Ontogenetic & reproductive state-dependent changes to the auditory inner ear and swim bladder of the plainfin midshipman fish (*Porichthys notatus*)

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Abstract

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Fish have become an important model for studying hair cell plasticity and development. Unlike mammals, the inner ears of fish continue to grow and add hair cells throughout their lifespan, and fish can regenerate hair cells after inner ear damage. The plainfin midshipman fish (*Porichthys notatus*) is a useful model for investigating auditory plasticity because, in addition to these more widely studied contexts of hair cell plasticity, the midshipman auditory system undergoes changes associated with the reproductive state. In chapter 1, I summarize the multiple contexts of hair cell plasticity in fish and the secondary adaptive functions of the gas-filled swim bladder as an auditory and vocal structure. I then discuss the specific questions explored in this dissertation regarding developmental and reproductive state-dependent changes to the auditory inner ear and swim bladder using the plainfin midshipman as a model. In chapter 2, I provide evidence for a reproductive state-dependent change in hair cell density and a possible cellular mechanism in vocal type I male midshipman. In chapter 3, I describe changes in sensory epithelium area, hair cell density, and total hair cell quantity in the auditory inner ear throughout ontogeny in plainfin midshipman fish. In chapter 4, I discuss a change in the morphology of the swim bladder and its

proximity to the inner ear in type I males related to reproductive state, indicating a possible change in adaptive use as an auditory structure to a vocal organ. Lastly, in chapter 5, I discuss the implications of the findings in this dissertation and future directions for studying auditory-related questions in the plainfin midshipman fish.

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GLOSSARY

AEP = auditory evoked potential

BM = body mass

CT = computerized tomography

GSI = gonadosomatic index

HC = hair cell

K = body condition (Fulton's K)

SC = support cell

SL = standard length

SMSI = sonic muscle somatic index

TC = total apical saccular cells

PREFACE

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Chapter 3 has been prepared in publication format.

Chapter 4 has been prepared in publication format.

Please excuse any redundancies in the text due to the original data chapters being prepared in publication format.

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Chapter 1. Background and Significance

1.1 The auditory periphery of fish

It has been argued that the inertial mode of hearing in fish is the ancestral state in all vertebrates and that understanding how acoustic information is received and processed in the fish periphery will enhance our understanding of all vertebrate hearing (Fay and Popper, 2000). Fish auditory inner ears are composed of mass-loaded otolith organs that mechanically transduce particle motion of a sound wave in water as opposed to ears of terrestrial vertebrates that respond to sound pressure (for review see Popper and Fay, 1993; Popper and Lu, 2000). Many fishes are also capable of indirectly detecting sound pressure (see section 1.3). The auditory inner ears of teleosts (ray-finned fishes) are composed of three mass-loaded otolith organs referred to as the saccule, lagena, and utricle. There is evidence that these end organs have vestibular functions in addition to their role in the auditory system (Popper and Fay, 1993).

As in all vertebrates, the site of auditory transduction in fish occurs at the hair cell which make synaptic connections with ganglion cells in the eighth cranial nerve. Unlike the mammalian cochlea, where a ganglion afferent fiber makes synaptic contact with one inner hair cell, a single ganglion afferent cell makes connections with multiple hair cells in the auditory end organs of fish (Furukawa, 1978; Edds-Walton and Popper, 2000). In some teleosts, there is evidence for tonotopy in the saccule, with most of the evidence coming from experiments on goldfish (e.g., Furukawa and Ishii, 1967; Smith et al., 2011), though the primary mode of frequency encoding in fish is thought to be by way of a temporal code that is encoded by the phase locking of afferent fibers (Fay, 1978; Sisneros et al., 2004; for review see Fay, 1981).

The saccule, lagena, and utricle maculae are composed of hair cells and support cells. On the apical surface of the maculae there are populations of hair cells with stereocilia bundles oriented in the same plane of maximum excitation, called orientation groups. There are multiple orientation groups in sensory maculae of every species studied, though the amount varies by species. Usually for each orientation group there is an opposing orientation group with a plane of maximum excitation 180 degrees in the opposite direction (Popper, 1978; Popper, 1981; for review see Popper and Lu, 2000; Popper and Schilt, 2008). It is hypothesized that these differing orientations of hair cells play a role in sound source localization as there is physiological and anatomical evidence indicating that a class of afferent fibers innervate hair cells oriented in a particular direction and best respond to a particular phase and direction of otolith oscillations, though there are exceptions (Furukawa and Ishii, 1967; Furukawa, 1978; Sento and Furukawa, 1987). Also, there is evidence from auditory afferent recordings that the orientation of the end organs themselves have differing planes of maximum excitation (Fay, 1984). Thus, there is a large body of evidence indicating that fish, unlike terrestrial vertebrates, possess a spatial filter for a sound direction in the auditory periphery.

