frequency response properties of three different age/size classes of fish. The results for this experiment inform the experiments described in chapters three and four.

1.6.2 When does fish audition begin and does auditory sensitivity undergo ontogenetic changes during larval development?

In chapter three I used the acoustically evoked behavioral response (AEBR) to determine when the plainfin midshipman begins responding to acoustic stimuli and when the lateral line develops. I also characterize the AEBR response profiles using puretone stimuli in four groups of developing larval and juvenile fish to learn if auditory sensitivity is plastic during the larval stage of development.

1.6.3 What structural changes occur in the saccule during development?

Chapter four describes the morphological techniques I used to answer this specific question. More specifically I analyze changes in saccular epithelial area and hair bundle number, density and length in larval, juvenile, and adult midshipman. I also investigate hair bundle orientation patterns as well. The results from this experiment lead to potential explanations as to the mechanisms for maintaining auditory sensitivity in *Porichthys notatus* throughout development.

The next three chapters provide empirical evidence for the ontogenetic mechanisms that help shape the hearing of the plainfin midshipman. These chapters also provide an

accurate representation of the difficulties and complexities of doing auditory research in larval and juvenile fish. The final chapter summarizes the conclusions from chapters two through four and elaborates on future work that can provide an even greater understanding of ontogenetic plasticity in this and other fish species. It is my goal in writing this dissertation to provide the reader with a deeper understanding of the ontogenetic processes and their effects on hearing in the plainfin midshipman.

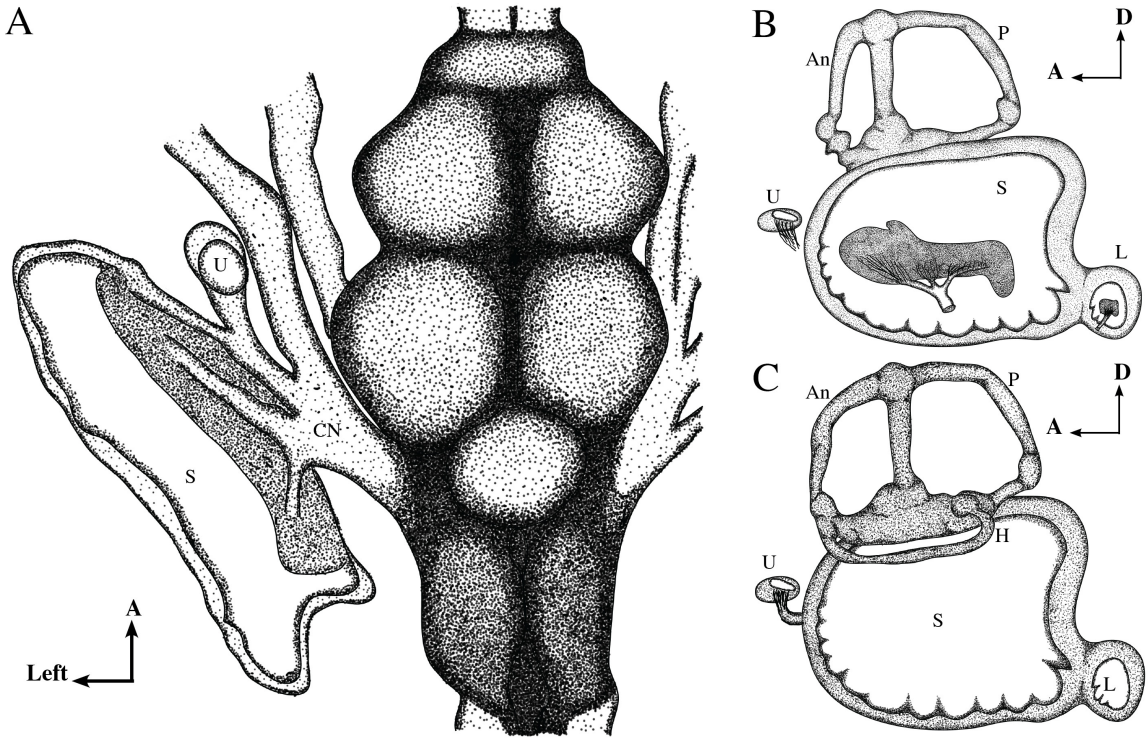


Figure 1.1 The inner ear in the plainfin midshipman. **A** depicts a dorsal view of the brain, auditory nerve (CN- VIIIth cranial nerve) and the inner ear (S- saccule, U- utricle). Notice the size of the saccule in relation to the brain. B and C show drawings or the right and left inner ears respectively in the plainfin midshipman. The three otolithic endorgans (S- saccule, L- lagena, and U- utricle) as well as the three semi-circular canals (An- anterior, H- horizontal, P- posterior) are visible.

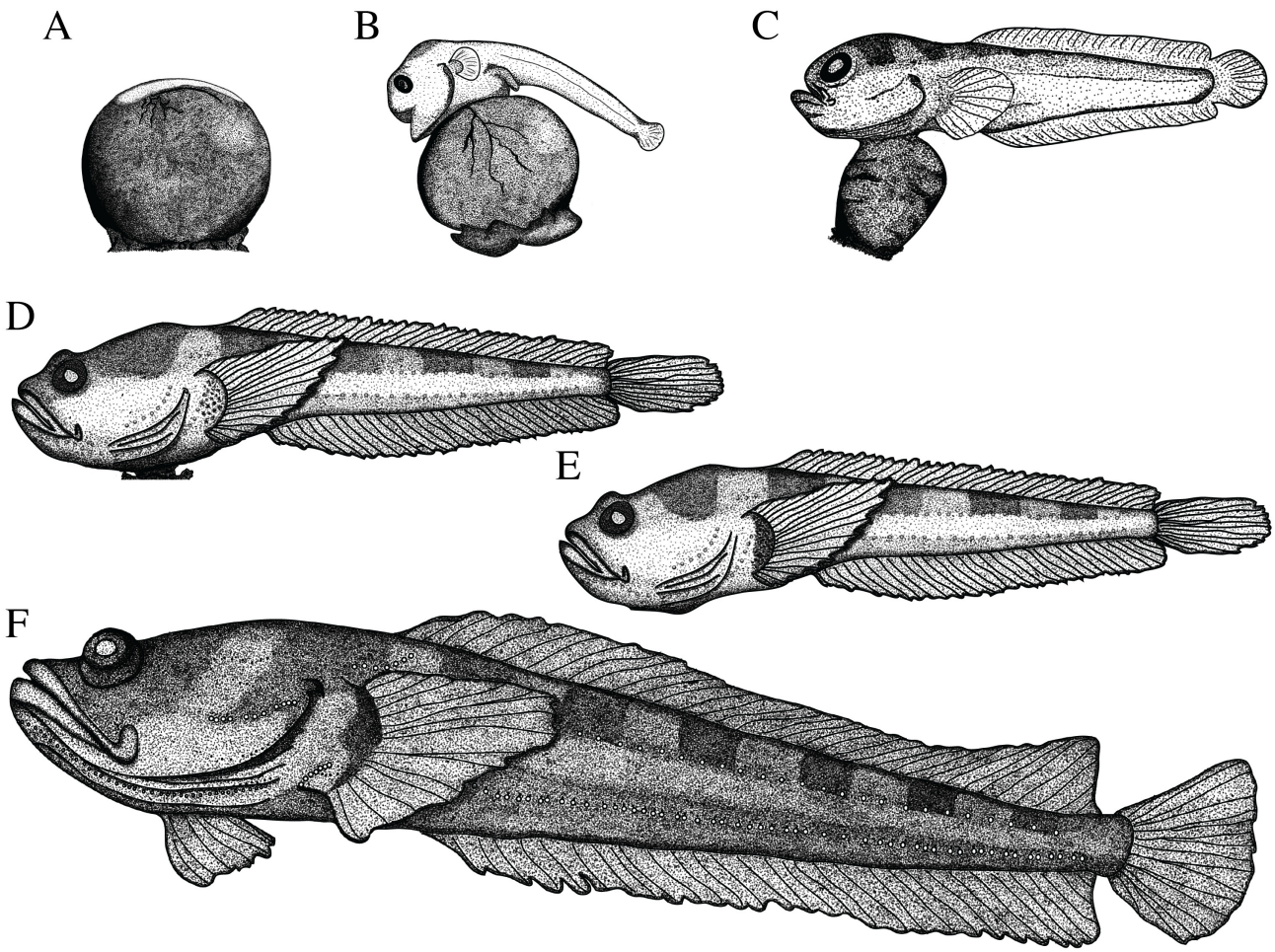


Figure 1.2 Life history stages in the plainfin midshipman. **A** depicts a developing embryo; **B** a 1.1 cm TL larval fish; **C** a 1.9 cm TL larval fish; **D** shows a 2.7cm TL large larval fish; **E** is a 3.0 cm TL free swimming small juvenile fish; and **E** depicts a nesting male adult midshipman.

Chapter 2. Ontogeny of auditory saccular sensitivity in the plainfin midshipman fish, *Porichthys notatus*

2.1 Summary

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

2.2 Introduction

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

The auditory system is an example of an important sensory system that is used to extract biologically important information from the natural environment. The acquired acoustic information can then be used to guide and coordinate behavior necessary for survival and reproduction. One auditory system that has become a model for investigating neural mechanisms of auditory perception that may be shared by all vertebrates is that of the plainfin midshipman fish (*Porichthys notatus*), in part because acoustic communication plays an important role in the social and reproductive behavior of this species (Bass and McKibben 2003; Sisneros 2009a). Female midshipman use their auditory system to detect and locate nocturnally active, ‘‘singing’’ males that produce multiharmonic advertisement calls to court and spawn with during the breeding season. While it is well established that the adult midshipman auditory system is adapted to encode the acoustic signals of conspecifics and functions in acoustic communication (Bass and McKibben 2003; Bass 2006; Sisneros 2009b), less is known about the response properties and

function of this sensory receiver system in the pre-adult life history stages. It remains unclear whether juveniles are vocally active during early life history stages and whether the auditory system of juveniles functions in acoustic communication similar to that of adults. Numerous studies have examined ontogenetic changes in the structure and function of the auditory sensory system in various other vertebrates including mammals, birds and amphibians (e.g., see Gray and Rubel 1985; Walsh et al. 1986; Mills et al. 1990; Dmitrieva and Gottlieb 1992; Geal-Dor et al. 1993; Brittan-Powell and Dooling 2000), but relatively few studies have examined ontogenetic changes in the neurophysiological response properties of the auditory system, especially in ancestral vertebrates such as fishes (Popper 1971; Corwin 1983; Kenyon 1996; Iwashita et al. 1999; Wysocki and Ladich 2001; Higgs et al. 2002, 2003; Egner and Mann 2005; Parmentier et al. 2009; Lechner et al. 2010).

Sisneros and Bass (2005) investigated age-related changes in the response properties of the midshipman peripheral auditory system and showed that the resting discharge rate and sensitivity at best frequency (BF) of saccular afferent neurons increased with age/size during ontogeny. Remarkably, this study represents the only investigation to date to report ontogenetic changes in the encoding properties of individual auditory neurons for any fish species, which in part is likely due to the difficult nature of these experiments and the rather robust stress tolerance of *P. notatus* for single unit recording methods. Although Sisneros and Bass (2005) provided important data on the changes in the response properties of saccular afferents during ontogeny, their study was to some extent limited because it only reported saccular afferent responses at one sound pressure level

(130 dB re 1uPa) due to the limited survival time of individual fish, especially for the smallest juvenile size class. More recently, Sisneros (2007) developed an evoked potential recording technique to determine the frequency response of hair cells within the saccule that can readily be used with smaller fish and is more amenable for investigating ontogenetic changes in auditory saccular sensitivity.

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

2.3 Methods and materials

2.3.1 Animal collection and care

We collected both male and female plainfin midshipman fish and grouped them into three classes based on sizes used in previous research (Sisneros and Bass 2005): adults, large juveniles, and small juveniles. Adults were defined as those fish greater than 9 cm standard length (SL), large juveniles were between 6.8 and 8 cm SL, and small juveniles were less than 3.2 cm. Adults and large juveniles were collected in mid February 2006 and in early March 2007 via otter trawl in Puget Sound near Edmonds, WA (n = 8 adults; R/V Kittiwake, Bio-Marine Enterprises) and in Monterey Bay near Moss Landing, CA (n = 24 adults, 12 large juveniles; R/V John H. Martin, Moss Landing Marine Laboratories),

respectively. Small juveniles (<3 cm SL) were collected from intertidal breeding areas during July and August as embryos attached to the underside of rocky nests in Tomales Bay near Marshall, CA (n = 42). After being collected, the small juveniles were temporarily maintained in coolers with aerated seawater until they could be transported to temporary holding tanks at the Bodega Marine Laboratory in Bodega Bay, CA and then finally transported to the University of Washington, Seattle, USA.

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

Because the reproductive status of the adult midshipman is known to affect hearing sensitivity (Sisneros and Bass 2003), the ratio of gonad mass to body mass (gonad somatic index or GSI; defined here as 100 * (gonad mass/body mass—gonad mass), according to Tomkins and Simmons 2002) was measured to determine the reproductive status of the experimental fish. All fish used in this study were in non-reproductive condition (Brantley et al. 1993; Grober et al. 1994; Bass et al. 1996).

2.3.2 Experimental procedures

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

2.3.3 Stimulus generation

Acoustic stimuli were generated using the sinusoidal output signal from a lock-in amplifier (Stanford Research Systems SR830) that passed the stimulus signal through an audio amplifier to an underwater loud speaker (UW-30, Telex Communications, Burnsville, MN). Prior to each experiment we tested the speaker's frequency response

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

To measure and compare the evoked iso-level responses of the saccule, we recorded the saccular potentials for each test frequency at a sound pressure level of 130 dB re 1μPa. In order to control for differences in the absolute magnitude of the evoked saccular potentials and compare the shape of the iso-level response profiles, we normalized the iso-level response data by expressing the saccular potential data relative to a value of 0 dB that was assigned to the maximum evoked potential at the corresponding stimulus frequency (i.e., best frequency). The normalized data were then used to construct the iso-level response profiles. The sound pressure level of 130 dB re1μPa was used in this study because it is consistent with biological relevant sound pressure levels of type I

midshipman calls (e.g., the male advertisement call or ‘‘hum’’) that have been recorded near nest sites (Bass and Clark 2003) and can be used for comparison with previous studies (Sisneros 2007, 2009b).

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

2.3.4 Saccular potential recordings

Methods for recording saccular potentials from the midshipman were adapted from previous studies in goldfish (Furukawa and Ishii 1967; Furukawa et al. 1972; Fay and Popper 1974) and were the same as those in previous midshipman studies (Sisneros 2007, 2009b). Saccular potential recordings were made using glass microelectrodes (tip diameter: 1–2 lm) filled with 3 M KCl (1–10 MΩ). Electrodes were visually guided into the endolymph of the saccule roughly 2–5 mm from the closest hair cell bed (saccular macula) in either the left or right saccule. The smallest juveniles tested were unable to survive a complete dorsal craniotomy, so the tissue above the skull dorsal to the saccule was removed and the electrode was inserted through the skull and into the saccule. In these small juveniles, the otoliths were readily visible through the thin translucent skull and were used as a reference to guide the placement of the electrode into the saccule. The electrode was placed in one of three positions within the saccule: rostral, middle, or caudal (Sisneros 2007). Analog saccular potentials were preamplified (109, Getting 5A), input into a lock-in amplifier (109, SR830, Stanford Research Systems) and then stored on a computer running a custom data acquisition Matlab script. The lock-in amplifier yields a DC voltage output that is proportional to the component of the signal whose frequency is locked to the reference frequency. The reference frequency was set to the second harmonic of the stimulation frequency (i.e., twice the stimulation frequency) while the sensitivity of the lock-in amplifier was set to 50 mV with a time constant of 100 ms. The lock-in amplifier filters out noise signals at frequencies other than the reference. We used the second harmonic of the stimulus frequency as the reference frequency because the greatest evoked potential from the saccule of teleost fishes occurs

at twice the stimulus frequency due to the nonlinear response and opposite orientation of hair cell populations within the saccule (Zotterman 1943; Cohen and Winn 1967; Furukawa and Ishii 1967).

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

2.3.5 Statistical analysis

Iso-level data was analyzed using a standard one-way ANOVA to determine the effects of size class and recording position on BF, the magnitude of the evoked potential, relative gain (sensitivity) and relative range of the saccular response. When a significant omnibus test resulted, the data were further analyzed using a Bonferoni posthoc test for multiple planned comparisons (Howell 2007). We were unable to use repeated measures ANOVA to analyze the average threshold tuning curve data, due to the amount and uneven distribution of missing values (missing data was concentrated at higher test frequencies).

Missing values resulted when we were unable to record an evoked potential at a particular test frequency within the experimental amplitudes used (97–154 dB re 1μPa). We instead used growth curve modeling (Llabre et al. 2004) to analyze the threshold tuning data. The effects of size class, recorded saccular region (rostral, middle, caudal), and sex on auditory threshold were determined using an ANOVA on the regression coefficients of the growth curve modeled data followed by Bonferoni posthoc test for multiple planned comparisons. Analyses were carried out on computer using SPSS and Systat statistical software with alpha set to 0.05 for all tests.

2.4 Results

2.4.1 Iso-level response of the saccular potentials

We recorded auditory evoked saccular potentials from three relative sizes of midshipman fish: small juveniles, ranging in size from 1.9 cm to 3.1 cm SL (mean SL = 2.5 ± 0.2 SD cm, n = 42); large juveniles, ranging in size from 6.8 cm to 8.0 cm SL (mean SL = 7.6 ± 0.4 SD cm, n = 12); and non-reproductive adults, ranging in size from 9.0 cm to 22.6 cm (mean SL = 13.8 ± 3.5 SD cm, n = 32) with GSIs that ranged from 0.036 to 1.486 (mean GSI = 0.238 ± 0.414 SD) for male midshipman, and from 0.234 to 12.809 (mean GSI = 1.413 ± 3.182 SD) for female midshipman. Iso-level response profiles of the evoked saccular potentials were generated from the presentation of pure tone stimuli that ranged from 75 to 1,025 Hz at 130 dB (re 1μPa). Figure 2.1 shows representative iso-level response curves of the evoked saccular potentials from the three size classes. In general, the iso-level profiles from the three size groups consisted of response curves that had BFs ≤ 85 Hz (BFs, defined as the frequency that evoked the greatest saccular potential) with

evoked potentials rapidly declining above BF to that of the baseline levels below threshold (noise levels) above 145–185 Hz. Because there were no differences in the BFs of non-reproductive adults collected in California (mean BF = 80 ± 2 SD Hz) and Washington (mean BF = 82 ± 3 SD Hz) (t test, $t = 0.29$, $p = 0.77$), the adult data were pooled and then used to compare with that of small and large juveniles collected from the California midshipman population. BFs ranged from 75 to 145 Hz for all three size groups, with the majority of BFs occurring at 75–85 Hz (small juvenile = 88%, large juvenile = 95%, adults = 91%). The mode of BFs based on the iso-level response curves did not differ among the three size classes (one-way ANOVA, $F = 0.876$, $df = 2, 102$, $p = 0.42$). Nor did the mode of BFs differ among the three recording positions, rostral, middle, and caudal (one-way ANOVA, $F = 0.069$, $df = 2, 102$, $p = 0.934$).

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

In order to compare the range of the response magnitudes or relative gain (sensitivity) of the evoked saccular potentials for each size class, the iso-level data were normalized and expressed relative to a value of 0 dB at the BF in each recording and then averaged to construct a relative gain plot (figure 2.2). Although there was no difference in the range of relative gain from 75 to 945 Hz between small juveniles (range 43 dB) and adults (range 37 dB), the range of relative gain across test frequencies for small juveniles was 12 dB greater than that of large juveniles (range 31 dB) (ANOVA, $F = 5.784$, $df = 2, 102$, $p<0.005$, Bonferoni post hoc test: between small juvenile and adults, $p = 0.27$; between small juveniles and large juveniles, $p<0.005$). Thus, there were no differences in the shape or range of the relative gain of the iso-level response profiles between the three size classes when expressed by recording position.

2.4.2 Auditory saccular sensitivity

Auditory threshold tuning curves were constructed for whole populations of hair cells in the rostral, middle, and caudal regions of the saccule in all three size classes of fish. Representative tuning curves from the three recording regions and size classes are shown in figure 2.3. In general, the threshold tuning curves consisted of profiles with lowest thresholds at frequencies ≤145 Hz that increased steadily to highest thresholds at frequencies above 545 Hz. Best frequencies (BF, defined as the frequency that evoked the lowest saccular potential threshold) ranged from 75 to 145 Hz for all three size classes, and the threshold at BF ranged from 97 to 139 dB (re 1μPa) for the three size classes. The distribution of BFs based on the threshold tuning profiles did not differ by saccular recording region (rostral, middle, or caudal) (two-way ANOVA, effect of

recording position, F = 0.14, df = 2, 96, p = 0.87) or by size class (two-way ANOVA, effect of size class, F = 0.897, df = 2, 96, p = 0.41; interaction of size class and recording position, F = 0.791, df = 4, 96, p = 0.53). Also, the threshold at BF also did not differ by recording position (two-way ANOVA, effect of recording position, F = 3.053, df = 2, 96, p = 0.052) or by size class (two-way ANOVA, effect of size class, F = 2.079, df = 2, 96, p = 0.131; interaction of size class and recording position F = 1.101, df = 4, 96, p =0.361).

The saccular threshold tuning curves for the three size classes are summarized in figure 2.4 and show an increase in auditory threshold above 85 Hz that gradual diminishes above 545 Hz. We applied a quadratic regression model to analyze the threshold tuning curve data because it provided the best fit for the majority of the data (mean $r^2 = 0.86 \pm 0.11$ SD, min $r^2 = 0.48$, max $r^2 = 0.98$). There were no differences in saccular tuning profiles between the three size classes of fish based on slope (ANOVA, F = 1.466, df = 2, 101, p = 0.236), intercept (ANOVA, F = 0.94, df = 2, 101, p = 0.394), or curvilinear component (ANOVA, F = 1.424, df = 2, 101, p = 0.246). A separate analysis of auditory threshold tuning for the three size classes based on saccular recording region was performed and revealed no differences in the saccular tuning profiles for each recording position among the three size classes based on slope (two-way ANOVA, effect of recording position, F = 1.751, df = 2, 95, p = 0.179; interaction of size class and recording position, F = 0.85, df = 4, 95, p = 0.497), intercept (two-way ANOVA, effect of recording position, F = 0.22, df = 2, 95, p = 0.803; interaction of size class and recording position, F = 0.829, df = 4, 95, p = 0.51), or curvilinear component (two-way ANOVA, effect of recording position, F = 1.036, df = 2, 95, p = 0.359), however, there

was a slight interaction effect between the size class and recording position on the curvilinear component of the modeled regression lines (two-way ANOVA, interaction of size class and recording position, $F = 2.632$, $df = 4, 95$, $p<0.05$). The overall similarity of the filter shapes of the threshold tuning profiles for the three size classes based on saccular recording position indicates that there is no difference in tuning across the saccule during ontogeny (see figure 2.5).

Although there were no differences in the saccular tuning profiles for the three size classes, there was an ontogenetic difference in the maximum detectable frequency by size class. We recorded evoked saccular potentials in all small juveniles (100%) at 265 Hz (n = 42 records) while only 40% and 2% of the small juvenile recordings contained evoked potentials at 545 and 745 Hz, respectively (see figure 2.6). Similarly, we recorded evoked saccular potentials in all large juveniles (100%) at 265 Hz (n = 20) while 50% and 5% of the large juvenile recordings contained evoked potentials at 625 and 865 Hz, respectively. In contrast, we recorded evoked saccular potentials in all adults (100%) up to 225 Hz (n = 43) while 49% and 12% of the adult recordings contained evoked potentials at 705 and 945 Hz, respectively. We were unable to record the evoked saccular potentials of fish from any of the three size classes at frequencies higher than 945 Hz using the sound levels reported in this study.