1.2 Plasticity and development of the auditory periphery in fishes.

Fish are perhaps one of the most robust vertebrate models for studying plasticity and development of the auditory periphery because plasticity occurs in a variety of contexts and development of the auditory inner ear continues from early development into the adult life history stage. Due to the nature of the original research presented in this dissertation project, I will focus almost exclusively on hair cell plasticity and development in this section. The differing contexts of hair cell proliferation in fish include hair cell addition due to continued growth of the inner ear end organs throughout life (Corwin, 1981; Popper and Hoxter, 1985;

Lombarte and Popper, 1994), regeneration of hair cells following inner ear or lateral line insult (for review see Monroe et al., 2015), and the more recently discovered hair cell density changes dependent on reproductive state in the adult life history stage (Coffin et al., 2012). Studying plasticity and development of the inner ear in all three of these contexts in fish may yield an understanding of the evolutionary origins of hair cell and synaptic development and plasticity in the auditory periphery of all vertebrates, including mammals.

1.2.1 Hair cell addition throughout ontogeny

In all fish species investigated to date, the sensory epithelia of putative auditory end organs continue to grow throughout early development and into the adult life history stage, and hair cells are continuously added as the macula grows (Corwin, 1981; Corwin, 1983; Popper and Hoxter, 1984; Lombarte and Popper, 1994; Chaves et al., 2016). This generally results in a reduction in hair cell density but an overall increase in total hair cell numbers throughout ontogeny (i.e., hair cells are added at a slower rate than overall macula growth) (Popper and Hoxter, 1984; Lombarte and Popper, 1994; Lombarte and Popper, 2004; Chaves et al., 2016; but see Lu and DeSmidt 2013, and Wang et al., 2015). Afferent ganglion cells are added at a much slower rate, and there is conflicting evidence in how these changes affect auditory sensitivity (Corwin, 1983; Popper and Hoxter, 1984). In a study in developing zebrafish (*Danio rerio*) Higgs et al. (2002) found that there was no change in auditory evoked potential (AEP) thresholds throughout development despite an increase in total saccular hair cell quantity. However, a more recent study in zebrafish found that AEP sensitivity correlated with hair cell density throughout ontogeny, and the developmental stage with the greatest density had the lowest AEP thresholds (Wang et al., 2015). For a more detailed description of the functional changes related to hair cell additon throughout development see chapter 3.

1.2.2 Hair cell damage and regeneration

Fish can regenerate hair cells following inner ear trauma introduced by ototoxins such as antibiotics or heavy metals and exposure to loud noises. Studies in fish and other non-mammalian vertebrates (i.e., birds and amphibians) have discovered two possible routes of hair cell regeneration, referred to as direct transdifferentiation and mitotic replacement. In direct transdifferentiation, support cells differentiate into hair cells independent of mitosis, while in mitotic replacement support cell division is required prior to differentiation into hair cells (for reviews see Oesterle and Stone, 2008; Brignull et al., 2009; Monroe et al., 2015). Due to their genetic tractability, short generation times, and transparency in early development, larval zebrafish are the primary fish model of hair cell death and regeneration and have given important insight into the underlying cellular mechanisms of both (Brignull et al., 2009; Monroe et al., 2015). In a study of the zebrafish lateral line it was found that there is a spike in support cell division following excessive hair cell death, indicating that division of support cells is important for hair cell regeneration. It is important to note, however, that hair cell regeneration due to normal hair cell turnover (i.e., independent of injury) may be due to transdifferentiation (Hernandez et al., 2007). In contrast, in the zebrafish saccule, early regeneration of hair cells following laser ablation (within 24 hours-post injury) occurs via direct transdifferentiation of saccular support cells (Millimaki et al., 2010).