2.5 Discussion

The aim of this study was to characterize the frequency response and auditory thresholds of saccular hair cells to behaviorally relevant stimuli throughout development in the

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

2.5.1 Evoked saccular potentials

In general, saccular potentials are thought to result from the summation of evoked receptor potentials produced by populations of hair cell populations within the fish saccule. The saccular potentials of midshipman and other teleost fishes are evoked greatest at twice the stimulus frequency due to opposite oriented hair cell populations in the saccule that produce two summed evoked potentials for each stimulus cycle of a pure tone (Flock 1965; Wersall and Flock 1965). This double frequency effect of the saccular potentials is thought to be primarily due to the nonlinearity in the generation of the summed hair-cell potential response, which avoids the complete cancelation of the two summed waveforms from the opposing sets of hair cell populations (Fay 1974). This study takes advantage of the double frequency response of saccular potentials by using a lock-in amplifier to yield an output signal that is ‘‘locked’’ or referenced to the second harmonic of the stimulus (i.e., an evoked response that is twice the stimulus frequency).

Based on the iso-level response profiles, the saccular potentials of small juveniles evoked at 130 dB re 1 μPa were greater in magnitude than that in large juveniles and adults (figure 2.1) and had a greater range of relative gain across test frequencies than that of large juveniles (figure 2.2). These findings are somewhat surprising considering that small juveniles have a smaller saccule and presumably fewer saccular hair cells compared to that of large juveniles and adults.

One possible explanation for the magnitude differences in the saccular potentials of small juveniles versus that of adults and large juveniles could be due to the size of the saccule and the recording position of the electrode. The adult saccule and its corresponding macula is approximately 20 times larger than that in small juveniles, and the magnitude of the recorded evoked saccular potentials should vary depending on the distance between the recording electrode and the sensory bed of hair cells within the saccule. Thus, for small juveniles the size of the saccule may have allowed the electrode to be positioned closer in proximity to hair cells to produce a greater evoked potential measurement. Congruently, the smaller size of the saccule and proximity of the recording electrode in relation to the auditory eighth nerve in small juveniles may have permitted the measurement of evoked potentials from the saccular afferent terminals innervating opposite oriented hair cell populations within the saccule. Alternatively, the differences in the evoked saccular potential magnitudes may have been due to a greater density of hair cells in the saccule of small juveniles as compared to that in adults and large juveniles. Although this explanation seems unlikely since small juveniles would presumably have fewer numbers of saccular hair cells, the possibility exists that those

hair cells may have been more densely arranged in the saccule. Future studies that examine the distribution, morphology, and orientation patterns of hair cells within the saccule of the midshipman will be required in order to resolve our observed ontogenetic differences in the isolevel response profiles of saccular hair cells.

2.5.2 Ontogenetic retention of saccular sensitivity

Perhaps the most surprising result of this study was that there was no change in auditory saccular sensitivity among the three size classes, which indicates that there is an ontogenetic retention of saccular sensitivity with age/size. We show that the auditory saccular hair cells are broadly tuned to low frequency auditory stimuli throughout development and that the saccular sensitivity of adults is similar to that of both small and large juveniles. These results are in contrast to previous findings, which demonstrated an ontogenetic increase in auditory sensitivity at BF and resting discharge rate at the level of the saccular afferent neurons (Sisneros and Bass 2005). Sisneros and Bass posited that their results could be explained by an age related increase in the number of saccular hair cells and/or convergence ratio of hair cells to afferent neurons to increase saccular afferent sensitivity. In general, teleost fish continue to add hair cells postembryonically throughout their lifetime (Platt 1977; Lombarte and Popper 1984; Popper and Hoxter 1984), but only a few studies have examined the relationship between hair cell addition and auditory sensitivity of the fish inner ear (Corwin 1983; Sento and Furukawa 1987). Corwin (1983) and Sento and Furukawa (1987) showed that an increase in auditory sensitivity was correlated with increases in the number of hair cells innervated by individual primary afferent neurons. However, Popper (1971) was unable to demonstrate

such changes in behavioral auditory sensitivity with age/size between two subadult groups of goldfish (*Carasius auratus*), which presumably exhibited hair cell addition with size/age. Popper and colleagues later proposed a model of fish hearing that is congruent with their results and predicts that hair cell addition with fish growth will maintain hearing sensitivity as the relative size and positions of different structures associated with fish audition change during ontogeny (Popper et al. 1988; Rogers et al. 1988). Future ontogenetic studies of hair cell addition coupled with developmental anatomical studies of the midshipman auditory periphery will provide valuable insight into the mechanisms that allow for increases in afferent sensitivity while retaining hair cell sensitivity within the saccule during development.

Alternatively, Sisneros and Bass (2005) posited that their reported ontogenetic changes in the response properties of saccular afferent neurons could have been due to changes at the level of the CNS via the efferent auditory pathway. Using the saccular potential recording technique (Sisneros 2007), we were able to show that the auditory sensitivity and tuning of saccular hair cells did not change during ontogeny which now leads us to suggests that the previously reported changes occurred either post-synaptic to the hair cell and/or via the saccular efferents. In the closely related oyster toadfish (*Opsanus tau*), efferent synaptic terminals are found both on the dendrites of afferent neurons and on the hair cells (Holstein et al. 2004). Activation of efferent neurons generally increases the resting discharge rate of afferents and reduces the sensitivity (gain) of the hair-cell receptor potentials in the semicircular canals to rotary stimuli (Boyle et al. 2009). In addition, efferent feedback can increase the signal-to-noise ratio of saccular responses in

conditions where a signal is masked by noise, which could potentially help unmask biologically relevant signals (Tomchik and Lu 2005, 2006). Similar mechanisms of efferent activation and central neural control may play a role in the tuning and sensitivity modulation of saccular hair cells and their afferent responses in the midshipman inner ear.

2.5.3 Functional significance of the ontogenetic retention of saccular sensitivity

The results of this study indicate that the saccule of small and large juvenile midshipman is best adapted to detect low frequency sounds (≤105 Hz) in their natural environment. The early development and retention of this low frequency sensitivity in juveniles is consistent with adaptations to increase survival by enhancing their ability to detect low frequency periodic stimuli often associated with potential predators and prey. Although there is no evidence that juveniles produce conspecific vocalizations for communication, we show that the saccule of juveniles, like that of non-reproductive adults, is well adapted to detect the low frequency components of midshipman vocalizations, similar to the findings of Sisneros and Bass (2005) for saccular afferent sensitivity. The detection and encoding of the low frequency components of midshipman vocalizations may very well be important for the interception and eavesdropping of conspecific vocalizations during social encounters. In adults, the detection and production of conspecifics vocal signals is very important for acoustic communication during social and reproductive behaviors (Bass et al. 1999; Bass and McKibben 2003). Our results show that soon after detaching from their natal rock, small free-swimming juveniles possess auditory saccular sensitivity similar to larger non-reproductive adults. It would be interesting to know at what stage of embryonic development does the saccule become functional, especially since the

developing embryos are thought to be repeatedly exposed to the rather high sound levels of the male's advertisement call over the duration of their embryonic development. Thus, future research will be necessary to determine when the midshipman auditory system becomes functional and how such auditory saccular sensitivity is retained during post-embryonic development.

In contrast to the similar tuning profiles of juveniles and adults, we show that there is an ontogenetic increase in the ability of the midshipman saccule to detect frequencies higher than 385 Hz with age/size (figure 2.6). The large increase in the percentage of saccular potential recordings that were above threshold for adults at frequencies from 425 to 945Hz indicate that adults have a higher probability of detecting frequencies > 385 Hz than small and large juveniles. This increase in the ability to detect higher frequencies is likely to be adaptive for adults in social communication. Previous work by Sisneros and Bass (2003) showed that adult females undergo a seasonal plasticity of peripheral frequency sensitivity that is dependent on seasonal shifts in circulating plasma levels of testosterone and estradiol (Sisneros et al. 2004a, b). Non-reproductive adults and juveniles have similar basal levels of circulating hormones. The similarity of saccular tuning between juveniles and non-reproductive adults is consistent with their shared steroid hormone profiles and the role of seasonal elevated steroid levels to induce an upward shift in peripheral frequency sensitivity. These seasonal changes in peripheral frequency sensitivity occur at the level of the saccular hair cell (Sisneros 2009b) and auditory afferents (Sisneros and Bass 2003) act to enhance the detection and encoding of the higher harmonic frequency components of the male's advertisement call. These

higher harmonic components are thought to propagate further than the call's fundamental frequency due to the physical and environmental constraints of the shallow water breeding habitat that limit sound transmission (Bass and Clark 2003; Fine and Lenhardt 1983). This novel form of auditory plasticity in the midshipman is thought to provide an adaptable mechanism that enhances the coupling between sender and receiver in this communication system and acts to increase the probability of mate detection and localization during the breeding season (Sisneros at 2004a).

2.5.4 Ontogenetic studies of hearing in other fishes

Relatively few neurophysiological and behavioral studies have been performed on the ontogeny of hearing in fishes. In cases where such studies have been performed, the results have often been contradictory and sometimes confusing. Behavioral studies in several species of teleosts have shown decreases in auditory threshold with age/size (Kenyon 1996; Iwashita et al. 1999). However, studies in the goldfish *Carasius auratus* using similar conditioning techniques concluded that threshold did not undergo ontogenetic shifts (Popper 1971). Similar cases exist for auditory physiology studies using the auditory-evoked potential (AEP) technique. Such AEP studies have demonstrated decreases in auditory threshold during ontogeny in the croaking gourami, *Trichopsis vittata* (Wysocki and Ladich 2001), and in the Lusitanian toadfish, *Halobatrachus didactylus* (Vasconcelos and Ladich 2008) while another study using the same technique showed an increase in hearing thresholds with age/size in the damselfish, *Abudefduf saxatilis* (Egner and Mann 2005). In contrast, similar AEP studies of the zebrafish, *Danio rerio*, have shown that auditory thresholds do not change during

ontogeny but the maximum detectable frequency did increase with size/age (Higgs et al. 2002, 2003). The above reported ontogenetic differences in auditory sensitivity are most likely due to species-specific differences related to their evolutionary history and environmental habitat, and direct comparison of the findings from the previous ontogenetic fish hearing studies is difficult at best due to the wide array of techniques used to determine auditory sensitivity. Future studies that employ more than one method (behavioral and physiological) to determine auditory sensitivity should help resolve differences that maybe attributed to technique rather than to species-specific differences.

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

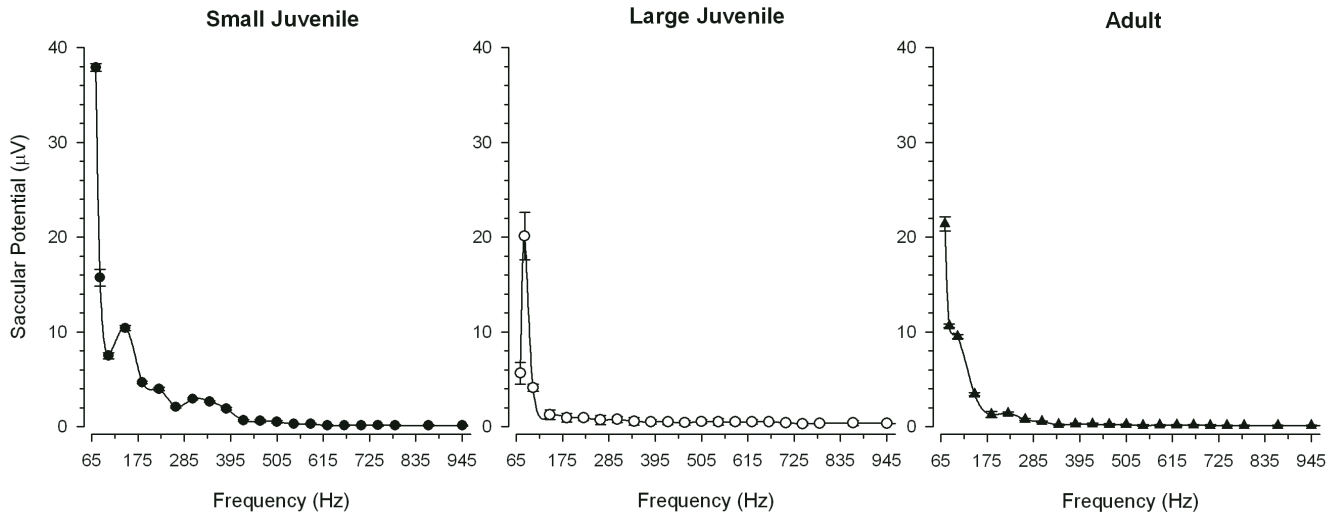


Figure 2.1 Representative iso-level response profiles of evoked saccular potentials to pure tones at 130 dB (re 1 μPa) recorded from small juvenile (solid circles), large juvenile (open circles), and adult (solid triangles) midshipman fish. The data plotted represent the mean evoked potential for 10 stimulus presentations and are plotted as means ± 1 SD (some SD bars are obscured by the symbols). Note the similar shape of the response profiles for the three size classes and that the greatest evoked saccular potentials occur at the lower frequencies.

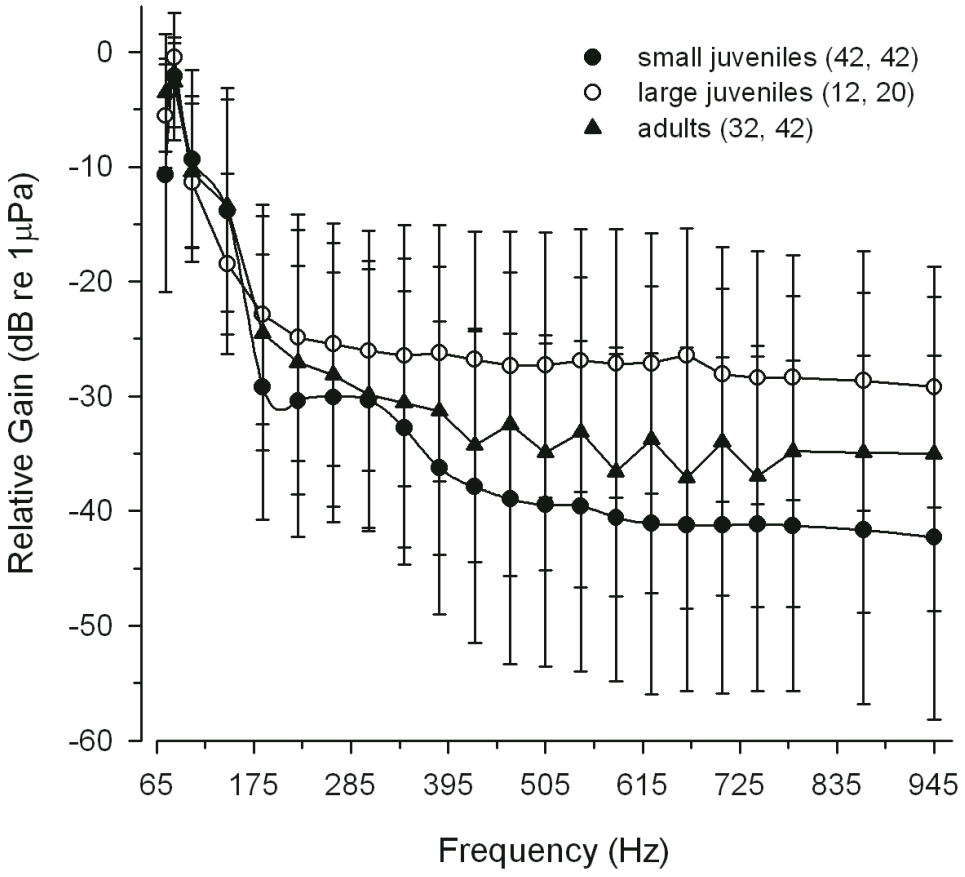


Figure 2.2 Relative gain plots of the evoked potentials recorded from the saccule of small juvenile (solid circles), large juvenile (open circles), and adult (solid triangles) midshipman based on the responses to iso-level pure tones at 130 dB (re 1 μPa). The iso-level response data were normalized to a relative value of 0 dB to control for the absolute sensitivity of the saccule from different recording positions and to compare across different animals. A relative value of 0 dB was assigned to the peak response for each recording and the remaining data for other frequencies were expressed in relative dB (re Best Frequency Sensitivity). Data are plotted as means ± 1 SD. The number of animals and records for each size class are indicated in parenthesis.

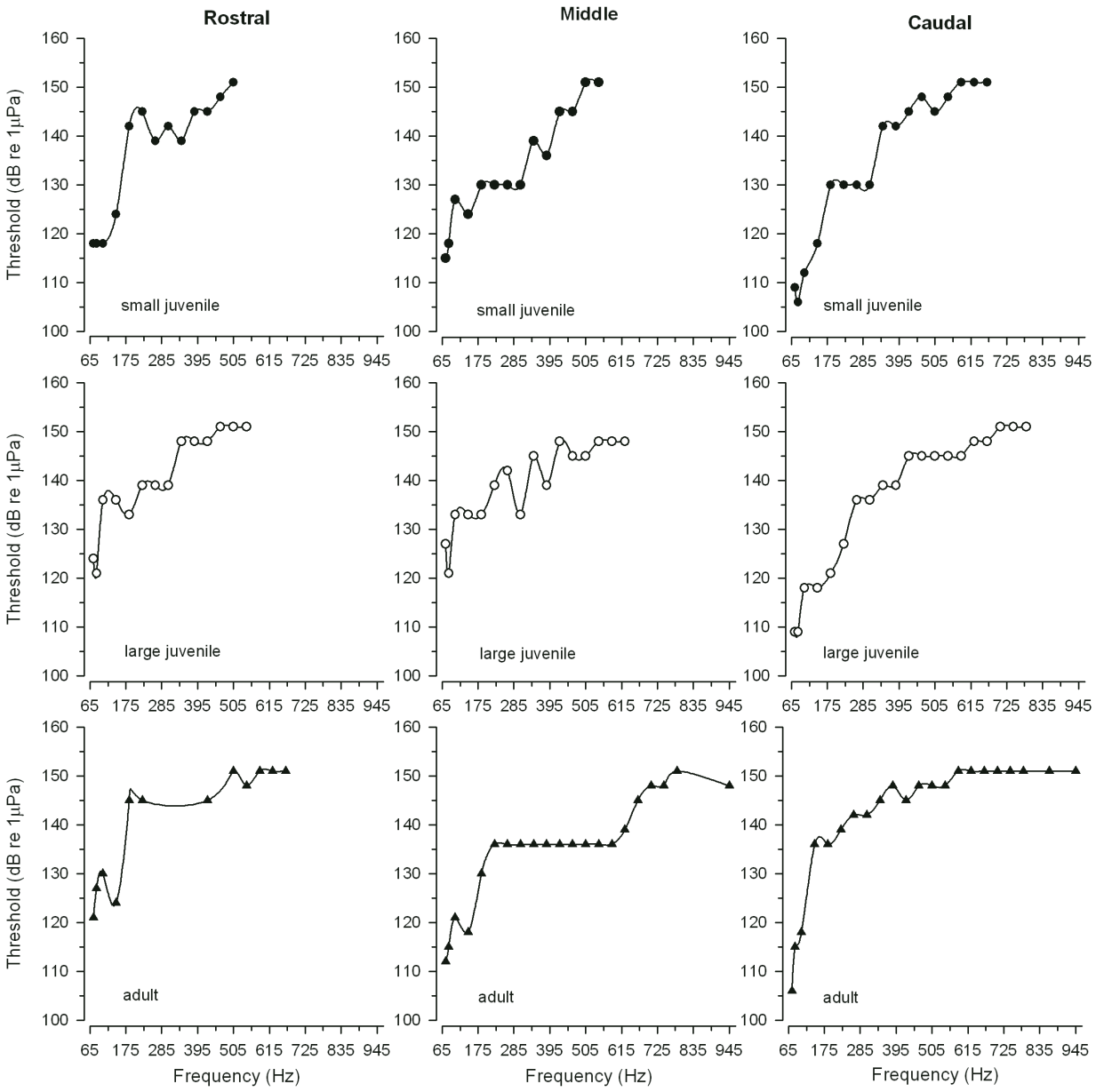


Figure 2.3 Representative examples of individual auditory threshold tuning curves for small juveniles (solid circles), large juveniles (open circles), and adults (solid triangles) based on the saccular potentials from three recording positions in the saccule: rostral (first column), middle (middle column), and caudal (third column). The auditory threshold for each frequency was determined as the lowest stimulus intensity in dB (re 1 µPa) that evoked a saccular potential greater than 2 SD above the background noise measurements.

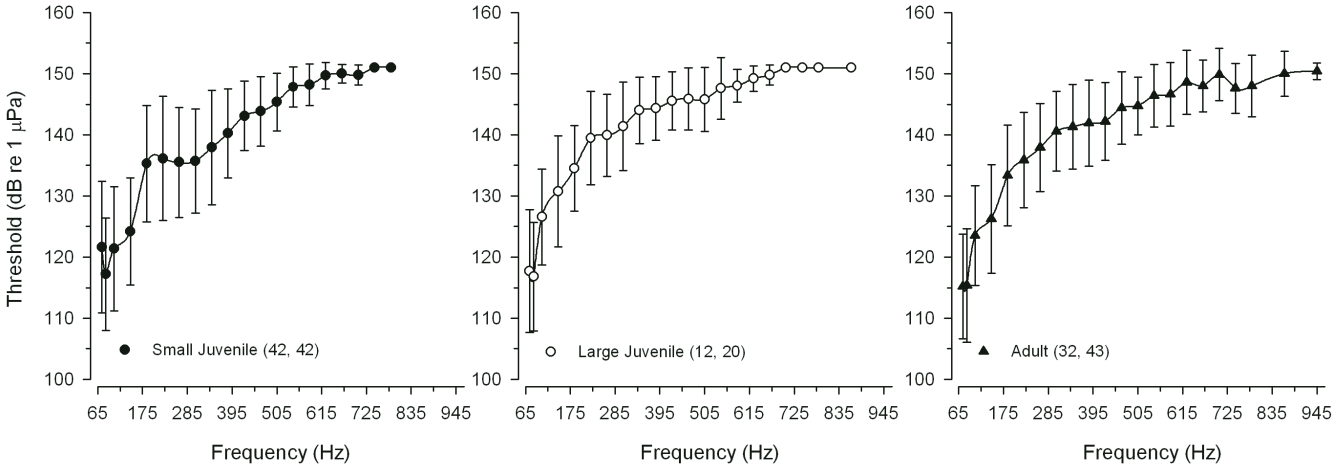


Figure 2.4 Auditory threshold tuning curves for small juvenile (solid circles), large juvenile (open circles), and adult (solid triangles) midshipman based on evoked saccular potentials. The auditory threshold for each frequency was determined as the lowest stimulus intensity in dB (re 1 μPa) that evoked a saccular potential greater than 2 SD above the background noise measurements. All data are plotted as mean ± 1 SD. The number of animals and records for each size class are indicated in parenthesis. Note the similar tuning filter characteristics for all three size classes.

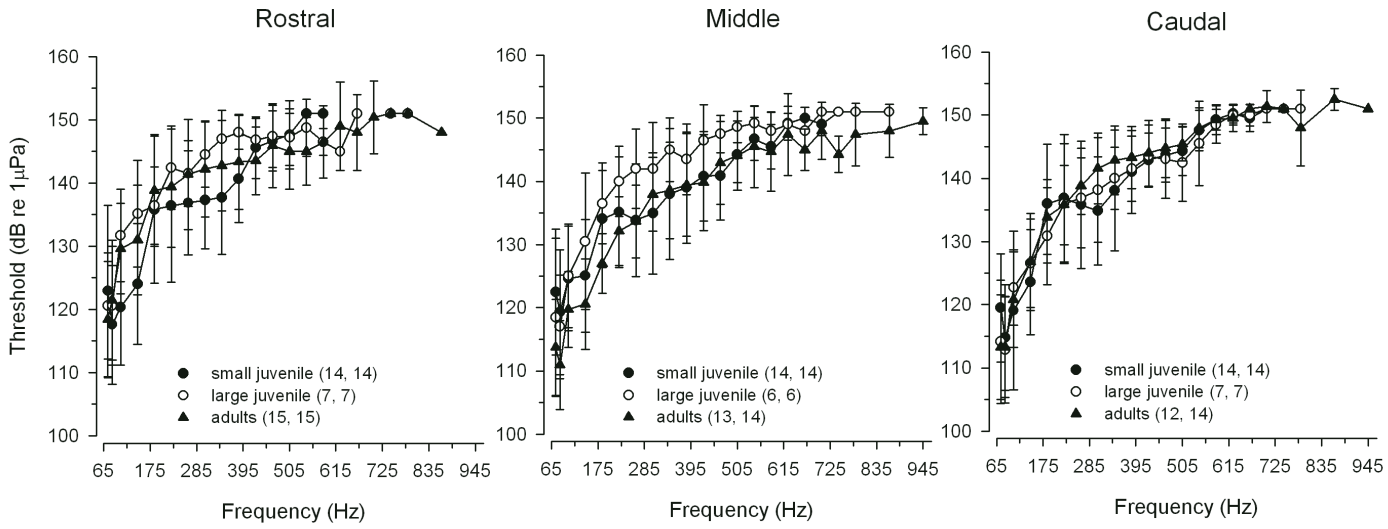


Figure 2.5 Auditory threshold tuning curves for small juvenile (solid circles), large juvenile (open circles), and adult (solid triangles) midshipman recorded from the rostral, middle, and caudal regions of the saccule. The auditory threshold for each frequency was determined as the lowest stimulus intensity in dB (re 1 μPa) that evoked a saccular potential greater then 2 SD above the background noise measurements. All data are plotted as mean ± 1 SD. The number of animals and records for each size class are indicated in parenthesis. Note the widely overlapping error bars and similar filter shapes for all size classes and recording positions. This overall similarity indicates that there is no difference in tuning across the saccular regions during ontogeny.

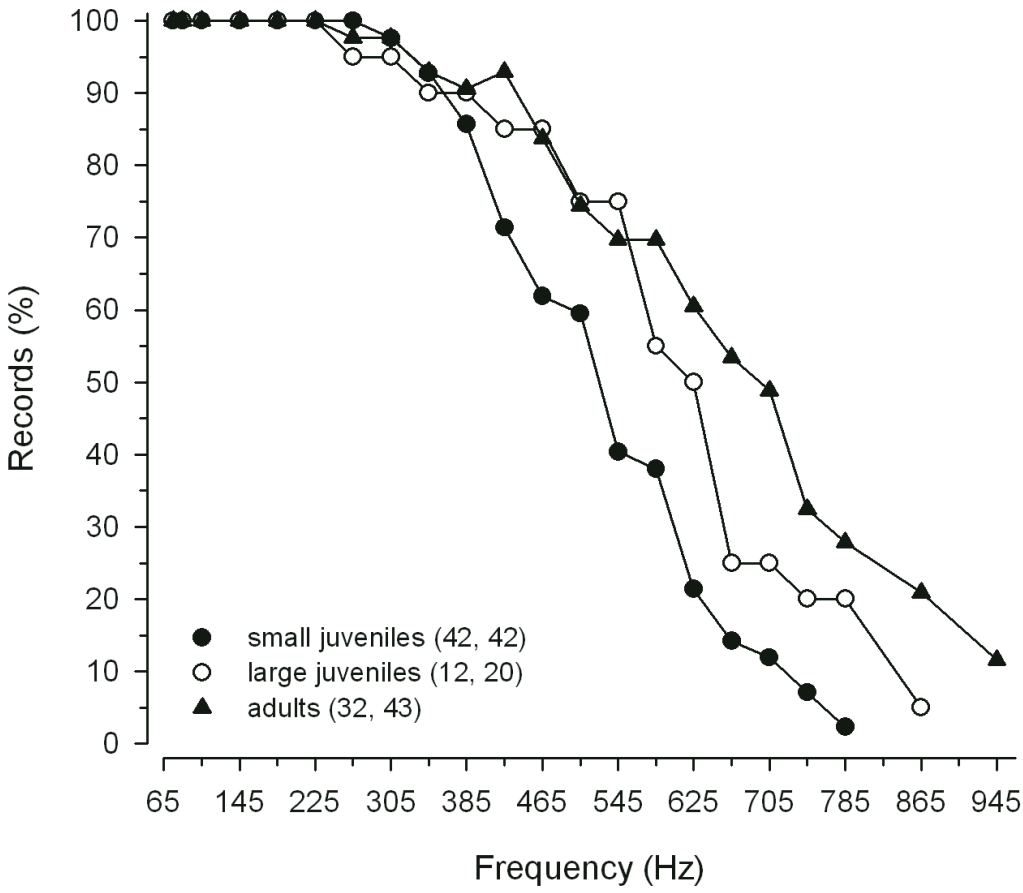


Figure 2.6 Distribution of the percentage of saccular potential recordings that were above threshold at given test frequencies for small juvenile (solid circles), large juvenile (open circles), and adult (solid triangles) midshipman. For example at 625 Hz, 20% of the records for small juveniles were above threshold at that frequency while 50% of the recordings for large juveniles were above threshold and 60% of the recordings for adults were above threshold. The number of animals and records for each size class are indicated in parenthesis. Note that the probability of detecting higher frequencies greater than 385 Hz increased with size/age.

Chapter 3. Development of the acoustically evoked behavioral response in larval plainfin midshipman fish, *Porichthys notatus*

3.1 Summary

The ontogeny of hearing in fishes has become a major interest among researchers studying fish behavior and sensory ecology. Most fish begin to detect acoustic stimuli during the larval stage which can be important for navigation and settlement, however relatively little is known about the hearing capabilities of pre-settlement larval fishes. We characterize the acoustically evoked behavioral response (AEBR) in the plainfin midshipman fish, *Porichthys notatus*, and used this innate startle-like response to characterize this species auditory capability during larval development. Age and size of the larval midshipman were highly correlated ($r^2 = 0.92$). The AEBR was first observed in larvae at 1.4 cm TL. At 1.8 cm TL all larvae responded to a broadband stimulus of 154 dB (re1 μPa)/ -15.2 dB (re 1g, z-axis). Lowest AEBR thresholds were 140-150 dB (re 1μ Pa) / -33 to -23 dB (re 1g) for frequencies below 225 Hz. Larval fish with size ranges of 1.9-2.4 cm TL had significantly lower best evoked frequencies than other size groups tested. We also investigated the development of the lateral line organ and its function in mediating the acoustically evoked behavioral response. The lateral line organ is likely involved in mediating the AEBR but not necessary to evoke the startle-like response. The midshipman auditory and lateral line systems are functional during early development while the larvae are in the parental nest and the auditory system appears to have similar tuning characteristics throughout all life history stages.

3.2 Introduction

Recent behavioral studies have provided evidence that the auditory system of larval fishes is active during early development and that sound cues may be an important for navigation and reef recruitment (Tolimieri et al. 2004, Simpson et al. 2005). The ability of fish to detect and localize sound during the larval stage may significantly affect mortality and successful recruitment of reef fishes to benthic habitats (Gagliano et al. 2008). Increasing evidence that fish use sound as a navigational cue in pre-settlement and settlement stages on coral reefs has sparked interest in determining the auditory capabilities of larval fishes (Leis et al. 2002, Leis et al. 2003, Leis and Lockett 2005, Simpson et al. 2008, Simpson et. 2010, for review see Leis et al. 2011). Understanding the behavioral impacts of sound on fish larvae has important implications for the conservation and management of commercially important fish species as well determining the impact of anthropogenic noise on the larval recruitment of fishes and invertebrates known to respond to biotic sounds (Jeffs et al. 2003, Montgomery et al. 2006, Popper and Hastings 2009, Stanley et al. 2011).

Despite the growing evidence that larval fishes can detect and localize sound, very little is known about the hearing capabilities of larval fishes. In one of the few physiological studies to investigate hearing in larval fish, Tanimoto et al. (2009) showed that auditory responsiveness can occur as early as 40 hours post fertilization in the zebrafish (*Danio rerio*). More recently, Wright et al. (2005, 2008, 2010) adapted the auditory evoked potential (AEP) technique to investigate the auditory sensitivity of coral reef fish larvae and showed that larvae have hearing abilities similar to that of post settlement juvenile

reef fish. Other studies using behavioral methods have found similar results in the hearing abilities of settlement stage larval fish and post-settlement juvenile fish (Simpson et al. 2010). In contrast, Wright et al. (2011) reported ontogenetic and interspecific differences in the hearing abilities of larval fishes with large variations in the auditory capabilities among species tested. While these initial studies are important, they fail to address basic questions such as when does the auditory system of larval fish first begin to respond to acoustic stimuli, and does the lateral line contribute to acoustic stimuli detection during larval development? More research is needed to determine whether ontogenetic changes in the fish auditory sense correspond to a general pattern of inner ear and auditory CNS development for all teleost fishes or if the hearing capabilities of larval fishes are species specific and/or environmentally dictated.

The limited seasonal availability of larval fishes combined with their delicate nature make studies of larval fish hearing difficult to conduct. Traditionally, non-invasive behavioral measures that rely on innate responses have allowed researchers to more reliably conduct fish hearing experiments. One such measure, the acoustically evoked behavioral response (AEBR), is well suited for the investigation of hearing in larval fishes. The AEBR is an innate behavioral escape or "startle-like" response that can be evoked by intense acoustic stimuli (Zeddies and Fay 2005). In most fishes, the startle response is mediated by large reticulospinal neurons known as Mauthner cells, which activate contralateral spinal motor neurons, and cause the fish to bend in a characteristic "C" shape away from the stimulus source during an escape (Zottoli et al.1999). Fish lacking or with reduced Mauthner cells exhibit startle-like responses that are less robust

without a complete c-start and are often longer in latency than typical Mauthner mediated startle responses (Meyers et al. 1998, Greenwood et al. 2010). Although startle audiograms may not be as sensitive as other measures, behavioral audiograms based on AEBRs are still a useful non-invasive measure for determining the auditory capabilities of delicate larval fish when other methods can not be used.

Here, we investigate ontogenetic changes in the AEBR in the plainfin midshipman fish (*Porichthys notatus*) as a means to characterize their auditory capability during larval development. The plainfin midshipman has become a neuroethological model for investigating the neural and behavioral mechanisms of audition in teleost fishes. The focus of this study is to use the AEBR as a measure to determine when the midshipman auditory system becomes functional and whether the lateral line also contributes to acoustic detection during early development. We test the hypothesis that larval midshipman fish are capable of detecting and responding to auditory stimuli during incubation in the nest, and that larval auditory sensitivity undergoes ontogenetic changes during development from embryos to free swimming juveniles. We interpret our findings as they relate to possible age-related adaptations of the midshipman auditory system for survival during early development.

3.3 Methods and Materials

3.3.1 Animal Collection and Care

We collected rocks with fresh midshipman eggs from the rocky intertidal zone at low tide during the summer breeding season (May- August) from field sites in Tamales Bay,

California and at Seal Rock Beach near Brinnon, Washington. Nest rocks with attached eggs were transported back to the laboratory at the University of Washington in coolers with fresh aerated seawater. In the laboratory, the rocks and eggs were placed in 190L seawater aquaria and kept at 15±2°C. The embryos/larval fish were allowed to develop until they were removed for experimentation.

We monitored and photographed the developing embryos and larvae daily. The photographs allowed us to document when each individual embryo hatched from the egg. We cleaned the embryos/larvae weekly using a small jet of water from a pipet to prevent fungal growth and any sediment build up. Larvae were selected for experimentation based on size. When a larval fish was removed for experimentation, we used a rounded blunt tip knife (approximately 1cm diameter) to separate the larval fish with yolk from the nest substrate. The fish were then carefully removed using a 5ml pipet and placed in a glass petri dish with chilled fresh seawater. The larvae and juveniles used in this study ranged in size from 0.6 to 3.3 cm total length (TL). The larval fish were divided into four groups based on TL: small, n=19, 1.5-1.7cm TL (1.6 cm TL average); medium, n=19, 1.9-2.4cm TL (2.1 cm TL average); large, n=12, 2.5-2.7 cm TL (2.6 cm TL average); and free-swimming juveniles, n=17, 2.8-3.2 cm TL (3.1 cm TL average).

When midshipman larvae were large enough to naturally detach from the rocky nest substrate, they were transferred to a smaller aquarium (3.8L) with chilled seawater (water temperature was 15±2°C) and quartz sand sediment, which provided substrate for the

juveniles to bury themselves during the day. Free-swimming juveniles were fed a diet of SELCO enriched deshelled live brine shrimp daily.

3.3.2 Life History Stage Terminology

The terminology of the various life history stages for embryonic and larval fish is very complex and many different classification systems exist to describe early fish development (Balon 1975, Shardo 1995, Bartsch et al. 1997, Martinez and Bolker 2003, Elliott et al. 2007, Martin et al. 2009). The majority of these classification systems apply specifically to larval marine fishes that undergo a pelagic developmental stage before metamorphosis into juvenile stages at the time of settlement (Kendall et al. 1984). Batrachoidid fishes, including midshipman, lay demersal eggs that undergo development in benthic nests without a pelagic larval stage (Greenfield et al. 2008). In *P. notatus*, females are attracted to males that "sing" or produce advertisement calls during the breeding season and will deposit their eggs in the nest of a suitable mate after successful courtship (Hubbs 1920, Greene 1924, MacGinitie 1935, Sisneros et al. 2009, Sisneros 2009a,b). The type I male guards and cleans the developing eggs until the external egg yolk is depleted and then larvae become free swimming juveniles at a size of approximately 2.5 to 3 cm TL (Arora 1948, Crane 1981). We are unaware of any life history terminology that adequately describes the larval development and parental care of batrachoidid fishes. Therefore, we define the midshipman embryonic stage as the developmental period from fertilization of the ova to when the developing embryos hatch. The larval stage is defined as the time period from hatching to when the larvae completely absorb their yolk and detach from the nest substrate. The juvenile stage is

defined as the time from when the juveniles become free-swimming after detaching from the nest until they reach sexual maturity. Our description of larval development and terminology is in agreement with what is conventionally defined and used in previous studies for Batrachoidid fishes (Gill 1907, Hubbs 1920, Crane 1981, Greenfield et al. 2008).

3.3.3 Post hatch larval growth analysis

We investigated the relationship between size and age in the plainfin midshipman. Because midshipman are difficult to breed in the lab, we chose to quantify post-hatch development and determine larval age from the date that embryos hatched from eggs; nests containing recently spawned eggs were collected from the field and incubated in the lab. Although age post-hatch doesn't give absolute age, it does give a useful reference time point of development and the age relative to hatching date. Because growth rates of developing fish are known to be temperature dependent (Villamizar et al. 2012, O'Brien et al. 2012, Grasman et al. 2012), we maintained the embryos and larva at a relatively constant temperature of 15 °C ± 2 °C, which is similar to summer water temperatures in the intertidal region at our field collection sites. In order to determine post-hatch age and development, we collected six large nest rocks with fresh eggs attached and brought them into the lab where we could monitor them daily in a controlled environment. These nests were photographed and individual eggs/embryos were noted and given a number so we could individually monitor the 38 developing embryos/larvae over the course of incubation. We noted the date when individual embryos hatched and measured the TL of

the larvae after removal for experimentation. These data were used to correlate post-hatch age with larvae size or TL.

3.3.4 Stimulus Calibration and Generation

Before each experiment the acoustic stimuli were calibrated so that each fish received the same stimulus sound level. Acoustic stimuli were calibrated for both sound pressure and particle acceleration. Although *P. notatus* primarily detects the particle motion component of sound, we calibrated our stimulus in terms of sound pressure to allow for a more straightforward comparison between this and previous studies. Furthermore, calibrating stimulus particle acceleration in all three axis (x, y, z) simultaneously can be difficult for an underwater speaker, however calibrating the stimulus produced by the speaker in terms of sound pressure can provide a more consistent measure of the stimulus. We also calibrated the z-axis of particle acceleration (the primary vector of stimulation along the dorsal-ventral axis of the animal) produced by the speaker using an underwater accelerometer (PCB Model 356A32). We verified that both sound pressure levels and the z-axis of particle motion were consistent across all test frequencies. We noted that a 3 dB (re 1µPa) change in sound pressure intensity did not translate to a corresponding 3 dB (re 1G) change in acceleration in the z-axis of particle motion (see figure 3.1 for the relationship between sound pressure level and particle motion in these experiments).

When calibrating the acoustic stimulus to characterize the AEBR, we placed a hydrophone (Bruel and Kjaer 8103) 10 cm above the speaker in the position normally occupied by the fish during an experiment. Stimulus generation was controlled using a

custom matlab script. We generated the stimulus using a lock-in amplifier (Stanford Research Systems SR830) that produced the analog signal and passed the signal waveform through an audio amplifier to an underwater speaker (UW-30, Telex Communications, Burnsville, MN). We monitored the amplified hydrophone output on an oscilloscope (Tektronix TDS 2002) and manually adjusted the output level on the audio amplifier until the peak intensity of the signal was 154 dB (± 2 dB) re 1μPa.

Similarly when calibrating the stimulus to test for the sensitivity of the AEBR, the hydrophone was also placed 10cm above the speaker. We generated the stimulus using a custom Matlab script and a digital to analog converter (CED 1401 MKII DAC-ADC) that passed the generated signal though a programmable attenuator (CED 3505) and an audio amplifier to an underwater speaker (UW-30, Telex Communication, Burnsville, MN). The amplified output from the hydrophone was measured and used with the custom Matlab script to automatically compensate for differences in sound intensity at the test frequencies. The calibration script adjusted the output voltage for each test frequency so that the sound pressure was of equal amplitude within ±2 dB re 1μPa. We then verified the speaker calibration by measuring the stimulus frequencies relative to each other using a spectrum analyzer (Stanford Research Systems SR780). During the experiment, we made adjustments to the sound intensity level by adjusting the programmable attenuator in 3 dB (re 1 μPa) steps (CED 3505).

3.3.5 Characterization and Onset of the Acoustically Evoked Behavioral Response

In order to characterize the AEBR and determine the size/age larvae begin to exhibit the AEBR, we detached 62 midshipman larvae from their natal rock and individually glued their external yolk to an acrylic disk (1.5 cm diameter, 0.75 cm thick) using cyanoacrylate glue. The animals used in these experiments included 38 larvae of known post hatch age ranging in size from 0.6 to 3.3 (mean size = 1.46±.58) cm TL and age from 1 to 47 (mean age = 20.87±12.3) days post hatch. After the yolk was attached and the animal was ready to be tested, the disk was submerged 10 cm above an underwater speaker in a round Nalgene tank that was resting on a vibration isolation table. The water temperature of the tank was maintained at 15 ± 2°C and the fish were provided with chilled aerated seawater throughout the experiment. The acrylic disk holding the fish was suspended above the speaker using an acrylic support structure that was attached to the vibration isolation table. Because midshipman are nocturnal, we performed all of the experiments in a darkened sound attenuation booth. Fish were given 5 min. to acclimatize to the water and recover from handling before any experiments were initiated. All experiments were video recorded for later analysis using a low light camera with a video capture rate of 30 frames/second.

We presented the fish with complex, broad-band, click stimulus that had a peak amplitude of 154 dB (± 2 dB) re 1µPa (-15.2 dB re 1G z-axis) 3 times per trial using a custom Matlab script. A subset of the animals (n=12) received stimulus presentations with either a 30 second, 1 minute, 2 minute or 5 minute inter-stimulus interval. Having multiple presentations of the same stimulus allowed us to determine the optimal inter-stimulus interval that we later used to test the frequency sensitivity of the AEBR. We

determined that a 2 minute inter-stimulus interval was the optimal interval that prevented stimulus habituation. The complex click stimulus was chosen because it was broadband and contained a high concentration of energy at frequencies below 200 Hz (see figure 3.2). Similar to other fishes that do not have specialized adaptations for hearing high frequencies, the plainfin midshipman is most sensitive to frequencies below 200 Hz (Alderks and Sisneros 2011). The midshipman AEBR consisted of quick posterior thrust of the pectoral fins followed by rapid undulation of the caudal fin (see figure 3.3). A positive AEBR was noted when the fish deflected it's caudal fin more than ½ the body length directly following a stimulus presentation. Because some movement of the pectoral fins and caudal fin is associated with opercular movement and normal ventilation of the gills, a positive AEBR was only considered when the caudal fin moved greater than 50% the fish's total length. This response criteria represents a conservative estimate as such large movements were only witnessed in response to intense acoustic stimuli or when fish were physically handled. Undulations of the caudal and pectoral fins during an AEBR commonly lasted several seconds, with durations up to 45 seconds. The caudal undulation component was the most reliable measure of the AEBR to intense acoustic stimuli. We were unable to measure the latency of the AEBR due to the lack of a high-speed camera. Other measures of response, such as ventilation rate, were not used because it was difficult to observe the opercular movements under low light conditions.

3.3.6 Frequency Sensitivity of the Acoustically Evoked Behavioral Response.

We tested the sensitivity of the AEBR to pure tone stimuli in order to determine how the auditory system of larval midshipman responds to tonal acoustic stimuli, which is similar

to the tonal components of the advertisement call that is produce by the male while in the nest. We adapted the experimental setup used previously to determine the developmental onset of the AEBR. The only difference in these experiments was that we used a parafilm support to suspend the larvae above the underwater speaker instead of the acrylic support structure. Small and medium midshipman larvae were glued (cyanoacrylate glue) directly to the parafilm support via their external yolk, whereas the large larvae and free-swimming juveniles were placed in a parafilm cup positioned on the parafilm support suspended above the underwater speaker. After gluing the larvae to the parafilm support, fish were given a 5 min. acclimatization period that allowed them to recover from handling. It was not possible to glue the large larvae or the juvenile fish to the parafilm support due to the lack of an external yolk. The parafilm support provided greater acoustic transparency then other alternatives that allowed fish to be maintained 10 cm above the underwater speaker. Fish were supplied with continuous flow of chilled, aerated seawater that was maintained at 15 ± 2°C. The experiments were performed in a darkened sound attenuation booth and videotaped for later analysis.

All fish were randomly presented a 100ms pure tone stimuli with a 6ms ramp at 75 Hz and 105 to 425 Hz with 40 Hz increments. Each stimuli presentation was followed by a 2 minute inter-stimulus interval. We varied intensities from 154 to 136 dB (re 1μPa) always beginning at 154 dB (re 1μPa) and decreasing in 3 dB steps. We measured the particle motion vector using a 3-axis accelerometer (PCB Model 356A32) and report our results in both sound pressure and particle motion. To measure the particle motion, we first calibrated the stimulus using the procedure described above, and then played the

calibrated stimulus through the underwater speaker with the 3-axis accelerometer (PCB Model 356A32) 10 cm above the underwater speaker (occupying the position of the fish). The z-axis was orientated so that it was facing the surface of the speaker in the same position as the dorsal- ventral axis of the fish. The X-axis was orientated in the same position as the rostral- caudal axis of the fish, while the y-axis of the accelerometer was orientated in the same position as the right- left axis of the fish. The accelerometer's output was then amplified (PCB model 482A16) and passed through the analog to digital converter (CED 1401 MKII DAC-ADC) and the particle acceleration calculated using a custom matlab script. We used the same response criteria that were used to characterize the AEBR (i.e. undulations of the caudal fin greater than ½ TL) and determine the AEBR thresholds. We then compared the overall AEBR threshold profiles for the four groups fish (small, medium and large larvae and free-swimming juveniles).