1.2.3 Reproductive state-dependent plasticity

Fish have become a useful model for studying reproductive state-dependent plasticity of the auditory system to increase sensitivity for enhanced detection of potential mates. An early example was found in the plainfin midshipman fish (*Porichthys notatus*) where physiological recordings of the saccular afferents (the fibers in the auditory nerve branch leading from the

saccule, the primary auditory end organ in teleosts) of reproductive females were better phase-locked to frequencies contained in the male's courtship signal (Sisneros and Bass, 2003). Later, it was found that this seasonal "tuning" of saccular afferents is mediated by gonadal sex hormones (testosterone and estradiol) as nonreproductive females implanted with these hormones had tuning curves similar to reproductive females (Sisneros et al., 2004). Later, it was found that reproductive female midshipman have increased sensitivity at the level of the hair cells in the saccule, as reproductive females had roughly 10 dB lower thresholds of evoked hair cell potentials at all tested frequencies (65-385 Hz) (Sisneros, 2009). This increased sensitivity at the level of the hair cells was later found to be related to a reproductive state-dependent increase in hair cell density independent of fish size (Coffin et al., 2012). More on the plainfin midshipman fish as a model for auditory plasticity in the context of reproductive state and communication is discussed in section 1.4.

1.3 Gas-filled swim bladder in fish

Up to this point, "direct stimulation" of the inner otoliths due to particle motion of a sound in fish has primarily been discussed, though many species can detect the pressure component of the sound wave via "indirect stimulation" from local particle motion produced by their gas-filled swim bladder. The swim bladder has multiple adaptive functions in fish including buoyancy regulation, respiration, aiding in sound detection, and sound production (Braun and Grande, 2008). The swim bladder has evolved as an organ for producing vocalizations in many species as well (for review see Popper and Fay, 1993; Popper and Lu, 2000; Braun and Grande, 2008).

1.3.1 Swim bladder as an accessory auditory structure

In many species, morphological adaptations increase the proximity of the swim bladder to the inner ear if there are strong selective pressures to increase the detection of sound pressure. Some species, known as otophysans, have evolved a Weberian apparatus which is a series of ossicles that mechanically couple the swim bladder to the inner ear. Clupeioformes (herring and anchovies), evolved otic bullae, or a gas-filled sac connected by rostral prominences of the swim bladder which resonate at high frequencies and increase the detectable frequency band in these species (Braun and Grande, 2008; Fine and Parmentier, 2015). Lastly, other species, including the plainfin midshipman fish, have evolved hornlike rostral extensions of the swim bladder which increase sensitivity of evoked hair cell responses in the saccule (Mohr et al., 2017; Colleye et al., 2019).

1.3.2 Swim bladder as a vocal organ

Many species vocalize by contracting their sonic muscles which attach either intrinsically or extrinsically to the swim bladder. In some species, the swim bladder is sexually dimorphic, and this difference often corresponds with vocal behavior (Courtenay, 1971; Fine et al., 1990; Mohr et al., 2017). In the batrachoididae family (toadfish and midshipman species), swim bladder-mediated vocalizations are especially important for courtship vocalizations of males (Bass and Marchaterre, 1989; Fine et al., 1990). This has been studied in detail in the oyster toadfish (*Opsanus tau*) and plainfin midshipman (*Porichthys notatus*) where males produce advertisement signals called “boatwhistles” or “hums”, respectively, by contracting their sonic muscles which intrinsically attach to the swim bladder (Fine et al., 1990; Bass and Baker, 1990). Both oyster toadfish and plainfin midshipman fish have sexually dimorphic sonic muscles and the vocal males have extremely large sonic muscles compared to females (Bass and Marchaterre, 1989; Fine et al., 1990).

1.4 Plainfin midshipman as a model for ontogenetic growth and reproductive state auditory plasticity

The plainfin mishipman fish (*Porichthys notatus*) has become a valuable model for investigating reproductive state and ontogeny-related changes to the peripheral auditory system and accessory auditory and vocal structures because production and reception of acoustic signals is important for reproductive success (for review see Forlano et al., 2015). Midshipman, like many other animals, reproduce at a specific time of year. In midshipman this occurs during the late spring and summer months, roughly April through July each year. In preparation for the breeding season, males and females undergo morphological changes as they prepare to spawn including gonadal recrudescence in both sexes (Brantley et al., 1993). There are two male reproductive morphs that utilize alternative reproductive tactics referred to as type I and type II males. Type I males are territorial and vocalize by contracting their sonic muscles which directly attach to the swim bladder wall to recruit gravid females to their nest in order to spawn. Type II males do not vocalize to attract females and instead steal fertilizations from type I males by satellite spawning (Brantley and Bass, 1994; Forlano et al., 2015). Sonic muscles in type I males hypertrophy in preparation for the mating season and then atrophy at its end (Sisneros et al., 2009). After spawning, females leave the nests and type I males do all parental care until larvae develop into free-swimming juveniles and leave the nest (Forlano et al., 2015).