3.3.7 Lateral Line Visualization

In order to determine if the mechanosensory lateral line is involved in mediating the AEBR, we first visualized the distribution and number of neuromasts using the vital dye DASPEI. DASPEI is a mitochondrial dye that is selectively taken up by metabolically active cells such as lateral line neuromasts. Thirty-five midshipman larval were immersed in a 0.005% concentration of DASPEI and chilled seawater for 15 minutes and then rinsed twice in chilled seawater. We then anesthetized the fish using 1.6mL MS-222 and visualized the active neuromast cells *in vivo* using a fluorescent dissecting microscope. To verify the results of the DASPEI staining, we used the post-fix actin stain phalloidin, which selectively stains the actin bundles in the cilia of the neuromast hair cells. Fish

were euthanized by overdose of MS-222. We removed patches of epidermal tissue from the operculum, anterior trunk, and dorsal cranium from the euthanized fish and fixed overnight in 5% paraformaldehyde. These patches were chosen because they represent areas where the anterior and posterior lateral line develops earliest. The tissue was then rinsed in 1xPBS and stained using 0.001% phalloidin for 20 minutes followed by two rinses of 1xPBS. We visualized the tissue using a fluorescent light microscopy at 40x. This data was used to help interpret the AEBR results.

3.3.8 Statistical Methods

Growth data were analyzed using a linear regression to determine the relationship between body length and posthatch age. Positive AEBR response data were used to identify the age at which the AEBR is first evoked as an indicator of the onset of audition. Positive DASPEI staining was used as an indicator for the development of the lateral line system. Both AEBR onset and DASPEI staining data were analyzed using non-linear regression to find the best-fit model for the data. Best frequency sensitivity data of the AEBR was analyzed using one-way ANOVA followed by a Bonferroni post-hoc analysis to analyze any differences. Frequency sensitivity data were analyzed using a multivariate analysis of variance (stimulus frequency was the dependent variable, and size class was the fixed factor) followed by a Bonferroni post-hoc analysis to analyze differences between the four size groups at each frequency. SPSS statistical software was used to perform all statistical analyses.

3.4 Results

3.4.1 Larval growth analysis

In order to determine the relationship between larva size and post-hatch age, we collected six nests with freshly laid eggs attached and monitored the development of the embryos/larvae daily, noting the date when larva hatched (day = 0). We recorded fish size in terms of total length (TL) for 38 larvae at various stages of post-hatch development and used a regression analysis to determine the size and post-hatch age relationship. Larvae hatched at a size range of 0.5 to 0.6 cm TL (mean = 0.54 ± 0.05 cm SD) and detached from the nest at a size range of 2.6 to 3.0 cm TL (mean = 2.86 ± 0.13 cm SD) at 15°$\pm$ 2° C. A linear model provided the best fit of the size-age data with a regression formula of $y = 23.3 x - 9.9$, $r^2=0.92$, $p < 0.001$, see figure 3.4. Because of the strong relationship between size and post-hatch age ($r^2 = 0.92$) of the midshipman we are able to estimate the age of fish used in this study by measuring the TL of the fish.

3.4.2 Onset and characterization of the acoustically evoked startle-like response

During the care and incubation of midshipman nests in the laboratory, we noticed that the larvae were capable of producing the startle-like AEBR to intense acoustic stimuli. We decided to characterize the AEBR and use it as a behavioral measure to test the auditory capabilities of midshipman larvae. The characteristic AEBR consisted of a fast posterior thrust of the pectoral fins followed by undulations of the caudal fin. The duration of the caudal fin undulations ranged from 1.5 to 45 seconds with a mean duration of 4.3 ± 3.5 SD seconds. Following the initial vigorous undulations of the caudal fin, the fish would then stop all body movement with the exception of the respiratory movements of the operculum and small spontaneous movements of the pectoral and caudal fins

Characterization of the AEBR in midshipman larvae exposed to broadband acoustic stimuli was performed using a complex click stimulus that had a peak intensity of 154 dB (re 1 μPa). We tested 62 midshipman larvae of various sizes (0.6 cm TL to 3.3 cm TL) to determine the developmental onset of the AEBR. Fish were individually tested and the AEBR to the startle stimulus was coded: 1 for a positive response and 0 for no response. A sigmoidal function (i.e., a 4 parameter sigmoid with the equation: f= y0+a/(1+exp(-(x-x0)/b)), $r^2 = 0.94$) provided the best fit for the relationship of the AEBR with TL. A response rate of 50% for the AEBR corresponded to a fish size of 1.5 cm TL (see figure 3.5). We did not observe AEBRs in fish smaller than 1.4 cm TL, and all fish (100%) greater than 1.8 cm TL responded to the intense acoustic startle stimuli. Our data suggest that the AEBR can first be evoked at a size of 1.4 cm TL or an estimated age of 23 days post-hatch development.

3.4.3 Development of the lateral line

In order to determine the relative contribution of the lateral line in mediating the AEBR, we investigated the relationship between AEBR and development of the lateral line in larval midshipman. We used the vital dye DASPEI to visualize the lateral line neuromasts in 35 midshipman larvae that ranged in size from 0.8 to 2.5 cm TL. The DASPEI dye allowed us to visualize both superficial and canal neuromasts. We recorded the presence of lateral line neuromasts as either yes or no (binary data), having one or more neuromasts, or not having any neuromasts, respectively. The percentage of fish having neuromasts at a given size is shown in figure 3.6 and the relationship of neuromast

DASPEI staining of the lateral line and body size (TL) was best fit according to a sigmoidal function (i.e., a 3 parameter sigmoid with the equation: f= a/(1+exp(-(x-x0)/b), $r^2 = 0.98$). Fish less than 1.6 cm TL were not observed to have any lateral line neuromasts; however 50% of the fish at a size of 1.8 cm TL had superficial neuromasts. All fish greater than 1.9 cm TL had at least one superficial neuromast present. The negative DASPEI staining results were verified using a post fix fluorescently conjugated phalloidin label. Our results show that lateral line neuromasts are first observed in larval fish at a size of 1.6 cm TL, which corresponds to fish > 27 days old post hatch.

3.4.4 Frequency sensitivity of the AEBR

AEBR response profiles were constructed for the four midshipman size groups (small, medium and large larvae and free-swimming juveniles). In general, thresholds for the AEBR were lowest at frequencies below 145 Hz and the sensitivity to tonal stimuli gradually decreased at higher frequencies gradually with highest thresholds found at the highest frequency that evoked a behavioral response (see figure 3.7). Significant threshold differences for the AEBR were observed at 75 Hz (MANOVA $F_{3, 38} = 4.6$, $p = 0.008$), 105 (MANOVA $F_{3, 38} = 3.8$, $p = 0.017$), and 145 Hz (MANOVA $F_{3, 38} = 4.4$, $p = 0.01$). Post-hoc analysis revealed that at 75 Hz the medium larvae had lower AEBR thresholds than that of small larvae (Bonferroni, mean difference = 6.1 dB, $p = 0.016$) and the free-swimming juveniles (Bonferroni, mean difference = 4.7 dB, $p = 0.042$). Medium larvae also had lower AEBR thresholds than that of small larvae at 105 Hz (Bonferroni, mean difference = 4.4 dB, $p = 0.023$) and free-swimming juveniles at 145 Hz (Bonferroni, mean difference = 4.7 dB, $p = 0.006$). No other differences in AEBR

thresholds among the four test groups were observed. Best evoked frequencies (BEF, defined as the frequency with the lowest threshold for the AEBR) ranged from 75 to 145 Hz (See Fig. 3.8). The AEBR threshold at BEF ranged from 133 to 151 dB (re 1μPa) or -32 to -14 $dB_{z\text{-}axis}$ (re 1G). The distribution of BEFs for the AEBR did not differ across size class (one-way ANOVA, $F_{3, 57} = 2.24$, p=0.095), however, the medium larvae (1.9-2.4 cm TL) did have significantly lower AEBR thresholds at BEF then all other size groups (one-way ANOVA, $F_{3, 57}= 7.64$, p = 0.00024; Bonferroni post-hoc comparisons: medium vs. small: mean difference = 6.7 dB, p = 0.00015, medium vs. large: mean difference = 4.8 dB, p = 0.026, medium vs. free-swimming: mean difference = 4.8 dB, p = 0.014). No other differences in AEBR threshold at BEF were observed between the size classes.

3.5 Discussion

3.5.1 The acoustic evoked behavioral response

The AEBR is an innate startle-like response that can be evoked by intense acoustic stimuli. This response observed in the plainfin midshipman shares many characteristics similar to that of startle responses observed in other fishes. Startle responses can be elicited by acoustic, visual, or tactile stimuli and serve the adaptive function of initiating escape responses. Escape behaviors are evolutionarily conserved due to their survival value in, but not limited to, predator-prey interactions (Eaton and Didomenico 1986, Foreman and Eaton 1993). In fishes, the acoustic startle response is mediated by relatively large brainstem reticulospinal neurons (RSNs) called Mauthner cells that receive information from ipsilateral sensory afferents and synapse with contralateral

spinal motor neurons (Eaton et al. 1991, Canfield and Rose 1993, Zottoli et al. 1999). When activated, the Mauthner cells depolarize and cause the contralateral motor neurons to fire synchronously and the fish bends into a characteristic "C" shape away from the stimulus source. The development and evolution of the c-start startle response has been studied in many fish species (Kimmel et al. 1974, Hale et al. 2002, Kastelein et al. 2008). The innate c-start startle behavior has also been exploited to better understand fish hearing (Cervi et al. 2012) and to determine the onset of hearing in larval fishes (Zeddies and Fay 2005, Higgs et al. 2004). However, not all fish have Mauthner cells. Fish without Mauthner cells exhibit startle-like responses that are often substantially longer in latency and lack the characteristic "C" shape body bend (Meyers et al. 1998, Greenwood et al. 2010). Removal of the Mauthner neurons results in escape behaviors that have similar characteristics to behavioral responses in fishes without Mauthner cells (Eaton et al. 1982, Zottoli et al. 1995, 1999, Greenwood et al. 2010). This non-Mauthner escape pathway is mediated by the MiD3cm RSN. (Eaton et al. 1984, Lee and Eaton 1991, Liu and Fetcho, 1999, Weiss et al. 2006, Kohashi and Oda 2008). Plainfin midshipman fish do not have Mauthner cells but do exhibit a longer latency AEBR. Both the acoustic startle and AEBR likely serve a similar function in the initiation of escape behaviors; however further research is necessary to determine if AEBRs are mediated via the MiD3cm neuron or analogous RSN or via a different reticulospinal hindbrain pathway in the plainfin midshipman.

3.5.2 Early ontogeny and auditory development

Development in the plainfin midshipman begins at spawning when female midshipman deposit their eggs in the nest of a suitable mate after successful courtship (Hubbs 1920, Greene 1924, MacGinitie 1935). Midshipman eggs are about 6mm in diameter at spawning and the embryos develop for approximately 11-16 days before hatching (Arora 1948). Flexion occurs before hatching and midshipman larvae resemble juvenile and adult midshipman as they develop (Watson 1996). Larvae remain attached to their natal rock during the larval stage detaching at approximately 25-30 days post hatch (Hart 1973). It is during this 6 to 8 week developmental period when the auditory system develops and becomes active.

In this study, we show that the AEBR can be used as a conservative measure to determine the age at which *P. notatus* first begins to respond to sound. Previous research indicated that both adult and juvenile midshipman were primarily sensitive to frequencies below 200 Hz (Alderks and Sisneros 2011, Sisneros and Bass 2005). Thus, in this study we used a broadband acoustic stimulus with a peak energy concentrated at 50-200 Hz with an amplitude of 154 dB (re1 μPa)/ -15.2 dB (re 1g, z-axis) to characterize the AEBR. Of the 62 larval fish tested with a size range of 0.6 to 3.3 cm TL, we were unable to evoke an AEBR in fish smaller than 1.4 cm TL. All larval fish greater than 1.8 cm TL exhibited the AEBR to the acoustic startle stimulus. We chose to use a range of size of 1.4 to 1.8 cm TL for our smallest test group, as this is likely when the auditory system first becomes active. Research on other fishes has shown that startle responses can be acoustically evoked when the auditory end organs and their innervation are fully developed (Higgs et al. 2004, Cervi et al. 2012). Using 50% AEBR response rate as our benchmark,

midshipman larvae begin hearing at 1.5 cm TL. Future work will be necessary to determine the relationship between the onset of hearing and the connectivity and development of the peripheral and central auditory systems in larval midshipman fish.

3.5.3 Role of the lateral line system in mediating AEBRs

The lateral line system detects information about hydrodynamic flows over the skin in fishes (Dijkgraaf 1962, Lane and Whitear 1982, Bleckmann 1985, Coombs and Braun 2003). It is composed of mechanoreceptive organs known as neuromasts that are distributed along the head and trunk of the animal (Jakubowski 1966, Denton and Blaxter 1976, Page 1977). Neuromasts are found either superficially on the skin or in canals and consist of sensory hair cells and nonsensory support cells covered by a gelatinous cupula that is directly exposed to the water (Allis 1889, Johnson 1917, Coombs et al. 1988). Adult plainfin midshipman have both canal neuromasts (located primarily on the head and operculum) and superficial neuromasts located on the head and four rows descending caudually along each side of the body trunk (Greene 1899). Midshipman appear to develop both superficial and canal neuromasts at the same time which is in contrast to zebra fish in which the canal neuromasts first develop about 30 days after the superficial neuromasts (Dambly-Chaudière et al. 2003, Webb and Shirey 2003). This simultaneous appearance of both superficial and canal neuromast types is of note because these two neuromast types have different ranges of frequency sensitivity; canal neuromasts are sensitive to higher frequencies up to 200 Hz (Denton and Gray 1988, Montgomery et al. 1995), whereas superficial neuromasts are sensitive to primarily low frequencies < 100 Hz (van Netten 1991, Coombs and Montgomery 1993, Bleckmann 1993). The lateral line

system develops soon after the AEBR is first observed in small midshipman larvae. Using the in vivo DASPEI and postfix phalloidin stains, we were unable to visualize the presence of any lateral line neuromasts in fish smaller than 1.6 cm TL while at a size of 1.8 cm TL only 50% of the examined fish had superficial neuromasts. The lateral line system did not appear fully developed until fish were greater than 2.0 cm TL.

It is likely that the detection of acoustic stimuli by the lateral line influences the AEBR, but our data suggests that lateral line input is not necessary to evoke the AEBR since some larvae (n = 3) without superficial or canal neuromasts exhibited AEBRs. However, both the superficial and canal neuromasts of the lateral line may be involved in mediating the startle-like behavior later in development since the inner ear and lateral line organ are thought to have overlapping receptive fields (Braun and Coombs 2000). Recently in the goldfish (*Carassius auratus*) the lateral line has been demonstrated to be involved in the encoding directional information and to affect the onset latency of the escape response (Mijany et al. 2011). It is possible that the onset latency of the AEBR decreases as the lateral line becomes more developed in *P. notatus*, however future research is necessary to determine if such an ontogenetic relationship exists. We should note that the medium-sized midshipman larvae (1.8 – 2.4 cm TL), which were the most sensitive to the startle stimuli, were within the size range when the superficial and canal neuromasts first appear and undergo significant proliferation. The medium sized larvae had significantly lower BEF sensitivities than both the small and large larvae. Future work should focus on AEBR sensitivity and development of the medium size larvae to determine the mechanisms for this apparent increase in hearing sensitivity.

3.5.4 AEBR thresholds

The AEBR is an innate response evoked by intense acoustic stimuli similar to an acoustic startle response (Young and Fechter 1983). The resulting behavioral thresholds and response profiles of the AEBRs represent a conservative measure of auditory sensitivity (Ouagazzal et al. 2006,). Although the thresholds for the AEBRs may not represent absolute hearing sensitivity, they are a useful measure of hearing when working with very delicate animals where other techniques may be too invasive.

The overall shape of the AEBR profiles revealed lowest thresholds at frequencies below 225 Hz and response sensitivity that gradually decreased at higher frequencies in all size groups tested. Auditory tuning profiles based on saccular potential and single unit recordings in free-swimming juvenile and adult plainfin midshipman are similar in shape to that of the AEBR profiles reported here (Alderks and Sisneros 2011, Sisneros and Bass 2005). It is likely that sensitivity to low frequency sound is important in *P. notatus* since it appears to be conserved throughout all life history stages from early larval to adult fish.

The sensitivity of the midshipman auditory system to low frequencies (<225 Hz) may be related to structure of the inner ear in midshipman. The saccule, the primary auditory end organ in the midshipman and most teleosts, is an otolithic organ that responds to the particle motion component of sound much like an accelerometer (Platt and Popper 1981, Popper and Tavolga 1981, Fay 1984). The saccule contains a dense otolith, much denser than the rest of the fish's body, which moves at a different phase and amplitude from the

saccular epithelium as sound passes through the ear (Dijkgraaf 1960, Fay and Popper 1975, Popper and Lu 2000). As the otolith and sensory epithelium move past each other, the resulting shearing motion bends the ciliary hair bundles generating a receptor potential and causing signal transduction (Fay and Popper 1974, Popper 1983, Popper and Fay 1993, Fay and Popper 2000). This system is inherently most effective at responding to low frequencies (Fay 1988, Popper and Fay 1999).

The medium-size midshipman larvae had significantly lower AEBR thresholds at BEFs as well as significantly lower AEBR thresholds at 75, 105, and 145 Hz test frequencies. There were not significant differences between any of the other size groups. The greatest mean difference between the medium and large larvae was at 75 Hz, which was only 4.7 dB. This result is significant, however it is a relatively small difference and likely does not reflect a behaviorally relevant difference in auditory sensitivity. One possible explanation for this difference is that inner ear hair cells are being added at a greater rate during the larval stage than in other life history stages (Popper and Hoxter 1990, Lombarte and Popper 1994, Landford et al. 1996). During early life history stages, dendritic arborization and ganglion cell numbers increase rapidly (Popper and Hoxter 1984). It is possible that in medium-sized midshipman larvae hair cell addition briefly outpaces ganglion cell addition such that the convergence ratio increases at a greater rate than in other larval stages. This possible increase in convergence may temporarily result in greater AEBR sensitivity. However, further research is necessary to determine the mechanisms responsible for these differences in AEBR sensitivities between these size groups of midshipman larvae.

3.5.5 Utility of the AEBR

Determining the auditory capabilities of larval fishes is important for our understanding of the behavioral impacts of sound on larval fishes. The characterization of the sound levels that elicit the AEBR in larval fish could have important implications for the conservation of commercially important fish species as well the impact of anthropogenic sound on larval recruitment of animals known to respond to biotic sounds. There is an increasing amount of evidence that larval fish use sound cues in navigating to suitable habitat for settlement (Leis et al. 2002, Leis et al. 2003, Leis and Lockett 2005, Simpson et al. 2008, for review see Leis et al. 2011). Intense repetitive acoustic sound generated from construction and development projects are often a source of anthropogenic noise that is introduced into the underwater environment (Halvorsen et al. 2012, Popper and Hastings 2009a). While these sounds may not lead to permanent damage or fish mortality, very little is known about the behavioral impacts of anthropogenic sounds on aquatic animals including fishes (Smith et al. 2006, Slabbekoorn et al. 2010, Kane et al. 2010). Anthropogenic noise generated by activities such as pile driving could easily exceed the sound levels necessary to evoke an AEBR (Popper and Hastings, 2009b). If anthropogenic noise reach levels that evoke AEBRs in larval fish, it is possible that such human activities could have a greater than expected impact on fish populations, especially in areas that are critical for larval development or settlement (Wysocki et al. 2007). This is especially the case for commercially important fish species that may be already stressed by other environmental or human related factors. This is an area that needs further research and could lead to better insight into the effects of anthropogenic

sound on aquatic animals and lead to the development of better policies that could minimize the human impact on commercially important fish species.

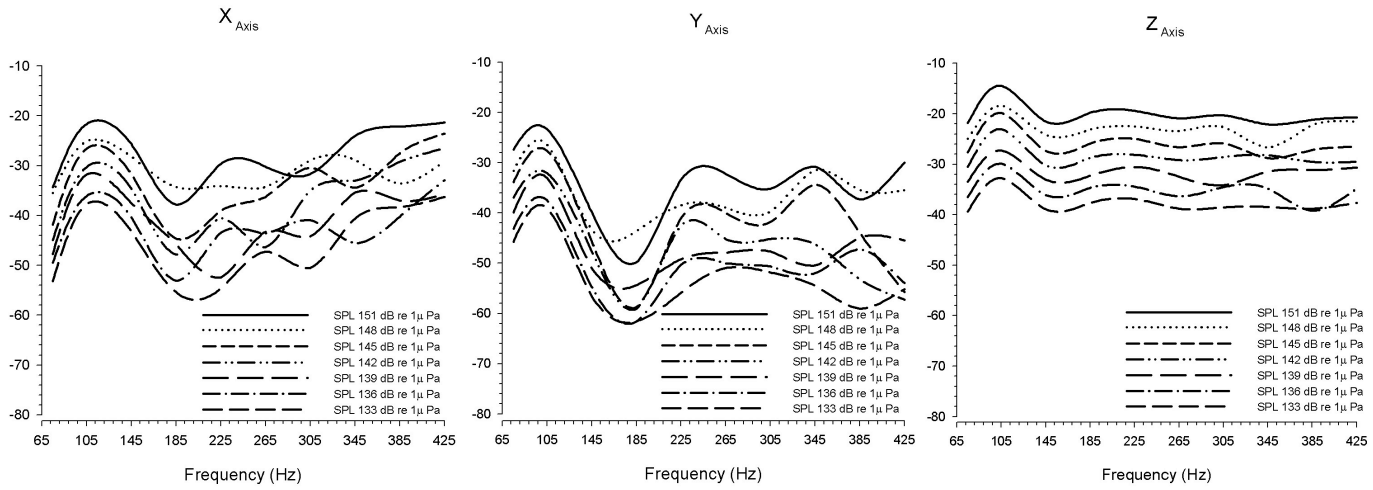


Figure 3.1 The relationship between particle motion (acceleration) and sound pressure in the experimental tank used to test the AEBR in midshipman fish. Particle motion was measured using a 3D accelerometer after calibrating stimulus frequencies using sound pressure such that all stimulus frequencies at a peak SPL within 2 dB of 154 dB re 1µPa. Here we display the particle motion measured in the X-, Y-, and Z-axes for all test frequencies and intensities. Note that the Z-axis represents the main axis of stimulation.