Midshipman have a sexual dimorphism in swim bladder morphology where reproductive females and type II males have horn-like rostral extensions, and reproductive type I males lack the horn-like rostral swim bladder extensions (Mohr et al., 2017). The lack of rostral extensions in type I males increases the distance to the inner ear and likely prevents stimulation of the otoliths, which

may be an adaptation to prevent stimulation of their own inner ears when vocalizing (Mohr et al., 2017).

As briefly discussed in section 1.2.3, midshipman females have been found to undergo a reproductive state-dependent tuning at the level of the afferent fibers which likely increases their chances of finding vocal reproductive type I males (Sisneros and Bass, 2003) for which they have a strong motivation to localize when full of mature eggs (McKibben and Bass, 1998). This physiological sensitivity was found to be mediated by gonadal sex hormones as both T and E2-implanted nonreproductive females had similar tuning curves to reproductive females (Sisneros et al., 2004). Reproductive state-dependent sensitivity has been described in females and type I males at the level of the hair cells (Sisneros, 2009; Rohmann and Bass, 2011), and this was found to be due in part to increased hair cell density in females (Coffin et al., 2012).

Midshipman are known to behaviorally respond to sound at the larval stage when they are still adhered to the nest (Alderks and Sisneros, 2013). There has been some previous work investigating changes to the auditory inner ear in plainfin midshipman fish throughout ontogeny by physiological recordings at different levels of the peripheral processing pathway with some conflicting results. When they recorded from single afferent fibers, Sisneros and Bass (2005) found that sensitivity increased as fish grew (i.e., adults had the greatest sensitivity). In contrast, when they recorded hair cell evoked potentials in the saccule, Alderks and Sisneros (2011) found that there is no difference in evoked thresholds throughout ontogeny and that small juveniles had greater magnitude evoked potentials than large juveniles and adults.

1.5 Questions addressed in this work

The primary aim of my dissertation research was to investigate changes in the structure of the inner ear and accessory auditory organs depending on reproductive state and throughout development related to auditory sensitivity. To answer these questions, I used confocal microscopy and computerized tomography (CT) scanning using wild-caught plainfin midshipman fish. Specifically, the primary questions I was interested in answering were:

1.) Does hair cell density increase in the auditory end organs of reproductive type I male plainfin midshipman? Rationale: Increased hair cell density correlates with physiological sensitivity at the level of the hair cells in females (Coffin et al., 2012). Reproductive type I males also have increased physiological sensitivity at the level of hair cells, but whether there is a change in hair cell density is unknown.

2.) How does hair cell density, number, and orientation of maximum depolarization change in the saccule throughout ontogeny? Rationale: Physiology has been investigated in the inner ear of plainfin midshipman throughout ontogeny (Bass and Sisneros, 2005; Alderks and Sisneros, 2011), but the structure of the inner ear has not been described. By investigating these features, I can more clearly elucidate how the auditory system functions from early development into the adult life stage

3.) Does the morphology of the swim bladder change in reproductive type I male midshipman to increase the distance to the inner ear, possibly affecting the function of the swim bladder? Rationale: Swim bladders are known to aid in auditory reception in female plainfin midshipman (Colleye et al., 2019), but whether this occurs in type I males is unknown. In the summer breeding season, type I males have large sonic muscles which may increase the distance of the swim bladder to the inner ear by pushing the rostral swim bladder extensions medially. In the winter, sonic muscle atrophies (Sisneros et al., 2004) which may lead to shorter distances of the

swim bladder to the inner ear and increase auditory sensitivity. Making morphological measurements are an important first step in determining whether there are changes that affect the function of the swim bladder.

More detailed explanations for the rationale of each question are discussed in the introductions of chapters 2, 3, and 4.

1.5.1 Does hair cell density increase in auditory end organs of reproductive type I male plainfin midshipman fish?

To answer this question, I stained sensory epithelia of the three auditory end organs (saccule, utricle, and lagena) with phalloidin, which binds specifically to f-actin, and analyzed hair cell stereocilia bundle density in discrete regions in reproductive vs. nonreproductive type I males. The phalloidin was conjugated to rhodamine and slides were imaged on a fluorescent confocal microscope. The results of this study are discussed in chapter 2.