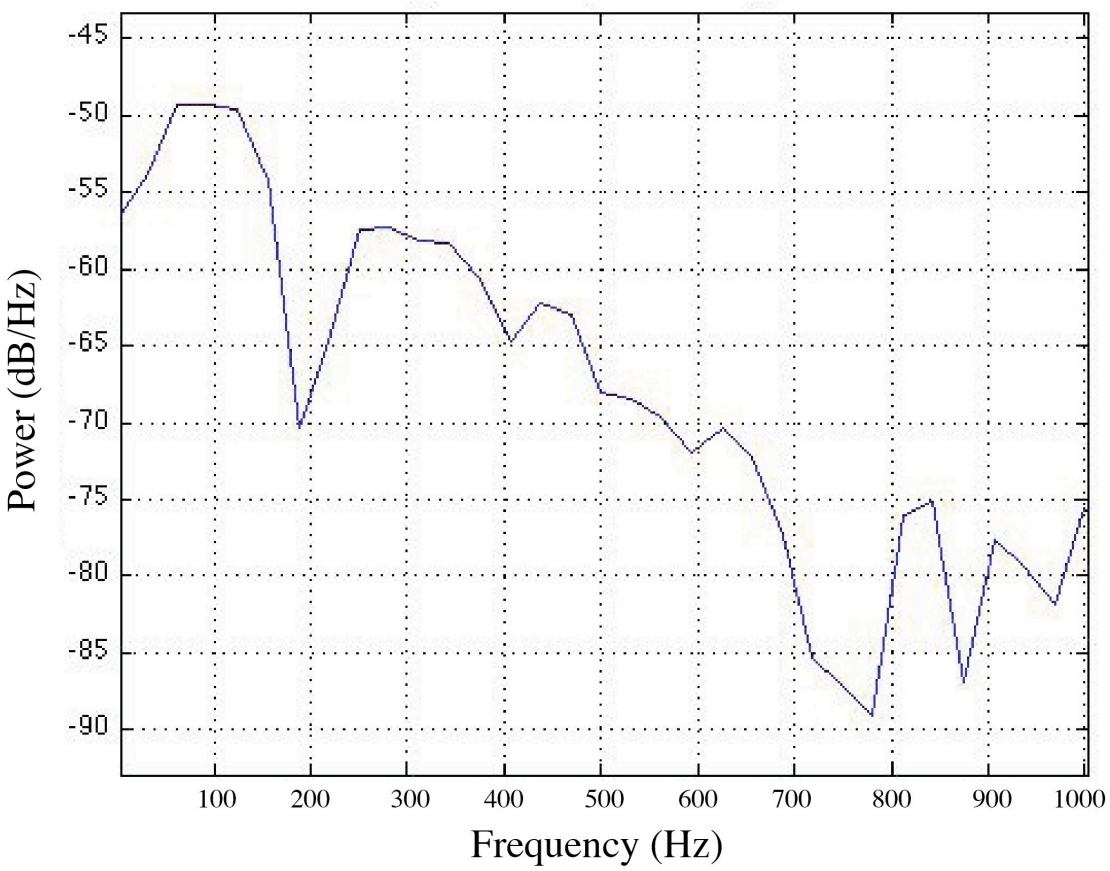


Figure 3.2 The power spectrum of the complex click stimulus used to identify the size/age for the onset of the acoustically evoked behavioral response. The majority of the energy in the stimulus is located below 700 Hz with peak energy between 50 and 200 Hz. This stimulus had a peak intensity of 154 dB (re 1µPa) or -15.2 dB (re 1g) in the Z-axis of stimulation. Juvenile and adult midshipman have greatest auditory sensitivity at frequencies below 300 Hz.

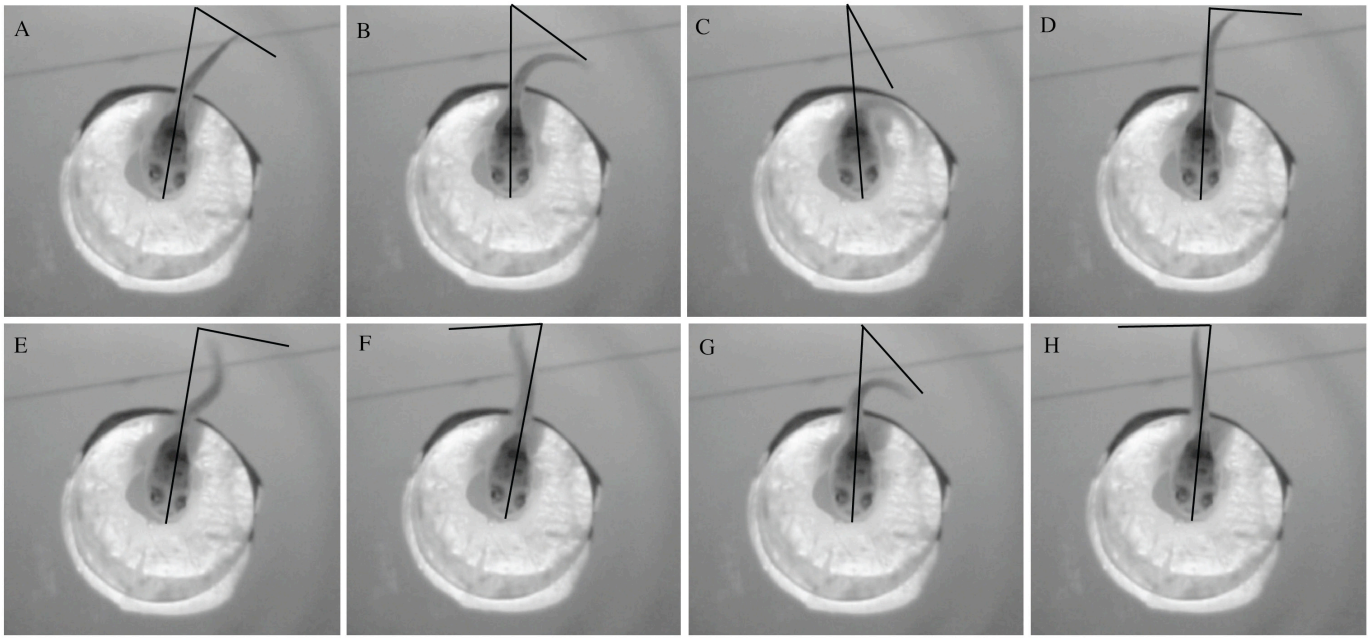


Figure 3.3 Video frame sequence of a representative acoustically evoked behavioral response (AEBR). The images show a 2.1 cm TL larval fish that responds to the complex click stimulus with repeated rapid undulations of the caudal body trunk and fin. The total response lasts for a duration of 2.5 seconds. Image A shows the position of the fish before stimulus presentation, and images B-H show the fish positions during and after stimulus presentation. Note that in image C the caudal fin is curved toward the head of the fish, almost forming a C shape. The longer bar represents the total length (TL) of the fish while the shorter bar represents the flexion point at ½ of the TL. This smaller bar also represents the minimum distance of movement by the fish's caudal region for a positive AEBR.

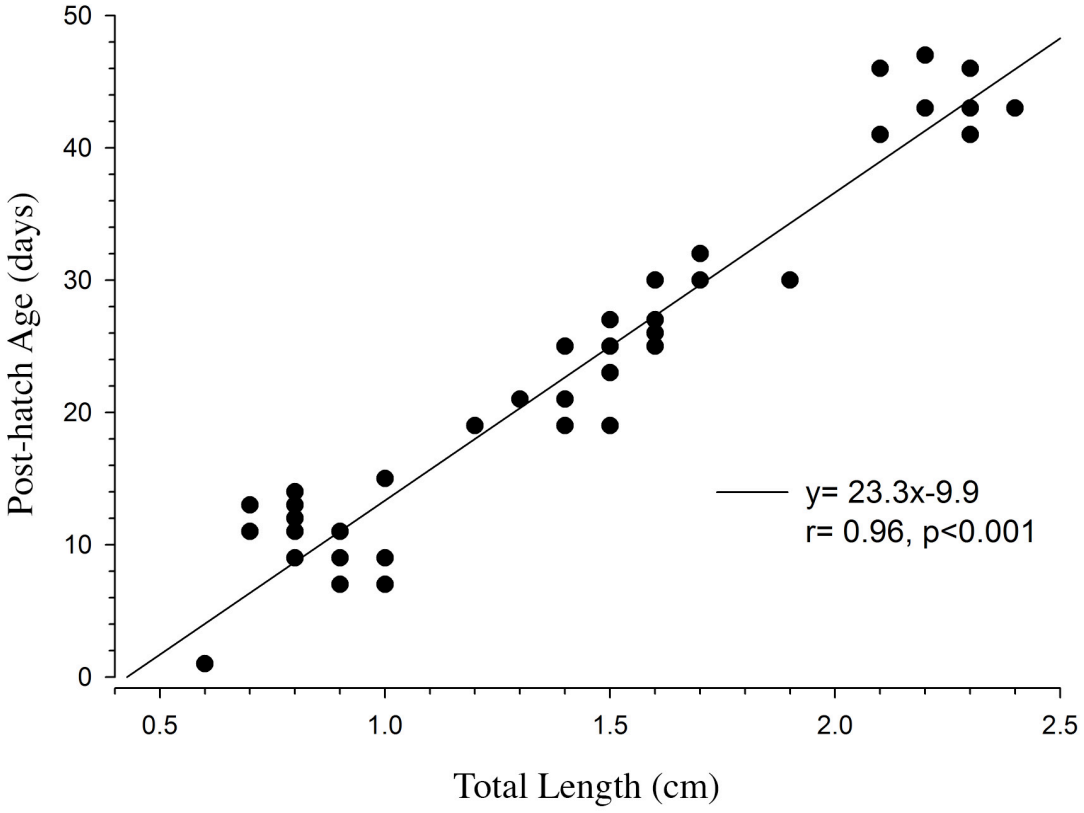


Figure 3.4 The relationship between size and post-hatch age of 38 midshipman larvae. Post-hatch age data of larva fish were recorded from 6 different nests and the age was then correlated with TL. Size and post-hatch age were highly correlated ($r^2 = 0.92$, $p < 0.001$).

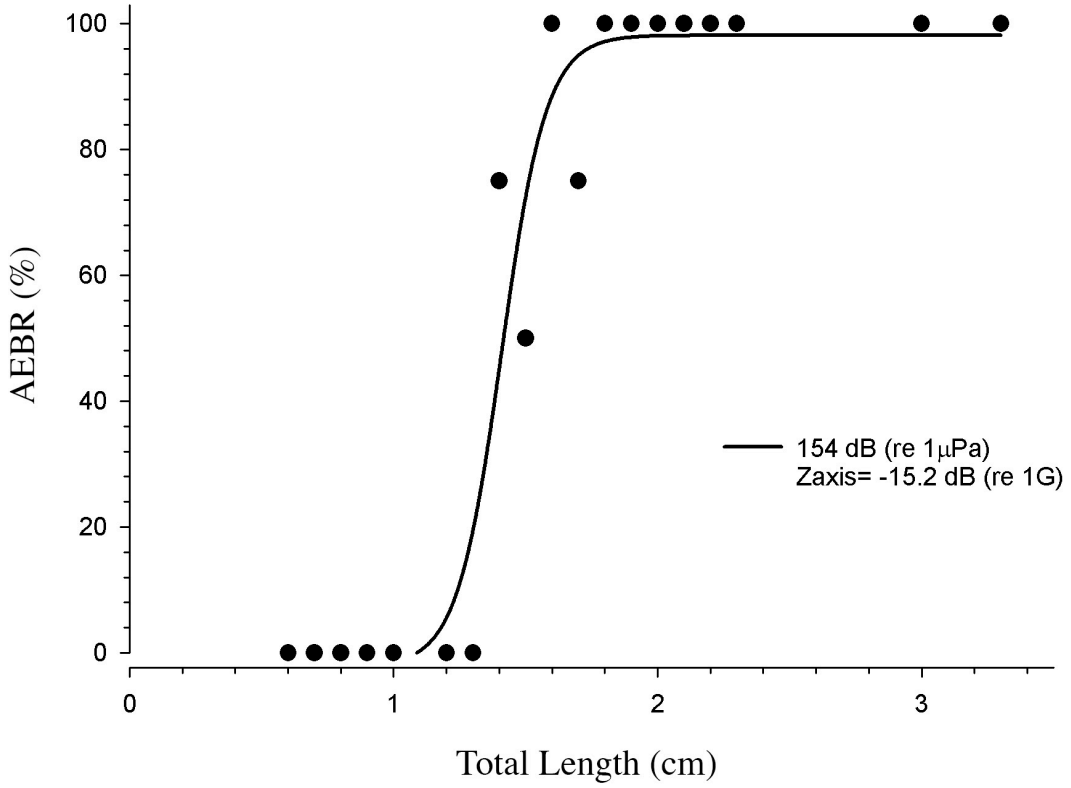


Figure 3.5 The acoustically evoked behavioral response (AEBR) of fish to a complex click stimulus with a peak SPL of 154 dB (re 1μPa) or -15.2 dB (re 1g) in the Z-axis of stimulation. The AEBRs are shown as the percentage of the tested fish (60 midshipman larvae and 2 free-swimming juveniles) that responded to the stimulus. Note that none of the small midshipman larvae less than 1.4 cm TL responded to the stimulus, whereas all of the midshipman larvae greater than 1.8 cm TL responded. Thus onset of the acoustically evoked behavioral response is estimated to occur between 1.4 – 1.8 cm TL. The solid line represents a best-fit sigmoidal curve.

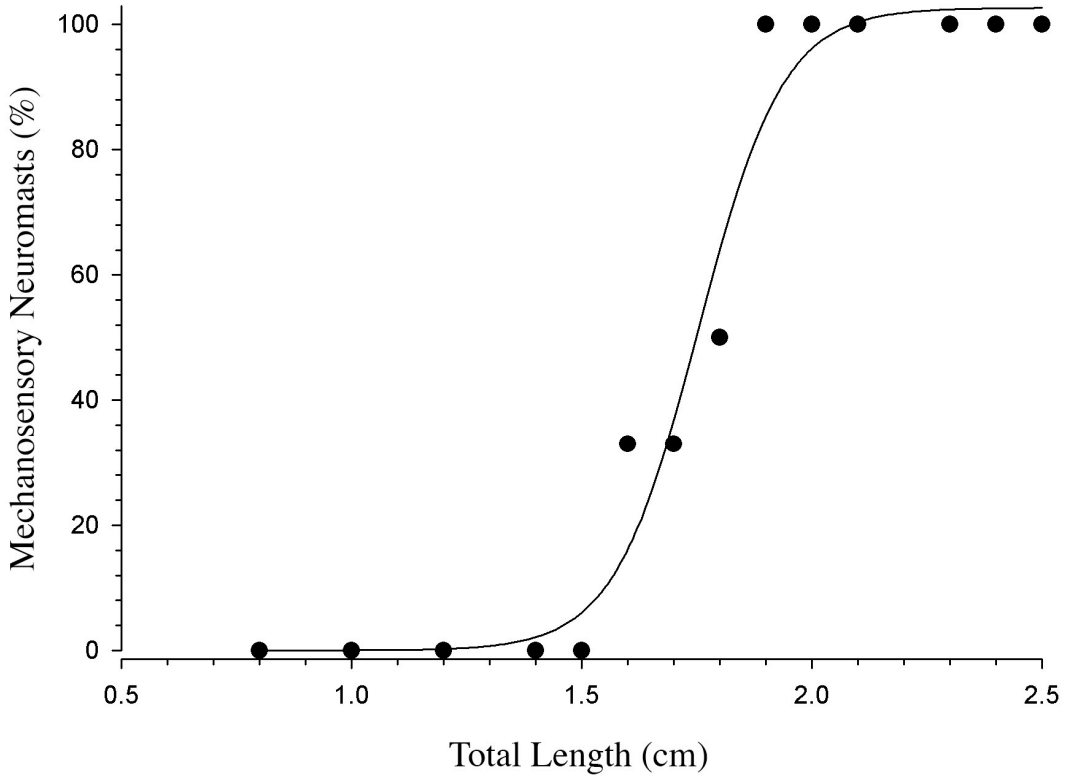


Figure 3.6 The presence of mechanosensory neuromasts as a function of fish total length (TL) in midshipman larvae. Mechanosensory neuromasts are shown as the percentage of fish examined that had neuromasts present. The presence of mechanosensory neuromasts was determined by the uptake of the vital dye DASPEI, which is taken up by energetically active cells such as lateral line neuromasts and can be visualized *in vivo*. Fish were scored base on DASPEI staining in a binary fashion (yes/ no): yes, for the staining of one or more neuromasts and no, for a lack of neuromast cell staining. Note that none of the small midshipman larvae less than 1.6 cm TL had any detectable neuromast cells, whereas all larvae greater than 1.8 cm TL had at least one neuromast cell with DASPEI staining. The solid line represents a best-fit sigmoidal curve.

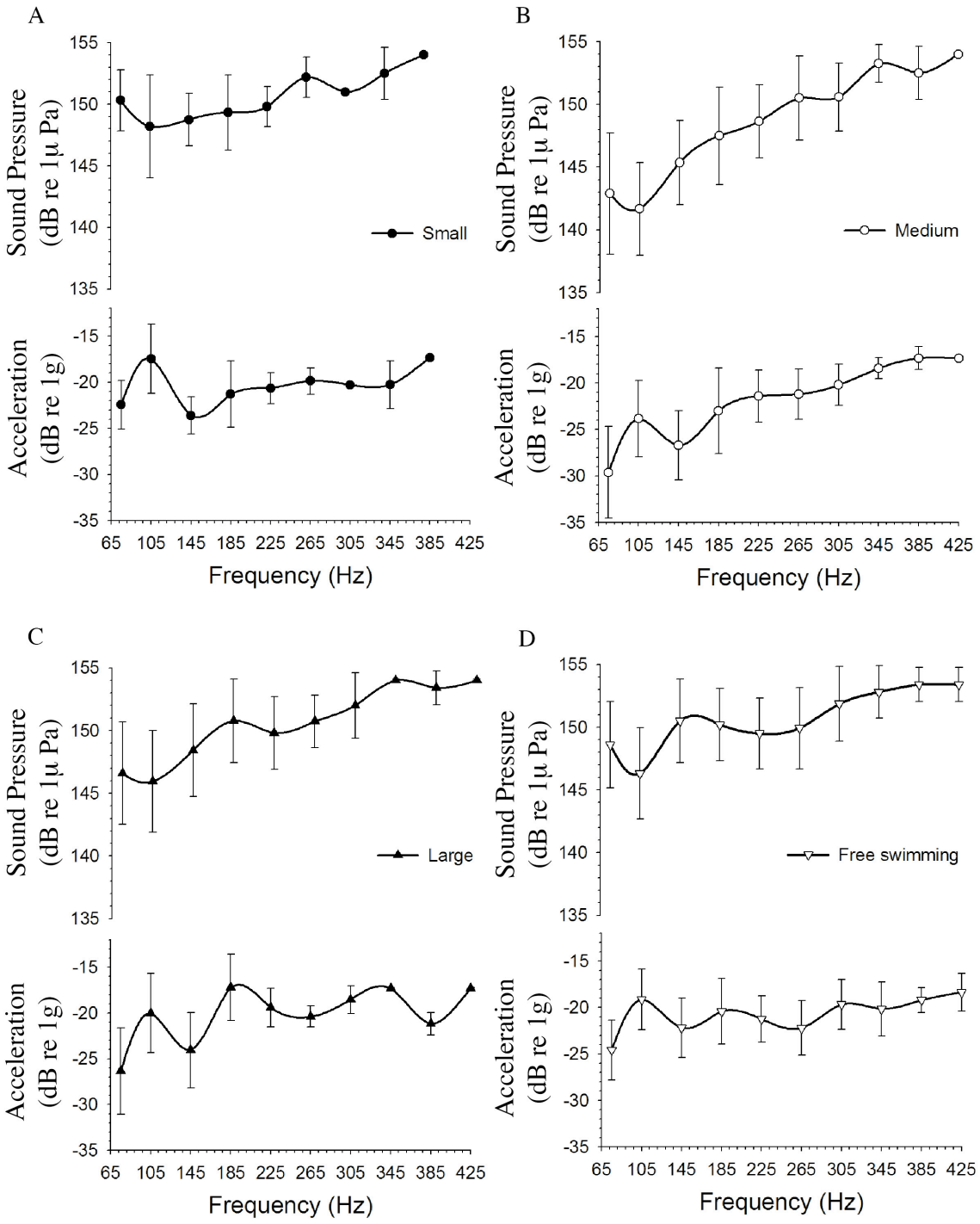


Figure 3.7 Acoustically evoked behavioral response (AEBR) profiles for the four size groups of midshipman larvae. The top portion of the graphs shows the response profiles in terms of SPL and the bottom portion of each graph is displayed in terms of acceleration (particle motion) in the Z (vertical)-axis of stimulation. Small midshipman larvae (A) are depicted by the line with solid circles, medium midshipman larvae (B) with open circles, large midshipman larvae (C) with solid triangles, and the free-swimming juveniles (D) with open triangles. Overall, the response profiles for all four groups were similar in shape with greatest sensitivity at the lowest test frequencies (<225 Hz).

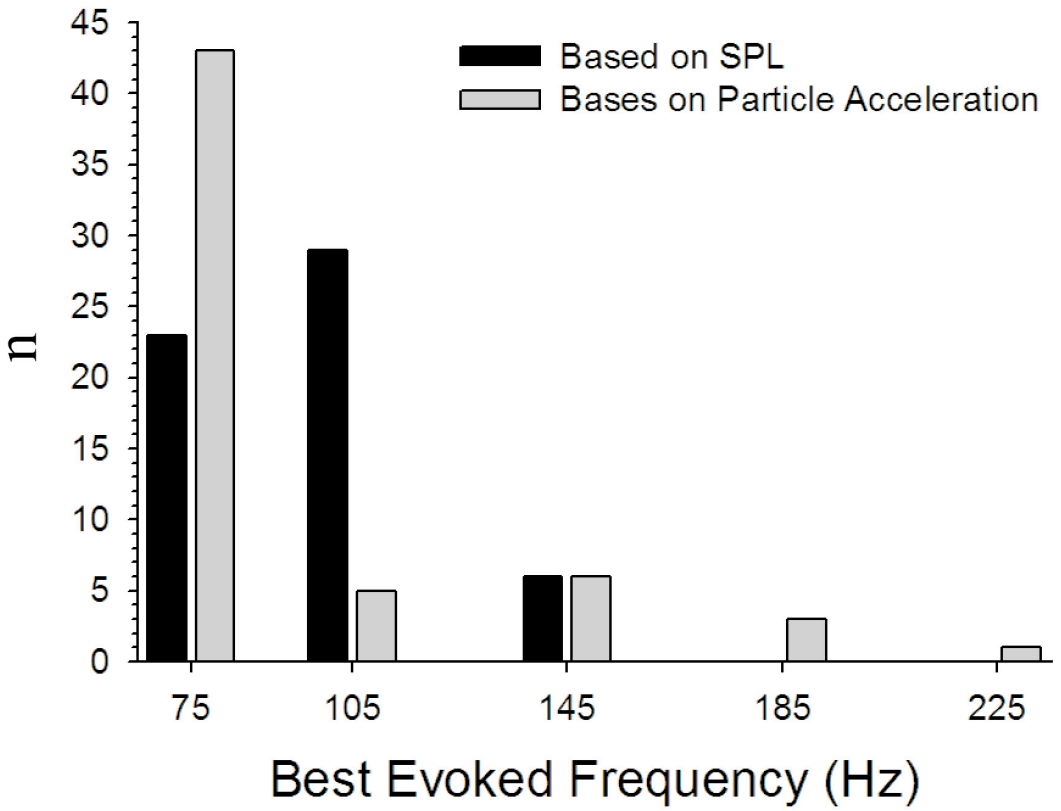


Figure 3.8 Best evoked frequency (BEF) histograms of the acoustically evoked behavioral response in midshipman larvae based on sound pressure level (SPL, black bars) and particle acceleration (gray bars). The distribution of the BEF for the AEBR is based on the individual AEBR profiles for all the midshipman larval groups tested. Note that the BEF is defined as the frequency with the lowest threshold to evoke the AEBR).

Chapter 4. Ontogenetic growth and morphology of the saccule in the teleost fish *Porichthys notatus*

4.1 Summary

Intraspecific structural differences of the inner ear have been examined comparatively across species and among the three otolithic endorgans within a given species in many teleost fishes. However, relatively few studies have examined how the saccule changes during ontogeny. The saccule, the main organ of hearing in most teleost fishes, undergoes profound growth during larval and juvenile stages. Here, we quantify the growth of the saccular epithelium and describe changes that occur in saccular morphology and ultrastructure during normal development in the plainfin midshipman fish, *Porichthys notatus*. The midshipman is a good neuroethological model for studying acoustic communication and hearing because male midshipman produce an advertisement call and successful spawning depends on female fish using this advertisement call to locate and select a suitable mate. Fish were divided into four developmental groups based on total length (TL) 'small' (1.4– 1.7 cm TL, n= 20) and 'medium' (1.8- 2.3 cm TL, n= 25) larval groups; a 'juvenile' (2.5- 3.1 cm TL, n= 30) group made up of late stage larvae with mostly absorbed yolk and juveniles; and an 'Adult' group (13- 19.6 cm TL, n= 12). The saccular epithelia were dissected, fixed overnight, and hair bundles were stained using fluorescently-conjugated phalloidin. We examined 87 total saccular epithelia using epifluorescent compound and confocal microscopy and found significant differences in hair bundle density, hair bundle length, epithelial area, and estimated total number of saccular hair cells. Hair bundle length, epithelial area, and total number of hair cells

increased with size/ age, whereas bundle density decreased with size/age. There was no difference in the pattern of orientation of hair cells between groups. We relate these changes in saccular morphology to previous physiology research on the ontogeny of auditory sensitivity in the plainfin midshipman during larval and juvenile development.