1.5.2 How does hair cell density, overall hair cell quantity, macula shape, and hair cell orientation change in the saccule throughout ontogeny?

Again, using rhodamine-conjugated phalloidin, I stained the saccular macula throughout development in the plainfin midshipman starting at the larval stage (still feeding on yolk) up to the adult stage of development. I imaged maculae on a fluorescent confocal microscope, and compared hair cell density, overall estimated hair cell quantity, saccular macula shape, and macula size between the developmental stages. The results of this study are described in chapter 3.

1.5.3 Does the morphology of the swim bladder change in type I males depending on reproductive state?

I imaged intact swim bladders of reproductive and nonreproductive type I males using computerized tomography (CT) scanning. Using the 3D rendering software Drishti, I measured the shortest distance of the swim bladders to the inner ear otoliths and also made morphometric measurements of the swim bladders themselves. These results are discussed in chapter 4.

The following original data chapters discuss reproductive state-dependent changes in hair cell density and swim bladder morphology in adult type I male plainfin midshipman. I also describe changes of the inner ear saccule in males and females related to ontogeny. The implications of these findings and future directions are discussed in chapter 5.

Chapter 2. Reproductive-state dependent changes in saccular hair cell density of the vocal male plainfin midshipman fish

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2.1 Summary

The plainfin midshipman fish (*Porichthys notatus*) is a nocturnal, seasonally breeding, intertidal-nesting teleost fish that produces social acoustic signals for intraspecific communication. Type I or "nesting" males produce agonistic and reproductive-related acoustic signals including a multiharmonic advertisement call during the summer breeding season. Previous work showed that type I male auditory sensitivity of the saccule, the primary midshipman auditory end organ, changes seasonally with reproductive state such that reproductive males become more sensitive and better suited than nonreproductive males to detect the dominant frequencies contained within type I vocalizations. Here, we examine whether reproductive type I males also exhibit reproductive-state dependent changes in hair cell (HC) density in the three putative auditory end organs (saccule, lagena, and utricle). We show that saccular HC density was greater in reproductive type I males compared to nonreproductive type I males, and that the increase in HC density occurs throughout the saccular epithelium in both the central and marginal epithelia regions. We also show as saccular HC density increases there is a concurrent decrease in saccular support cell (SC) density in reproductive type I males with no overall change in total cell density (i.e., HC + SC). In contrast, we did not observe any seasonal changes in HC density in the utricle or lagena between nonreproductive and reproductive type I males. In addition, we compare the

saccular HC densities in reproductive type I males with that of reproductive females and show that females have greater saccular HC densities, which suggest a sexually dimorphic difference in HC receptor density between the two sexual phenotypes, at least during the summer breeding season.

2.2 Introduction

Auditory sensory epithelia in mammals stop growing after early postnatal development and the hair cells (HCs) that compose the mammalian auditory epithelia lack the capacity to regenerate after cell damage or death (for review see Brignull et al., 2009). In contrast, auditory epithelia in fishes continue to grow beyond postembryonic development and into the adult life history stage (Corwin, 1981; Popper and Hoxter, 1984; Lombarte and Popper, 1994), and the HCs in adult fish are capable of regenerating after injury or death (Lombarte et al., 1993; Millimaki et al., 2010; for review see Warchol, 2011; Smith and Monroe, 2016). Previous fish postembryonic studies have demonstrated that HC addition can occur at the edges or in the "marginal zones" of sensory epithelia in elasmobranch fishes (Corwin, 1981; Corwin, 1983) and throughout the entire sensory epithelia including inner "central zones" at some distance away from the edges of sensory epithelia in teleost fishes (Popper and Hoxter, 1990; Lanford et al., 1996). Postembryonic HC addition is also known to occur in all three of the putative auditory end organs (saccule, lagena, and utricle) in teleosts throughout ontogeny and into the adult life history stage (Lombarte and Popper, 1994; Chaves et al., 2017).