4.2 Introduction

The ability to continually grow new hair cells at any life history stage in response to acoustic trauma (Lombarte and Fortuno. 1992) or as a normal part of the growth of the ear is a well established feature of the teleost auditory system (Corwin 1981, Presson and Popper 1990, Lombarte and Popper 1994, Lanford et al. 1996, Coffin et al. 2012). However, relatively few studies have examined hair bundle proliferation as a function of ontogenetic growth (Corwin 1983, Popper and Hoxter 1984, 1990, Lombarte and Popper 1994, Higgs et al. 2001). Other interspecific structural differences, such as: hair bundle orientation patterns, area of the sensory epithelium, shape and size of the otolith, hair bundle length and density, etc., have been demonstrated between otolithic endorgans in the same species as well as between species (Flock 1964, Dale 1976, Popper 1977, Platt 1977, Popper 1981, Popper and Hoxter 1981, Platt 1983, Platt and Popper 1984, Edds-Walton and Popper 1995, Lu and Popper 1998). However the structure-function relationship between these differences is poorly understood (Popper and Fay 1993).

The plainfin midshipman, *Porichtys notatus* has become a useful model for investigating vocal behavior and auditory physiology largely due to the fact that the fish's spawning

behavior relies on vocal communication (Bass 1996, Bass et al. 1999, McKibben and Bass 2001, Sisneros et al. 2009, Sisneros 2009). Recently *P. notatus* has been used to investigate ontogenetic plasticity in the peripheral auditory system. Sisneros and Bass (2005) found an ontogenetic increase in the resting discharge rate and sensitivity at best frequency (BF) of saccular afferent neurons. Alderks and Sisneros (2011) demonstrated that auditory threshold and frequency response of the midshipman saccule is established early and retained throughout development. The plainfin midshipman makes an ideal model for investigating ontogenetic changes in saccular structure since its auditory physiology is well understood and it provides interesting predictions about possible structure-function relationships.

Here we describe changes that occur in the saccule of the teleost fish *P. notatus* during normal development. The purpose of this study was to answer the question, what structural changes occur in the saccule of the plainfin midshipman during development? Specifically, we address the hypothesis that there are changes in saccular morphology and ultrastructure during developmental. We relate differences in hair bundle density, hair bundle length, epithelial area, and the total number of saccular hair cells, as well as measurements of orientation patterns to previous ontogenetic studies of hearing sensitivity in the plainfin midshipman and discuss possible mechanisms for maintaining frequency sensitivity in the saccule throughout all life history stages. To our knowledge this is the first study to examine changes in saccular morphology and ultrastructure during larval fish development.

4.3 Methods and Materials

4.3.1 Fish collection and animal care

Nonreproductive adult fish were collected by otter trawl (R/V Kittiwake; Bio-Marine Enterprises) in Puget Sound near Edmonds, Washington at depths of 60–100 m during January 2012 and December 2012. Rocks with attached plainfin midshipman eggs and larvea were collected by hand at low tide from the nests of parental (type I) males at Seal Rock Beach near Brinnon, Washington during July-August 2011 and July 2012. All fish were transported to the University of Washington where they were housed in saltwater aquaria maintained at 14–16°C. Developing larval fish were left attached to the rocks until used in this study or they naturally detached having obtained a sufficient size to adopt a free swimming state. Adult fish were fed a diet of live goldfish or guppies every 2–4 days and free-swimming juvenile fish were fed SELCO enriched brine shrimp daily. Larval fish did not require feeding. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

Fish were divided into 4 groups (small, medium, large, adult) to allow for comparisons with previous research (Alderks and Sisneros 2013, Alderks and Sisneros 2011). The small sized fish group (n=20) contained larval fish that ranged in size from 1.4– 1.7 cm total length (TL). The medium sized fish group (n= 25) consisted of larval fish that ranged in size from 1.8- 2.3 cm TL. The large sized fish group (n= 30) was composed of detaching late-stage larval and free-swimming juvenile fish that ranged in size from 2.5-3.1 cm TL. Adult fish (n=11) ranged in size from 13- 19.6 cm TL. We define larval fish

as having hatched, but remaining attached to the natal rock, and juvenile fish as having detached from the rock, but not yet having achieved sexual maturity. This is consistent with previous research in the plainfin midshipman (Alderks and Sisneros 2013). As reproductive status is known to affect both auditory sensitivity as well as hair bundle density and length, only non-reproductive adult fish were used. (Coffin et al. 2012, Sisneros 2007). Reproductive state of adult fish was verified by calculating gonadosomatic index (GSI), a measure of the relative reproductive state according to Tomkins and Simmons (2002) and compared to GSI measurements in plainfin midshipman of known reproductive state (Sisneros et al. 2009, Sisneros et al. 2004).

4.3.2 Tissue processing and fluorescent labeling

The methods used for tissue processing and morphological analysis are similar to those used by Coffin et al. (2012). Briefly, fish were euthanized with an overdose of buffered ethyl p-aminobenzoate (benzocaine). Both saccules were exposed by way of a dorsal craniotomy. In the adult fish, the head was then removed and placed in 4% paraformaldehyde. The larval and juvenile fish were placed directly in the 4% paraformaldehyde without decapitation. In both cases paraformaldehyde was allowed to perfuse into the openings and the heads/ fish were fixed overnight at 4°C. After fixation heads/ fish were rinsed in 0.1 M PBS and either processed immediately or stored (up to 3 days) in 0.1 M PBS at 4°C. The ears were dissected from the head and the saccular epithelia were carefully trimmed following removal of the saccular otoliths. The trimmed saccular epithelia were returned to 0.1 M PBS, and labeled with phalloidin (Alexa Fluor 488 phalloidin, diluted 1:100; Invitrogen) for 20 minutes. Phalloidin is a

mushroom toxin that binds actin and is useful for visualizing the stereocilia of hair bundles. Whole epithelia were mounted with Fluoromount-G (Southern Biotech) and coverslipped. Because the dissection of larval and juvenile fish is very difficult, both saccular epithelia from each fish were stained, mounted, and visualized to maximize the likelihood of getting undamaged tissue for analysis. In cases where both saccular epithelia were adequate for analysis, only one epithelium per fish was used.

4.3.3 Morphological analysis

Hair bundle density analysis. Images (40X) of each epithelium were taken using a Leica DMR microscope equipped for epifluorescence. Hair bundle counts were performed in seven nonoverlapping 2,500 μm^2 regions of each saccule (see figure 4.1) using ImageJ (v.1.45s). We chose 2,500 μm^2 regions because in the saccules of the smallest larval fish used in this study (1.4 cm TL), 2,500 μm^2 regions were the largest that would still allow for seven nonoverlapping areas. The seven regions chosen allow us to compare our results with previous studies done in the plainfin midshipman (Coffin et al. 2012) and the sampling strategy is based on the strategy used by Smith et al. (2006) and Oxman et al. (2007). Bundle count data were analyzed by two-way ANOVA with epithelial region and size class as factors. Since we planned to compare the same saccular epithelial regions between the four size classes of fish, an *a priori* one-way ANOVA followed by Bonferroni post-hoc analysis was used to determine significant paired comparisons across saccular epithelial regions. SPSS statistical software (Ver. 14) was used to perform all statistical tests.

Hair bundle length analysis. We also quantified hair bundle length for a subset (n=12) of phalloidin labeled saccules from each size class. Images were collected of the same 7 distinct saccular regions used in the hair bundle density counts on a Leica TCS SP5 II confocal microscope with a 63X oil objective. Optical sections were taken at 200 nm intervals and images were collected with Leica Application Suit AF software. Max-intensity images were opened with Fiji (1.47n) and ten hair bundles were measured per image using the Simple Neurite Tracer plugin, with the measurement path following the length of the longest stereocilium of each bundle from the tip to the cuticular plate. We measured only intact (not splayed) bundles with clearly visible stereocilia. As with hair bundle density, hair bundle length data was analyzed by two-way ANOVA with 7 epithelial regions and size class as factors. For each saccular epithelial region we conducted a one-way ANOVA followed by Bonferroni post-hoc analysis to determine significant among the four size classes.

Hair bundle orientation analysis. We qualitatively mapped hair bundle orientation in a subset (n=8) of phalloidin-labeled epithelia from each size group. Bundle orientation is the direction of polarization from the shortest stereocilia to the kinocilium (Flock, 1964; Popper, 1981). In a phalloidin stained saccule, the kinocilium position appears as a dark hole at the level of the cuticular plate with the fluorescent apical surface or the stereocilia visible. This is because the kinocilium is a tubulin structure and phalloidin does not label it, however the dark hole allows the kinocilium position to be definitively identified (Lu and Popper, 1998). Epithelia were viewed on a Leica DMR microscope using a 63X oil objective and bundle orientation was mapped onto drawings of each epithelium. The

drawings from each epithelium were compared to create an overall orientation pattern for each of the size groups and then orientation patterns were compared among groups.

Measurements of saccular epithelial area. Multiple overlapping images from individual saccular epithelia were combined in Photoshop CS3 Extended (Adobe Systems inc.) using the difference method to create an image of the entire saccular epithelium. Images of the saccular epithelium were then opened and analyzed in ImageJ (1.45s). We used calibrated scale bars to set the image scale in ImageJ (1.45s) and traced the edges of the saccular epithelium using the tracing tool. The area of the encircled area was then measured using the measure tool for all fish from each size class. Small, medium, and large fish represent a continuous range of size and growth while developing in the nest. We analyzed the growth of the saccular epithelium from these three size groups using linear regression to determine if there is a relationship between size (TL) and epithelial area. Because of the lack of data from intermediary sizes between the large size class and adult fish, we excluded adults from this regression analysis. We also compare the size of the saccular epithelium from all size groups using a one-way ANOVA with a Bonferroni post-hoc analysis to determine differences between the four size classes.

Estimates of total hair bundle numbers. We estimated the total number of hair bundles using the data from the hair bundle density counts and total saccular epithelial area measurements. We use the equation (Popper and Hoxter 1984):

$$H = \sum C_i \times \frac{A}{\sum P_i}$$

to estimate the total number of hair bundles (H) where C_i represent the number of hair bundles in each of the seven areas measured and A represents the total area of the saccular epithelium and P_i represents the area of the seven distinct areas where hair bundle counts were performed. We compare the estimated total number of hair bundles across size groups using a one-way ANOVA with a Bonferroni post-hoc analysis.

4.4 Results

We performed the morphological analysis on 86 total fish divided into 4 developmental groups based on TL. The small group (n=20) were 1.6 ± 0.1 cm TL (mean ± SD), medium group (n= 25) were 2.0 ± 0.2 cm TL, the juvenile group (n= 30) were 2.8 ± 0.2 cm TL, and the adult group (n=11) were 16.1 ± 2.4 cm TL.

4.4.1 Growth of the saccular epithelia

Overall the epithelial area of the saccule underwent substantial growth during development. The area of the epithelia was 279,009 ± 48,252 μm^2 (mean ± SD) in the small group, 374,168 ± 68,144 μm^2 for medium fish, 599,979 ± 89,965 μm^2 in the large group, and 12,148,140 ± 5,331,652 μm^2 for adult fish. A significant difference was found in the total area of the saccular epithelia (ANOVA $F_{(3,82)}= 126.42$, $p < 0.001$). Bonferroni post-hoc analysis revealed that the adult group had significantly greater saccular epithelial area $p< 0.001$ than all other groups (see figure 4.2). There was not a significant difference in the area of the saccular epithelium between the small, medium, and juvenile groups, however saccular epithelial area is highly correlated with size (TL) during these developmental stages ($r^2= 0.882$, $p<0.001$, see figure 4.3a). Overall the

growth of the saccular epithelia fits the cubic equation: $f(x)= 382515x^3-27499x^2+3063x-298529$ ($r^2= 0.977$, $p< 0.001$, see figure 4.3b).

4.4.2 Hair bundle density measurements

Hair bundle counts were performed in seven nonoverlapping 2,500 μm^2 regions (see figure 4.1). Our initial analysis revealed significant main effects of both saccular area (ANOVA $F_{(6,492)}= 47.88$, $p < 0.001$) indicating that hair bundle density is variable across the sampled regions, and developmental group (ANOVA $F_{(3, 82)}= 77.53$, $p< 0.001$). We conducted an *a priori* analysis of the group effects at each of the saccular regions (one-way ANOVA for each of the 7 saccular regions, followed by Bonferroni post-hoc analysis) and found that adult fish have a lower density of saccular hair bundles than small, medium and large groups at all 7 areas examined (AvL, AvM, AvS all seven regions $p< 0.001$, see figure 4.4). On average, adults had 33% fewer hair bundles per sampling area than the other developmental groups examined, with the greatest differences in hair cell density being seen in areas 4 and 5 (35 and 40% fewer respectively). Small larval fish have a lower density of hair bundles than the medium sized group in areas 6 and 7 (caudal region, $p= 0.013$, 7% less; $p= 0.001$, 12% less respectively) and also a lower density of hair bundles than the large group in area 7 ($p< 0.001$, 12% less). No other differences in hair bundle density were observed.

4.4.3 Adults have more total hair bundles

We estimated the total number of hair bundles similarly to Popper and Hoxter (1984).

The small group of fish had 5742± 1014 (mean ± SD) total estimated hair bundles, the medium group had 8263± 1690 total estimated hair bundles, the large group had 12,596± 1879 total estimated hair bundles, and adults had 171,705± 74,307 total estimated hair bundles. Adults have significantly more hair bundles than all other developmental groups (ANOVA $F_{(3,82)}$= 125.08 p< 0.001, Bonferroni post hoc analysis AvL, AvM, AvS all p< 0.001, see figure 4.5). On average the total number of hair cells increased proportionally with epithelial area. The total number of estimated hair bundles is highly correlated with epithelial area (r^2= 0.996, p< 0.001, see figure 4.6a) and TL (r^2= 0.925, p< 0.001, see figure 4.6b). There were no differences in number of hair bundles between the small, medium, and large groups. Alderks and Sisneros (2013) have published data on the growth of larval fish at 15 ± 2 °C. Our larval fish were also housed at 15 ± 2 °C which allows us to estimate the age (days post-hatch) for larval fish that hatched and were maintained in our lab (n=45). Using this data and our estimates of total hair bundle density we estimate that larval midshipman add 258 new hair cells every day. It was not possible to include adults in this analysis because we could not determine their age.

4.4.4 Adult fish have greater hair bundle length than larval fish

We measured bundle length in three saccules from each of the four developmental groups (total n=12) in seven nonoverlapping 2,500 μm^2 regions (see figure 4.1). Our initial analysis reveled significant main effects of both saccular area (ANOVA $F_{(6,18)}$= 2.15, p = 0.046) indicating that hair bundle length is variable across the sampled regions with slightly shorter hair bundles in the rostral region, and developmental group (ANOVA $F_{(3, 116)}$= 510.442, p< 0.001). We conducted an *a priori* analysis of the group effects at each

of the saccular regions (one-way ANOVA for each of the 7 saccular regions, followed by Bonferroni post-hoc analysis) and found that adult fish have longer bundle lengths than all other developmental groups in all seven areas (Bonferroni post hoc analysis AvL, AvM, AvS all comparisons $p< 0.001$, see figure 4.7). Additionally small larval fish have shorter hair bundle lengths than medium larval fish (Bonferroni post hoc analysis SvM areas 1-5 $p< 0.001$, area 6 $p= 0.0018$, area 7 $p= 0.0027$). Small larval fish also have shorter hair bundle lengths than juvenile (the large group) fish (Bonferroni post hoc analysis SvL area 1 $p< 0.001$, area 2 $p= 0.011$, area 3 $p= 0.037$, area 4 $p= 0.0047$, area 5 $p= 0.0017$, and area 7 $p= 0.0127$). In area 5 medium larval fish have larger hair bundle lengths than juvenile fishes (Bonferroni post hoc analysis MvL, $p= 0.0017$). Overall adults had the largest hair bundles (mean ± SD, 11.03± 1.64 microns); juveniles and medium larval fish had the next largest hair bundle lengths (6.82± 1.34 and 7.3± 1.27 microns respectively); and small larval fish had the shortest hair bundles (5.79± 0.94 microns).

4.4.5 Hair bundle orientation is conserved throughout ontogeny

We mapped hair bundle orientation patterns in the saccules of all four developmental groups (n= 2 per group, n=8 total) to determine the overall orientation pattern and identify potential ontogenetic variation in hair bundle orientation. Overall the orientation pattern most closely matched the standard four-quadrant orientation pattern (Popper and Combs 1982), however, there was variation from this pattern in the rostral and caudal tips of the saccule (see figure 4.8). In general hair cells in the dorsal rostral quadrant were orientated caudally, hair cells in the ventral rostral quadrant were orientated rostrally, hair

cells in the dorsal caudal quadrant were orientated dorsally, and hair cells in the ventral caudal quadrant were orientated ventrally. There were no differences observed in the hair bundle orientation patterns across ontogenetic group.

4.5 Discussion

In this study we attempt to answer the question, what structural changes occur in the saccule of the plainfin midshipman during development? We tested the hypothesis that changes in saccular morphology and ultrastructure are developmental stage dependent in larval, juvenile and adult fish. There were significant differences in hair bundle density, hair bundle length, epithelial area, and the total number of saccular hair cells. We also examined hair bundle orientation patterns and found no differences in the orientation patterns of hair cells in the saccule of the plainfin midshipman across the four size groups examined.

4.5.1 Adult fish have saccules with greater epithelial area and more total hair cells.

It has been repeatedly demonstrated that as teleost grow, the inner ear and total number of hair bundles also increase (Corwin 1981, Presson and Popper 1990, Lombarte and Popper 1994, Lanford et al. 1996, Coffin et al. 2012). This feature of the teleost auditory system has also been demonstrated throughout ontogeny (Corwin 1983, Popper and Hoxter 1984, 1990, Lombarte and Popper 1994, Higgs et al. 2001). Our study however, is the first to examine changes in saccular hair cell numbers during larval stages (see figures 4.5 & 4.6). Estimates of the rate of hair cell proliferation in the auditory endorgans of fishes range from 1-3 hair cells per day in macula neglecta in the thornback ray, *Raja clavata* (Corwin

1983) to 302 hair cells per day in the saccule of the European hake, *Merluccius merluccius* (Lombarte and Popper 1994). In *P. notatus* we estimate that hair bundles are added at a rate of 258 per day (94,170 per year) in the saccule during the larval stage. It is likely that hair cell proliferation slows as the animal approaches maturity. Coffin et al. (2012) found an average of 40 PH3- labeled (cells undergoing mitosis) when examining cell proliferation in the saccule of adult plainfin midshipman. In the zebrafish, *Danio rerio*, Higgs et al. (2001) found that hair cell proliferation stops at 10 months with new hair cells replacing dying hair cells.

The saccular epithelial area also grows post-embryonically with size (see figures 4.2 & 4.3). This is consistent with what is known in fishes (Popper and Fay 1993, Popper and Fay 2011). Lombarte and Popper (1994) found that saccular area grew isometrically with TL finding a very similar function to describe the growth of the saccular epithelium as we report (see figure 4.3b). It is likely that this same pattern will be observed in all fishes that use the saccule as the primary auditory endorgan. In fishes where other auditory endorgans are primarily used for hearing, one would expect to see similar growth in the area of the sensory macula. Corwin (1983) describes such growth in the area of the macula neglecta, the primary auditory endorgan in elasmobranchs, in *Raja clavata*, however he does not quantify this growth. Further comparative work will be necessary to determine if this is a typical developmental pattern in fishes.

4.5.2 Larval and juvenile fish have greater saccular hair bundle density.

Hair bundle counts were performed in seven nonoverlapping 2,500 μm^2 regions (see figure 4.1). These distinct seven regions were chosen to allow us to compare our results with previous studies in the plainfin midshipman (Coffin et al. 2012). The 2,500 μm2 counting areas allowed for seven nonoverlapping regions in all fish including the smallest larval fish. We found that larval and juvenile fish had on average 33% more hair bundles per given area than adult fish (see figure 4.4). This observation is consistent with physiology experiments that found greater evoked saccular potentials in juvenile midshipman (Alderks and Sisneros 2011). Having a greater density of hair cells in the juvenile and larval midshipman would account for this observation as more hair cells would have been in the receptive field of the recording electrode and thus resulted in a greater summed evoked potential.

These results are also consistent with previous work in the oscar, *Astronotus ocellatus,* and the European hake, *Merluccius merluccius*, where smaller fish also had a greater density of hair bundles (Popper and Hoxter 1984, Lombarte and Popper 1994). In contrast Higgs et al. (2001) found no differences in hair bundle density with size in the zebrafish, *Danio rerio* however, they did find that hair cells continued to be added until the fish were 10 months old. It is possible that the contrasting observations represent different developmental strategies. All four fish species with available data represent a wide range in phylogeny and ecology among teleost fishes. Further research is necessary to determine if these observations really are distinct developmental strategies and how common those strategies are among fishes.

4.5.3 Hair bundle length

In the goldfish, *Carassius auratus*, it has been shown that hair bundle length varies along the rostral caudal axis and that these changes also have implications for auditory sensitivity (Smith et al. 2011). Differences in hair bundle length have been associated with differences in frequency sensitivity with longer hair bundles being more sensitive to low frequency (Popper and Combs 1982, Platt and Popper 1984, Sugihara and Furukawa 1989, Popper and Fay 1999, Lanford et al. 2000). Our data indicates that adults have longer hair bundles then other developmental groups and that the smallest larval fish examined have the shortest hair bundles, which would be consistent with differences in frequency sensitivity. However, previous research in juvenile and larval midshipman has demonstrated that auditory sensitivity is established early in the larval stages and maintained throughout later life history stages (Alderks and Sisneros 2011, Alderks and Sisneros 2013).

Our results are similar to measurements taken in adult female midshipman (Coffin et al. 2012). Coffin et al. (2012) reported slight difference in hair bundle length along the rostral caudal axis with shorter hair cells in the rostral portion of the saccule. They also found that reproductive animals had a greater proportion of shorter hair cells than non-reproductive animals, which they attributed to possibly being immature developing hair cells. This would be consistent with our finding shorter hair cells in small larval fish. Previous work has demonstrated that behavioral responses to acoustic stimuli first appear in fish 1.4 cm TL (Alderks and Sisneros 2013), so it is possible that these shorter hair cells represent developing hair cells that are just beginning to transduce sounds. Further

research is necessary to determine the frequency sensitivity of individual hair bundles and determine the structure function relationship of bundle length in the plainfin midshipman.

4.5.4 Hair bundle orientation

We found no difference in the orientation patterns of hair cells in the saccule of the plainfin midshipman. Hair bundle orientation has been hypothesized to contribute to sound localization (Schuijf 1975, Hawkins and Sand 1977, Buwalda 1981) and different hair bundle orientations have been observed in fishes with different hearing thresholds (Popper 1977, 1981, Popper and Fay 1993, Popper and Lu 2000). There is a great diversity in the orientation patterns of teleost fishes and the functional significance of these differences are not well understood. A common hair cell orientation pattern among developmental stages is perhaps one mechanism to maintain directional auditory sensitivity of the saccule in the plainfin midshipman.

4.5.5 Mechanisms for maintaining auditory frequency sensitivity

In *P. notatus*, auditory sensitivity has been shown to be conserved throughout ontogeny (Alderks and Sisneros 2011, Alderks and Sisneros 2013). There are several possible mechanisms that might contribute to maintaining auditory frequency sensitivity throughout ontogeny. One possible mechanism, as described above, is that the orientation pattern of hair cells in the saccule is established early in larval development and maintained throughout all life history stages. Although hair cell orientations vary widely in fishes and the functional significance of this variability is poorly understood, hair cell orientation has been suggested as a possible mechanism for producing

differences in directional auditory sensitivity (Popper and Coombs 1982, Popper and Fay 1999). Any effects hair bundle orientation might have on auditory sensitivity would not cause ontogenetic differences in auditory sensitivity in the midshipman because bundle orientation remains unchanged.

Another possible mechanism for maintaining frequency sensitivity in the plainfin midshipman is the increased density of hair bundles in juvenile and larval groups. As the saccule grows, hair cells and primary afferent neurons are added (Corwin 1983, Popper and Hoxter 1984). The addition of both hair cells and ganglion cells is not proportional, so that the convergence ratio increases. An increase in neural convergence has been shown to be involved in improvements in physiologic sensitivity (Corwin 1983, Sento and Furukawa 1987). It is possible that as hair bundle density decreases, the effects of neural convergence are reduced. It is of note that Sisneros and Bass (2005) found an ontogenetic increase in sensitivity at best frequency (BF) in the auditory nerve of the plainfin midshipman, so neural convergence is likely to occur during development. It is also possible that saccular hair cells are intrinsically tuned, and that tuning is consistent across developmental stages. More research is necessary to determine the rate of proliferation of auditory ganglion cells and the level of neural convergence as well as the physiologic consequences of the observed saccular changes more centrally in the plainfin midshipman.

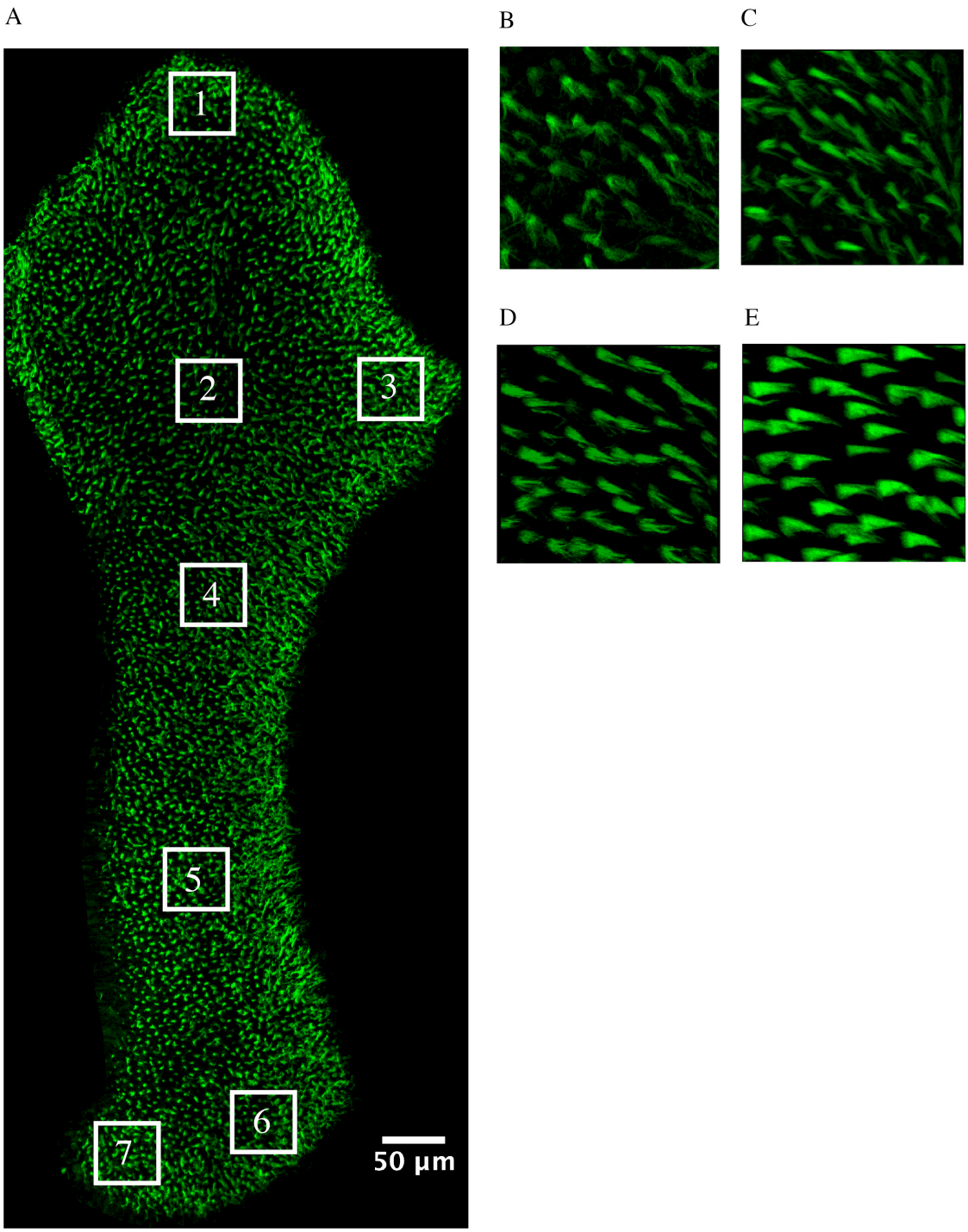


Figure 4.1 Seven non-overlapping 2,500 µm^2 sampling areas in the saccule of the plainfin midshipman are depicted in **A**. **B- E** are representative 2,500 µm^2 confocal images from area 4; **B** is a small larval fish, **C** is a medium larval fish, **D** is a juvenile fish, and **E** is from an adult fish. Note the differences in both number of hair bundles in each of the four representative images and the length of the hair bundles.

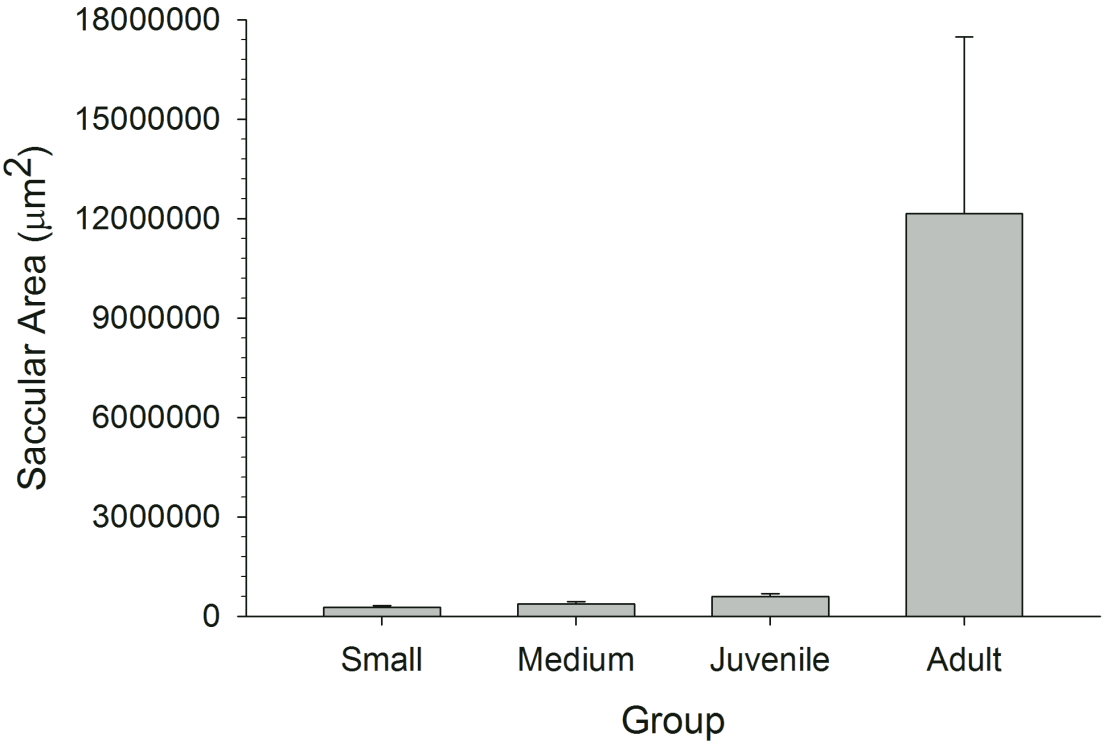
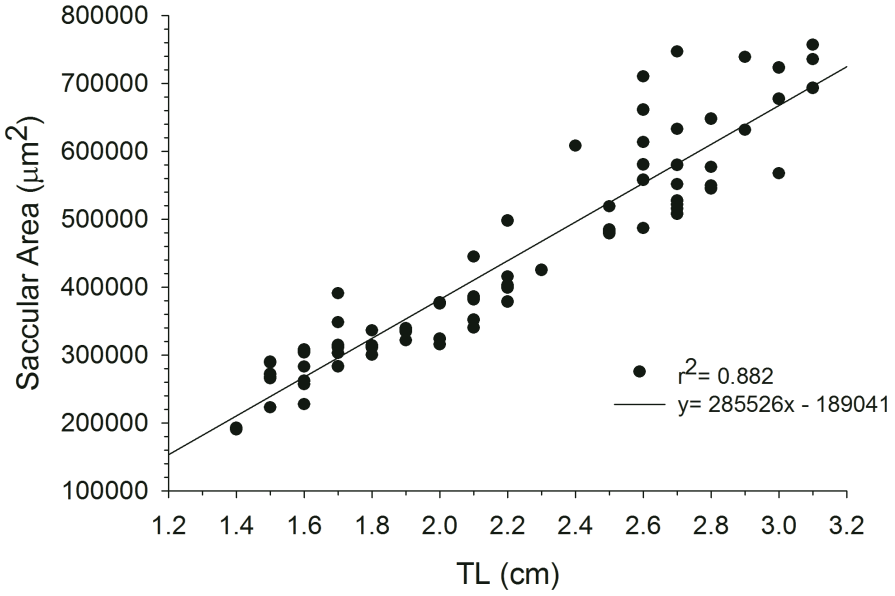


Figure 4.2 The mean total area of the saccular epithelium in the four size groups examined. The error bars represent ±1 SD. The area (mean ± SD) of the epithelia was 279,009 ± 48,252 μm^2 in the small group, 374,168 ± 68,144 μm^2 for medium fish, 599,979 ± 89,965 μm^2 in the large group, and 12,148,140 ± 5,331,652 μm^2 for adult fish. The adult group had significantly greater saccular epithelial area than all other groups (ANOVA $F_{(3,82)}$= 126.42, $p < 0.001$, Bonferroni post-hoc $p< 0.001$). There was not a significant difference in the area of the saccular epithelium between the small, medium, and juvenile groups.

A.



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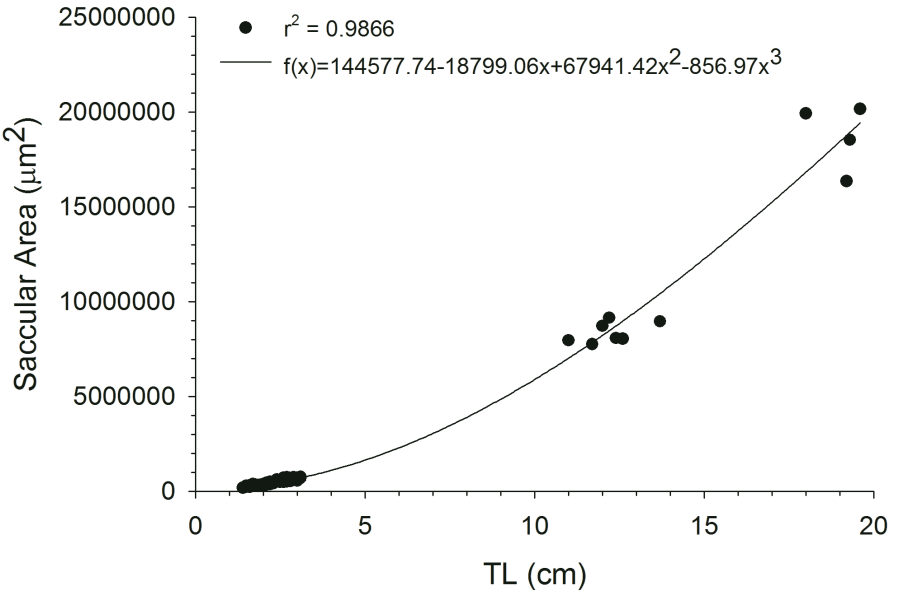


Figure 4.3 The relationship between saccular area and size (TL). **A** represents the growth of the saccule during larval development. The line represents a best-fit regression. The relationship between epithelial area and TL is approximately linear in the larval stage (r^2= 0.882, p< 0.001). **B** represents the growth of the saccule over all ranges sampled. The relationship best fits a cubic function (r^2= 0.987, p<0.001).

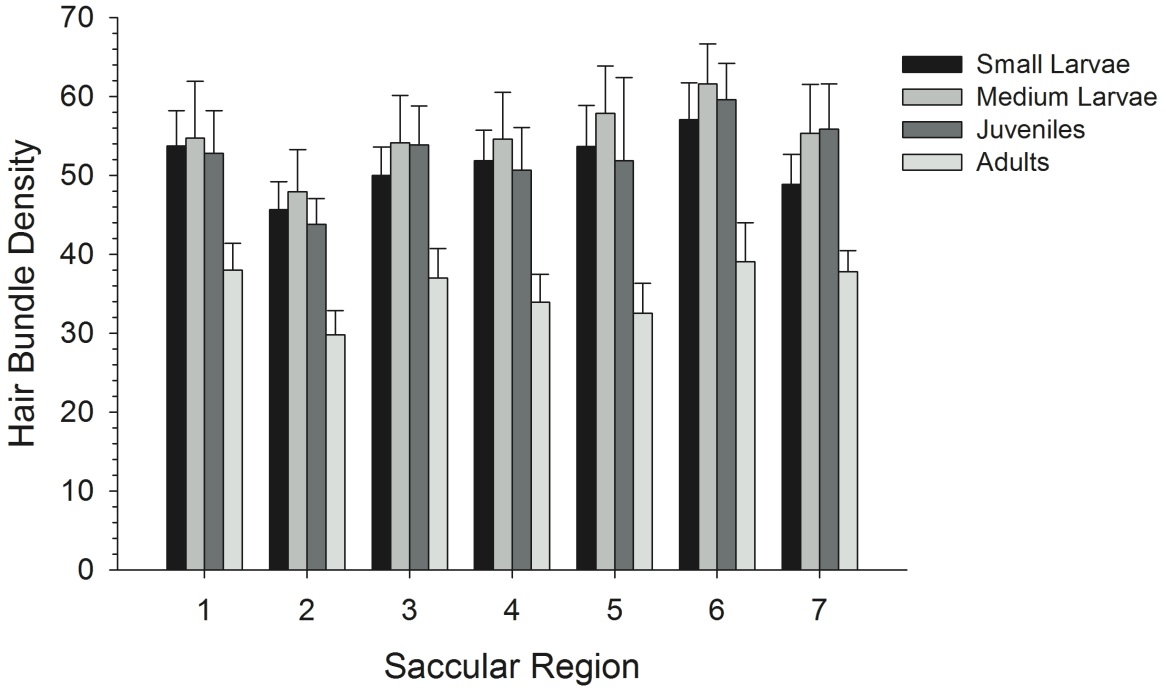


Figure 4.4 Hair bundle density measured in each of the seven non-overlapping saccular areas and four developmental groups. Each bar represents the mean ±1 SD. Adults had significantly fewer hair bundles in all seven of the saccular areas than small larvae, medium larvae, and juvenile groups (ANOVA $F_{(3, 82)}= 77.53$, $p< 0.001$, Bonferroni $p< 0.001$ for all comparisons). Small larval fish have a lower density of hair bundles than the medium sized group in areas 6 and 7 (Bonferroni $p= 0.013$, $p= 0.001$ respectively) and also a lower density of hair bundles than the juvenile group in area 7 (Bonferroni $p< 0.001$). No other differences in hair bundle density were observed.

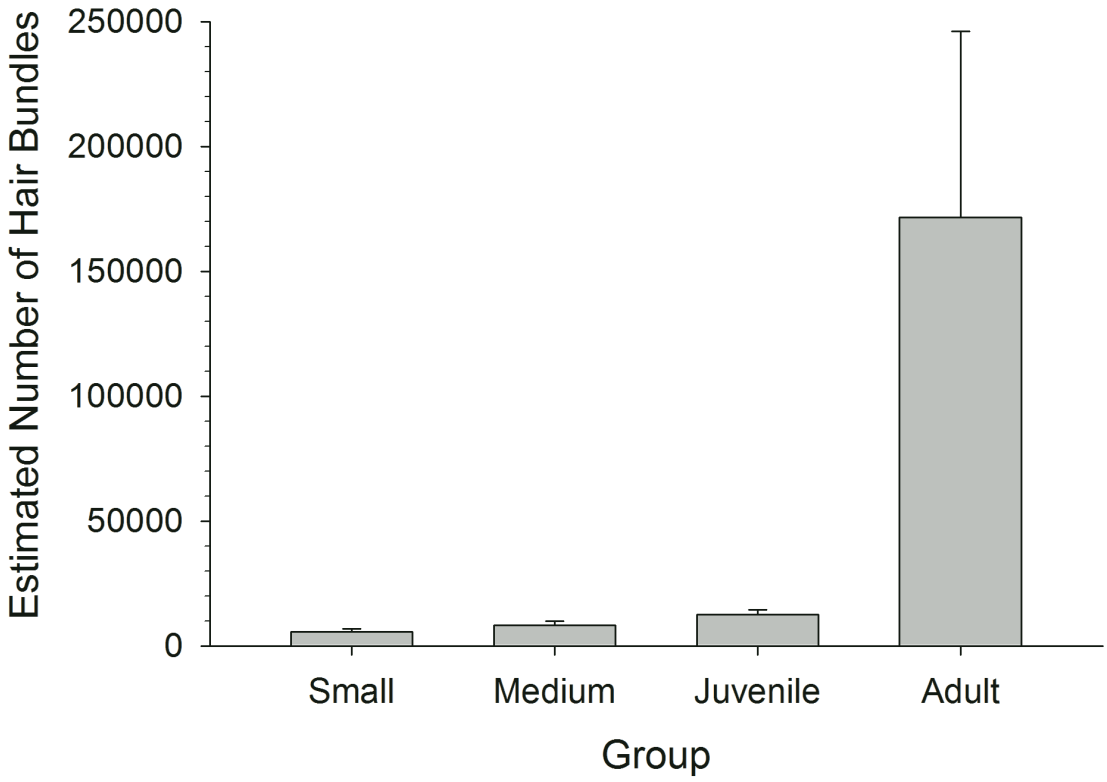
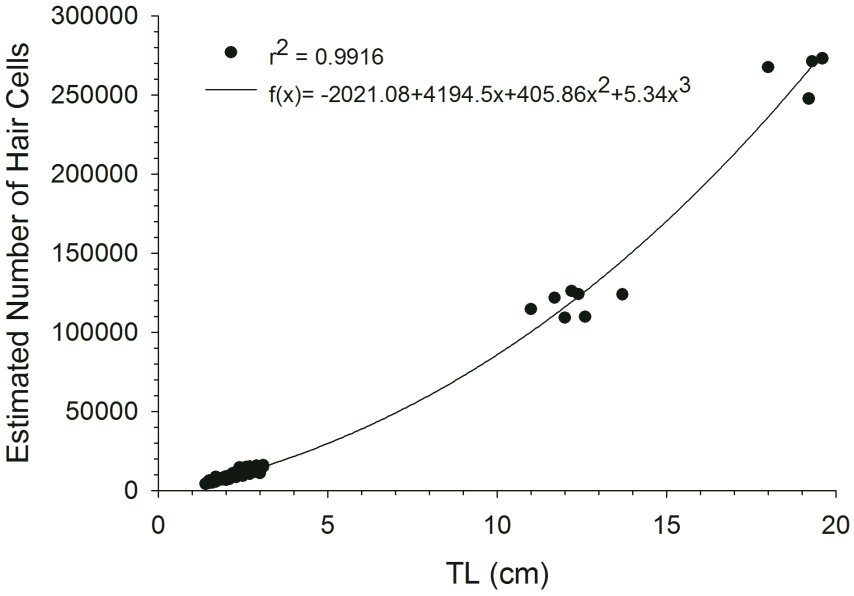


Figure 4.5 The estimated total number of saccular hair cells in the four developmental stages. Each bar represents the mean ±1 SD. The small group of fish had 5742± 1014 (mean ± SD) total estimated hair bundles, the medium group had 8263± 1690 total estimated hair bundles, the large group had 12,596± 1879 total estimated hair bundles, and adults had 171,705± 74,307 total estimated hair bundles. Adults have significantly more hair bundles than all other developmental groups (ANOVA $F_{(3,82)}= 125.08$ $p< 0.001$, Bonferroni all comparisons $p< 0.001$). There were no differences between other groups.

A.



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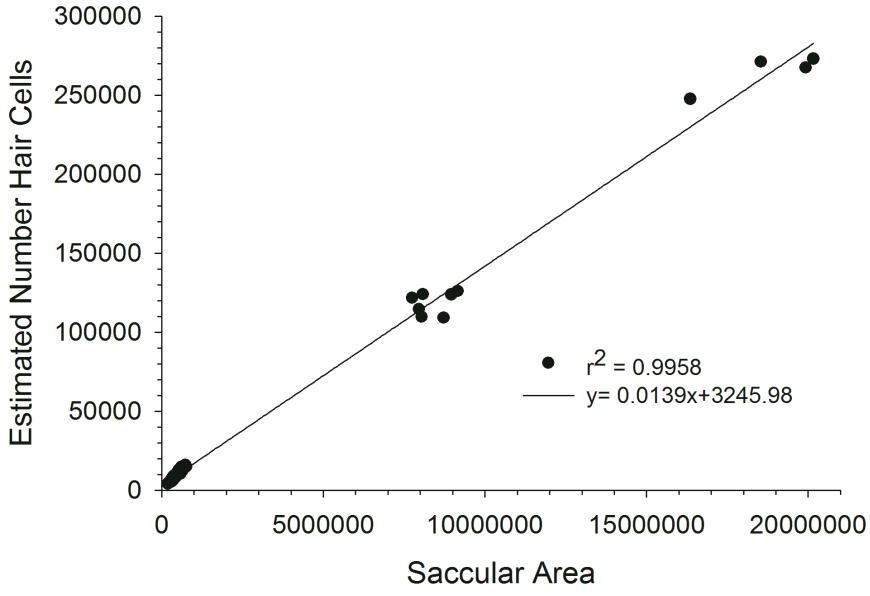


Figure 4.6 The relationship between estimated total number of saccular hair cells and size (TL) in **A** and saccular epithelial area in **B**. The lines represent the best-fit regression in both **A** and **B**. The relationship between total number of saccular hair cells and TL is approximated by a cubic function (r^2= 0.992, p< 0.001) and the relationship between estimated total number of saccular hair cells and saccular epithelial area is a linear relationship (r^2= 0.996, P< 0.001).