A novel form of HC addition that is posited to be adaptive for the reception of social acoustic signals was previously reported for the plainfin midshipman fish (*Porichthys notatus*) (Coffin et al., 2012). Female midshipman were found to exhibit a seasonal change in HC addition, measured as an increase in HC density, that was specific to the saccule, the main

auditory end organ in midshipman and most other teleost fishes, while no seasonal changes in HC densities were found in the lagena or utricle. The seasonal increase in saccular HC density was concurrent with seasonal changes in auditory saccular HC sensitivity such that reproductive females were 2.5 to 5.5 times (~8 to 15 dB re 1 µPa) more sensitive than nonreproductive females across a broad range of frequencies (Coffin et al., 2012). This increased auditory saccular sensitivity in females is thought to be adaptive for the enhanced detection and localization of conspecific mates during the breeding season. Although such changes in saccular HC sensitivity were first reported for female midshipman, seasonal plasticity of saccular HC sensitivity has also been reported in the nesting, vocal "type I" male midshipman, which produces multiharmonic advertisement calls to attract females for spawning during the breeding season (Rohmann and Bass, 2011). Whether seasonal changes in saccular HC sensitivity in type I males are related to seasonal changes in saccular HC density remain unknown.

The primary aim of this study was to determine if vocal type I male midshipman exhibit seasonal, reproductive-state dependent changes in HC density in the putative auditory end organs (saccule, lagena, and utricle) of the midshipman inner ear. We examine whether seasonal changes in HC density only occurs in the saccule as previously reported in females (Coffin et al., 2012). A secondary aim of the study was to determine whether the density of support cells (SCs) in the saccular epithelium changes seasonally with reproductive state in type I males. We also examine and compare saccular HC density in summer reproductive females with that of summer reproductive type I males to determine whether there are sexually dimorphic differences in the density of auditory receptors between type I males and females during the breeding season. We compare our findings of HC density changes in type I males with that previously reported for

females (Coffin et al., 2012) and interpret our results as they relate to possible adaptations for the reception of social acoustic signals during the breeding season.

2.3 Materials and Methods

2.3.1 Tissue collection

2.3.1.1 Animal collection/care: We collected winter, nonreproductive type I male midshipman via otter trawl in Monterey Bay near Moss Landing, CA in February 2018 (R/V John H. Martin, Moss Landing Marine Laboratories) at depths from 85-100 m. We hand-collected summer reproductive type I males from their nests at low tide in Tomales Bay near Marshall, CA in May 2018. We also hand-collected reproductive females from male nests at low tide in the Hood Canal near Brinnon, WA in June/July 2018. After collection, we relocated all fish to 50-gallon recirculating saltwater tanks at the University of Washington with a 12/12-hour light/dark cycle for reproductive fish collected during the summer and 8/16-hour light/dark cycle for nonreproductive fish collected during the winter. We fed animals de-shelled shrimp every 3-4 days and held them for 2 weeks or less before tissue collection. We recorded morphometrics, including standard length (SL,the length of the rostral-most point of the head to the caudal-most point of the tail, excluding the caudal fin), body mass (BM), body condition (Fulton's K, calculated by 100 x the quotient of BM divided by SL^3), and gonadosomatic index (GSI, calculated by 100 x the quotient of wet gonad mass divided by BM minus gonad mass) for all animals.

2.3.1.2. Tissue collection: Prior to sacrifice and tissue collection, we anesthetized midshipman in 10% benzocaine saltwater bath. Once fish were fully anesthetized, we transcardially perfused them with teleost ringer solution followed by fixation with 4% paraformaldehyde dissolved in

0.1 M phosphate buffer (PB). We immediately extracted the brains and intact inner ears separately following dorsal craniotomy and post-fixed the collected tissue in 4% paraformaldehyde in 0.1 M PB for 1 hour. We then washed tissue with 0.1 M PB and decoupled saccule, utricle, and lagena sensory epithelia from the otoliths by cutting around the end organ capsule and removing the otoliths and otolith membrane with forceps. We stored the auditory sensory epithelia in 0.1 M PB with 0.3% sodium azide for up to 6 months at +4°C prior to phalloidin staining. All animal care and tissue collection procedures were approved by University of Washington Institutional Care and Use Committee.

2.3.2 Morphology

2.3.2.1 Phalloidin staining: We washed saccule, utricle, and lagena sensory epithelia twice with 0.1M phosphate buffered saline (PBS) and incubated them for 1 hour with phalloidin conjugated to rhodamine (Thermo Fisher Scientific Invitrogen, cat. no. R415) diluted 1:40 in 0.1 M PBS. We washed epithelia again with 0.1 M PBS and then placed them on coverslips (oriented with apical sides against the coverslip) and whole mounted them in Fluoromount-G mounting media (Southern Biotech, cat. no. 0100-01) onto slides. We sealed slides with nail polish and stored them at +4° C in the dark until fluorescence imaging.