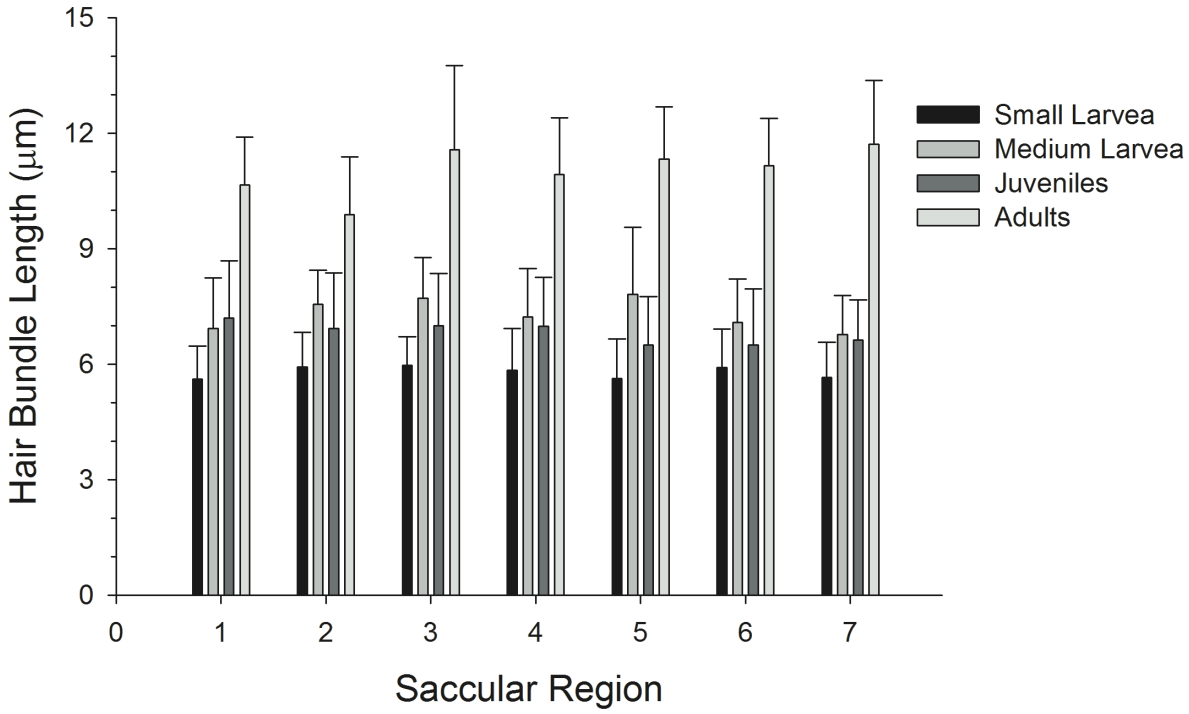
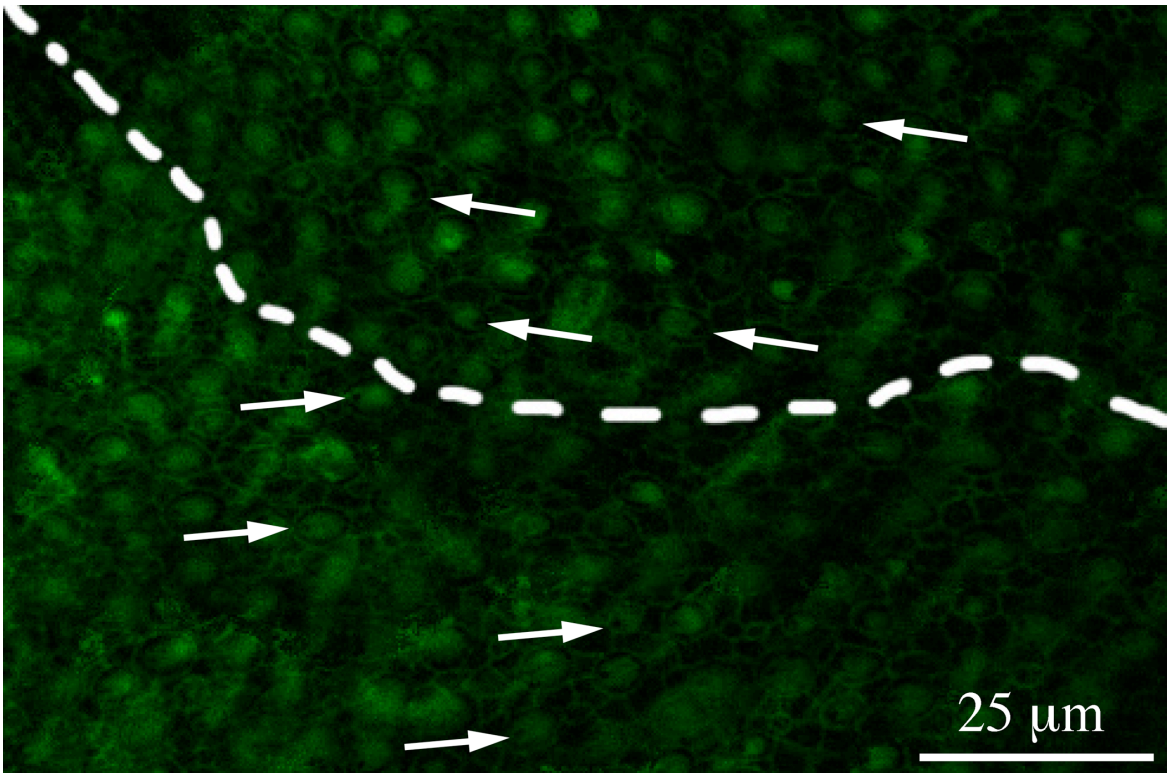


Figure 4.7 Hair bundle length in each of the seven non-overlapping saccular regions for each developmental group. Each bar represents the mean ±1 SD. adult fish have longer bundle lengths then all other developmental groups in all seven areas (ANOVA $F_{(3, 116)}$= 510.442, p< 0.001, Bonferroni p< 0.001 for all comparisons). Additionally Small larval fish have shorter hair bundle lengths than medium larval fish (Bonferroni areas 1-5 p< 0.001, area 6 p= 0.0018, area 7 p= 0.0027) and juvenile fish (Bonferroni area 1 p< 0.001, area 2 p= 0.011, area 3 p= 0.037, area 4 p= 0.0047, area 5 p= 0.0017, and area 7 p= 0.0127). In area 5 medium larval fish have larger hair bundle lengths than juvenile fishes (Bonferroni p= 0.0017). No other differences were observed.

A.



B.

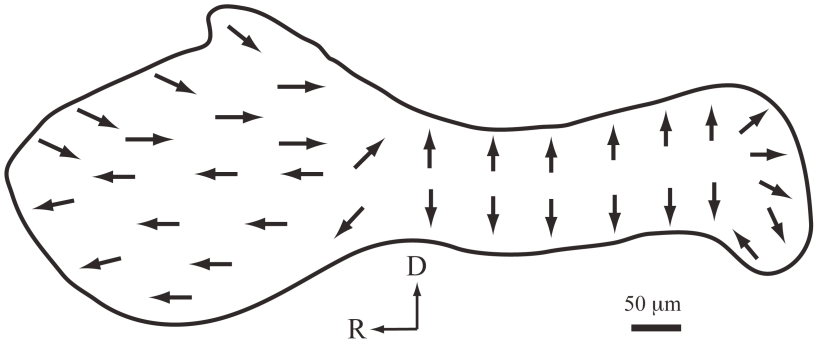


Figure 4.8 Hair bundle orientation patterns in the saccule of the plainfin midshipman. **A** Shows a single plane confocal image from the rostral end of the saccule, taken at the level of the cuticular plate. The white dotted line divides hair bundles from two distinct orientation groups. The white arrows show the kinocilia insertions into the cuticular plate. **B** Is the generalized hair bundle map observed in the midshipman saccule. The black arrows indicate the polarizing direction of the hair bundles. There were no differences in hair bundle orientation pattern observed between developmental groups.

Chapter 5. Summary and Future Directions

The goal of this dissertation was to gain insight into ontogenetic mechanisms that shape hearing in fishes. I used an ethological perspective and a variety of electrical physiological, behavioral, and morphological techniques to investigate auditory sensitivity and changes in inner ear structures in larval and juvenile plainfin midshipman fish. In chapters two through four I have provided empirical evidence for ontogenetic mechanisms that help shape hearing in the plainfin midshipman. These chapters also provide an accurate representation of the difficulties and complexities of doing auditory research in larval and juvenile fish. In this final chapter I will summarize the conclusions from chapters two through four and elaborate on future work that would provide an increased understanding of ontogenetic processes at work in this and other fish species.

5.1 Auditory sensitivity in juvenile fishes

I addressed the question: "Does the auditory threshold or frequency response properties of saccular hair cells change during ontogeny?" in chapter two by characterizing the auditory-evoked potentials from the saccule of *P. notatus* and compare the saccular hair-cell frequency response properties among three different age/size classes of fish. I demonstrated that the frequency response and auditory threshold of the midshipman saccule is established early in development before juveniles leave their natal nests and threshold sensitivity is retained throughout ontogeny. I did however find that juvenile midshipman have a greater magnitude evoked saccular potential at BF than adults. These results were surprising because the saccule undergoes a large amount of growth and adds hair cells throughout life. It was these physiological findings that lead me to

investigate changes in the saccular morphology and ultrastructure reported on in chapter four. I concluded that there is an ontogenetic retention of auditory saccular sensitivity with size/age in the plainfin midshipman fish. Additional neurophysiological studies, such as patch clamping of individual saccular hair cells and single unit recordings of auditory afferents, are needed to reveal the possible mechanisms enabling the ontogenetic retention of auditory saccular sensitivity.

Additionally adult midshipman are more likely to detect frequencies greater than 385 Hz than juvenile fish. The increase in the ability of the midshipman saccule to detect frequencies higher than 385 Hz with age/size, may be important for the detection of social acoustic signals during the adult life history stage. Adult plainfin midshipman produce three vocalizations, the hum (an advertisement call) and the growl (an agonistic call) are produced only by nesting males during the breeding season; and the grunt (an agonistic call) is produced by all adult midshipman year round. All three calls have low fundamental frequencies, but contain significant amounts of energy in higher frequency harmonics. It is possible that detection of these higher frequency components allow the adult midshipman to better evaluate potential rivals and mates. Further research is needed to determine the behavioral significance of these results.

5.2 AEBR and larval fish hearing

In chapter three I addressed the question: "When does fish audition begin and does auditory sensitivity undergo ontogenetic changes during larval development?" I used the acoustically evoked behavioral response (AEBR) to determine when the plainfin

midshipman begins responding to acoustic stimuli and I also characterize the AEBR response profiles using puretone stimuli in four groups of developing larval and juvenile fish to learn if auditory sensitivity changes during larval development. I found that larval fish first respond to acoustic stimuli at 1.4 cm TL (23 days post-hatch age). The lowest AEBR thresholds were found at frequencies below 225 Hz in all fish. Larval fish with size ranges of 1.9-2.4 cm TL had significantly lower best evoked frequencies than other size groups tested although the differences were small and likely do not reflect a behaviorally relevant difference. From this series of experiments I conclude that the midshipman auditory and lateral line systems are functional during early development while the larvae are in the parental nest. I also conclude that the auditory system appears to have similar tuning characteristics throughout all life history stages.

Future work should focus on the role of the lateral line in the AEBR. We were able to assess when the lateral line first developed and demonstrated that the lateral line is not necessary in order to evoke an AEBR, however we were unable to ablate the lateral line in late staged larvae or juvenile fish, which would have provided valuable insights into how the lateral line is involved in the detection of acoustic stimuli and if it modulates the AEBR. Additionally, future research using a different behavioral paradigm, such as pre-pulse inhibition, to assess frequency sensitivity would generate behavioral auditory response profiles more closely resembling absolute auditory thresholds. Our characterization of the AEBR can be thought of as the first step in adapting a pre-pulse inhibition paradigm for use with the plainfin midshipman model.

5.3 Saccular growth during development

Chapter four describes the morphological techniques I used to answer the question: "What structural changes occur in the saccule during development?" I analyzed saccular epithelial area, hair bundle number, density and length, and hair bundle orientation patterns in larval, juvenile, and adult midshipman. I found that hair bundle length, epithelial area, and total number of hair cells increased with size/ age, whereas bundle density decreased with size/ age. There was no difference in the pattern of orientation of hair cells between groups. The results from this study help explain the results I found in chapters two and three and provide insights into the ontogenetic mechanisms at work in plainfin midshipman.

Future research could provide additional insights into the mechanisms responsible for the ontogenetic retention of saccular sensitivity. Specifically, morphological examination of the connections between the saccular hair bundles and ganglion cells of the auditory nerve paired with electrical physiology of hair bundles and/or auditory nerve, would provide a much more complete understanding of how the auditory system of the plainfin midshipman develops. Of particular interest would be auditory nerve ganglion cell proliferation and dendritic arborization as well as patch clamping studies of individual saccular hair bundles and the auditory nerve to gain an understanding of how the saccule and auditory nerve connect and to determine the intrinsic tuning properties and channel dynamics of hair bundles and the auditory nerve. Such studies would also determine the structure function relationships of any observed ontogenetic changes in peripheral auditory structures.

5.4 Additional future directions

The previous sections have focused on questions raised from the results of my empirical studies discussed in previous chapters. In this section I briefly discuss broader avenues for potential future research.

5.4.1 Development of the central auditory pathway

Recent developments in molecular physiology have made it much easier to investigate the central auditory pathway in fishes. Immediate early genes (IEGs), such as Fos genes, are rapidly activated in response to cellular activity and involved in the transcription of other gene products. Recently, IEGs have been used as an indicator of cellular activity in the nervous system using immunohistochemistry to visualize IEG proteins. Using IEG activity after sound exposure it is possible to identify areas of the central nervous system involved in auditory processing. Once the potential differences in activity of various auditory nuclei have been identified, morphological analysis paired with physiology could identify the structural functional relationships between different cell types and processing that takes place in different auditory nuclei.

5.4.2 Development of the lateral line

The lateral line is known to respond to low frequency (< 200 Hz) sounds that overlap with the best hearing range of the plainfin midshipman. Additional research should investigate the morphological development and physiology of the lateral line to better understand the role of the lateral line in detecting acoustic stimuli.

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Curriculum Vitae

Education:

University of Washington, Seattle, WA
Ph.D. Animal Behavior, June 2013
Dissertation: Ontogeny of hearing in the plainfin midshipman, *Poricthys notatus*

Brigham Young University, Provo, UT
B.S. Zoology / minor in German August 2005

Research Interests:

By understanding the world around us through scientific investigation, we can become more effective and wise stewards of the earth. My primary research interests lie in the intersection between sensory neurophysiology, ecology and behavior; the interaction of neuroethology and the environment. All animals have specific problems to solve, and many animals use unique sensory adaptations or have learned unique behaviors to overcome those problems. More specifically, by using neuroscience techniques in a behavioral context, I hope to gain a greater understanding of the mechanisms that lead to specific behaviors in animals. Many of the mechanisms for the transduction and neural processing of sensory signals are conserved throughout all vertebrate groups, so by studying more ancestral vertebrates and using a comparative approach, we can gain a greater understanding of our own sensory processing and physiology. I feel that science is a collaborative effort and has the broadest impact when scientists with similar interests, but different backgrounds work together on common questions.

Research Experience:

Doctoral Candidate: Animal Behavior, University of Washington, Seattle, WA (Fall 2006- Present)
I am examining the ontogeny of hearing sensitivity in the plainfin midshipman using electrical physiology, neuroanatomical, and behavioral techniques. My research aims to determine at what stage of development the auditory system becomes functional, and to investigate the mechanism(s) responsible for ontogenetic plasticity affecting auditory sensitivity.
Advisor: Joseph Sisneros

Post-bachelorette Volunteer: Bimini Biological Field Station, Bahamas (Fall 2005)
Under the direct supervision of the field staff I helped collect tracking data on juvenile lemon sharks using ultrasonic telemetry and passive acoustic monitoring stations. I helped survey the marine life around the island using larval settlement traps, gillnetting, and long-lining. I learned how to implant and read RFID tags, operate and maintain ultrasonic tracking equipment, how to perform basic ecological field surveys, boat handling, and how to record morphometric data.
Advisors: Brian Franks, Samuel Gruber

Undergraduate Mentored Research: Brigham Young University, Provo, UT (Spring Semester 2005)
Working with a group of two other students while visiting the Oregon Institute of Marine Biology, we developed a series of experiments to learn about the basic ecology of the lined chiton, *Tonicella lineatta*. I learned how to analyze fecal pellets, perform two choice preference tests, perform mark recapture experiments, and I improved dissection skills learned in laboratory classes.
Advisor: Lee Braithwaite

Undergraduate Technician: BYU Paleontology Museum, Provo, UT (Fall 2003- Winter 2005)
I worked with a team of undergraduate and professional technicians to stabilize and prepare various dinosaur bones for use in research. One of the fossils I prepared lead to further research on dermestid beetle traces found on dinosaur bones. I learned how to prepare and stabilize a wide variety of fossils, identify important structures, catalogue and store prepped specimens, operate heavy equipment, and work with a team to accomplish large tasks.
Advisors: Kenneth Stadtman, Rodney Scheetz

Undergraduate Student Volunteer: Recombinant DNA Lab, Provo, UT (Winter Semester 2000)
I worked on a project to determine the genetic divergence between Yellowstone sub-species of cutthroat trout. Using fin clips, I learned protein digestion, DNA purification and extraction, I used PCR to amplify the DNA, and analyzed the resulting DNA fragments using gel electrophoresis.
Advisor: R. Paul Evans

Teaching Experience:
Teaching Assistant: duties included: operating audiovisual equipment, making copies, leading quiz sections, grading, leading review sessions, and write test questions.
Psych 101 Introduction to Psychology (Fall 2006)
Psych 202 Biopsychology (Spring 2007, Winter 2013)
Psych 300 Animal Behavior (Winter 2007)

TA Fellow: duties included: all normal duties of a teaching assistant, organize and lead a group of graduate and undergraduate teaching assistants for a large lecture class, schedule rooms for review sessions, act as the primary liaison between the instructor and support staff.
Psych 101 Introduction to Psychology (Spring 2012)

Primary Instructor: duties included: organizing the class, lecturing, developing all activities, assignments, and quizzes, assessing grades, and addressing all student concerns.
Psych 330 (Fall 2010-Winter 2012, Fall 2012, Spring 2013)

Mentoring Experience:
Kiel Shaub: Undergraduate volunteer, University of Washington (Spring 2008- Summer 2009)
I developed the analysis procedures and trained Kiel how to analyze video data for a NSF funded research project on sound source localization in plainfin midshipman. This project led to a publication in the Journal of the Acoustical Society of America.

Andrew Acob: Undergraduate volunteer, University of Washington (Summer 2009- Summer 2010)
I helped Andrew develop a research plan and complete a research project as part of a Howard Hughes scholarship on the behavior of type II "sneaker" male plainfin midshipman using playback experiments. Andrew presented the data from this project at the SACNAS National Conference and the University of Washington Undergraduate Research Symposium.

Research Publications:

Alderks, P.W. and Sisneros, J.A. (*in review*) Development of the acoustically evoked behavioral response in the plainfin midshipman.

Zeddies, D.G., Fay, R.R., Gray, M.D., **Alderks, P.W.**, Acob, A., and Sisneros, J.A. (2012) Local acoustic particle motion guides sound-source localization behavior in the plainfin midshipman fish, *Porichthys notatus*. J. Exp. Bio. 215:152-160.

Alderks, P.W. and Sisneros, J.A. (2011) Ontogeny of auditory saccular sensitivity in the plainfin midshipman fish, *Poricithys notatus*. J. Comp Physiol A. 197:387-398.

Zeddies, D.G., Fay, R.R., **Alderks, P.W.**, Shaub, K.S. and Sisneros, J.A. (2010) Sound source localization by the plainfin midshipman fish, *Porichthys notatus*. JASA 127:3104-3113.

Sisneros, J.A., **Alderks, P.W.**, Leon, K. and Sniffen, B. (2009) Morphometric changes associated with the reproductive cycle and behaviour of the intertidal-nesting, male plainfin midshipman *Porichthys notatus*. J. Fish Bio. 74:18-36.

Abstracts:

Alderks, **P.W.**, Coffin, A.B. and Sisneros, J.A. (2013) Ontogenetic changes in saccular morphology in the teleost fish *Porichthys notatus*. Abstract for poster presentation at 50 Years of Underwater Bioacoustics symposium, Mote Marine Laboratory, Sarasota FL.

Alderks, P.W. and Sisneros, J.A. (2011) Ontogeny of early audition in the plainfin midshipman, *Porichtys notatus*. (Invited speaker at ASA Conference, Seattle, WA.) J. Acoust. Soc. Am. 129: 2473.

Zeddies, D., Fay, R.R., **Alderks, P.W**., Gray, M.D., Coffin, A.B., Bandiwad, A., Mohr, R.A., Brown, A.D., Rogers, P. and Sisneros, J.A. (2011) Localization of monopole and dipole sound sources by midshipman fish (*Porichtys notatus*). (Abstract for talk at ASA Conference, Seattle, WA.) J. Acoust. Soc. Am. 129: 2474.

Zeddies, D., Fay, R.R., **Alderks, P.W.**, Acob, A. and Sisneros, J.A. (2010) Sound source localization of a dipole by the plainfin midshipman fish (*Porichthys notatus*). (Abstract for talk at ASA Conference, Baltimore, MD.) J. Acoust. Soc. Am. 127: 1886.

Acob, A., **Alderks, P.W.** and Sisneros, J.A. (2009) Auditory Physiology and Behavior of the "Sneaker" Male Plainfin Midshipman Fish (*Porichthys notatus*). Abstract for poster presentation at SACNAS National Conference, Dallas, TX.

Alderks, P.W. and Sisneros, J.A. (2009) Ontogeny of auditory saccular tuning in the plainfin midshipman fish, *Porichthys notatus*. Invited speaker at Acoustical Society of America Conference,

Portland, OR.

Zeddies, D., Fay, R.R., **Alderks, P.W.**, Shaub, K and Sisneros, J.A. (2009) Sound source localization by the plainfin midshipman fish, *Porichthys notatus*. (Abstract for talk at ASA Conference, Portland, OR.) J. Acoust. Soc. Am. 125: 2488.

Zeddies, D., Fay, R.R., **Alderks, P.W.**, Shaub, K. and Sisneros, J.A. (2008) Sound source localization by the plainfin midshipman fish (*Porichthys notatus*). Abstract for talk at Acoustical Society of America Conference, Miami, FL.

Alderks, P.W. and Sisneros, J.A. (2008) Ontogenetic retention of auditory saccular tuning in the vocal plainfin midshipman fish, *Porichthys notatus*. Abstract for poster presentation at Society for Neuroscience Conference, Washington, D. C.

Manuscripts in Prep:

Alderks, **P.W.**, Coffin, A.B. and Sisneros, J.A. (*in prep.*) Ontogenetic changes in saccular morphology in the teleost fish *Porichthys notatus*.

Alderks, P.W., Bandiwad, A., Mohr. R.A. and Sisneros, J.A. (*in prep.*) Temporal pattern of female size in the intertidally breeding plainfin midshipman fish, *Porichtys notatus*, during the breeding season.

Vasconcelos, R.O., Ramos, A., Fonseca, P.J., Sisneros, J.A., **Alderks, P.W**. and Amorim, M.C.P. (*in prep.*) Development of the auditory sense parallels vocal differentiation in a highly vocal fish, *Halobatrachus didactylus.*

Coffin, A.B., Zeddies, D.G., Fay, R.R., Brown, A.D., **Alderks, P.W.**, Bandiwad, A., Mohr, R., Gray, M., Rogers, P.H. and Sisneros, J.A. (*in prep.*) Use of the swim bladder and lateral line are not necessary for near-field sound source localization by fishes.

Professional Societies:

Society For Neuroscience

Animal Behavior Society

American Society of Ichthyologists and Herpetologists

Honors and Awards:

Travel award to present at 50 Years of Underwater Bioacoustics symposium.

Bolles fund award Spring 2013

Alcor Fellowship Summer 2011

Trainee, NIH Auditory Neuroscience Training Grant from July 2007- July 2010.

Brigham Young University Dean's Honor Roll, 2005

Office of Naval Research Scholarship for the best ocean related project at the International Science and Engineering Fair, administered by the American Academy of Sciences 1999

Finalist, Intel International Science and Engineering Fair 1997, 1998, & 1999

Eagle Scout 1994

References:

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