2.3.2.2. Hair cell imaging: The apical surface of HCs are highly reactive to phalloidin because HC stereocilia and the cuticular plate contain a high density of F-actin to which phalloidin binds. We imaged all epithelia on a Leica SP5 inverted confocal microscope (Leica Microsystems, Buffalo Grove, IL) with excitation/emission spectra set at 560/570-620nm. We imaged stereocilia bundles in z-series using a 40 x 1.25 NA oil immersion objective in 7 discrete saccular regions (see Fig. 2.1, top left panel) and 3 discrete utricle and lagena regions (n = 11-13) (see Fig. 2.2) similar to those described previously by Coffin et al. (2012). These auditory

epithelia regions were selected to include both marginal and central regions in both rostral/caudal and dorsal/ventral areas of the sensory epithelia. We captured z-series in steps of 0.5 um via Leica Application Suite Advanced Fluorescence software (Leica Microsystems).

2.3.2.3 Support cell imaging: SCs contain a circumferential belt of F-actin close to the apical surface which binds phalloidin. Because this F-actin belt is located near the apical surface, we could image apical SCs in conjunction with HCs and defined SCs as cell outlines lacking stereocilia. We counted SCs and HCs in region 2 (a central region) and region 3 (a marginal region) (see Fig. 2.1, top left panel) in the saccules of nonreproductive (n = 8) and reproductive (n = 8) type I males using 63 x 1.4 NA oil immersion objective. Sample sizes were slightly smaller (n = 8) in SC/HC counts at this higher magnification compared to HC-only counts (n = 11-13) because HC stereocilia obscured the ability to visualize SCs in some of the samples which we did not attempt to analyze.

2.3.2.4 Hair cell/support cell quantification: We quantified all HCs and SCs using Image-J software. To quantify HCs, we stacked z-series for each region into a single image and total HCs were counted in a 100µm X 100µm square area. We defined a HC as a separate stereocilia bundle. We included every bundle that was partially contained within each 100µm X 100µm square area in our analysis. Because we imaged SCs at a higher magnification than for HC-only counts, these counts were made in a 50µm X 50µm square area. SCs that were only partially represented in each 50µm X 50µm square area we also included in all analyses. In a separate analysis, we also counted HCs within each 50µm X 50µm square area to determine the spatial relationships between SC and HC densities and to better understand the relative densities of these two cell types at the apical layer.

2.3.2.5 Statistical analyses: To eliminate the possibility that morphometrics such as SL, BM, and K were a confound in this study we analyzed potential differences by conducting independent samples t-tests for each morphometric between reproductive and nonreproductive type I males and then between reproductive type I males and females separately. We also used an independent samples t-test to determine if GSI differed between reproductive and nonreproductive type I males. We analyzed differences in HC density between reproductive and nonreproductive type I males in separate two-way ANOVAs for each end organ with reproductive condition and end organ epithelial region as the independent variables and then conducted separate *a priori* t-tests to examine differences in each of the 7 saccular regions between reproductive and nonreproductive type I males (i.e., we only compared region 1 of nonreproductive males with region 1 of reproductive males and region 2 of nonreproductive males with region 2 of reproductive males, etc). We used the same statistical methods to compare saccular HC density between reproductive females and reproductive type I males. Because the three largest reproductive type I males exceeded the size range of reproductive females (see Fig. 2.5, 2.6), we excluded the three largest males from the ANOVA and *a priori* t-tests comparing HC density in type I males vs. females. We took this conservative approach to ensure there was no confounding effect of SL on HC density. Using counts from the 50µm x 50µm square areas in saccular regions 2 and 3, we analyzed differences in SC density, HC density, and total apical cell density (HCs + SCs) in 3 separate two-way ANOVAs across reproductive and nonreproductive type I males . Lastly, we ran 2 separate multiple regression analyses with SL and sexual phenotype (reproductive females vs. reproductive type I males, and reproductive vs. nonreproductive type I males) as predictor variables and HC density as the criterion variable.

2.3.2.6. Saccule area measurements: To estimate HC quantities in type I males and females, we imaged whole saccules at 10 x 0.4 NA dry objective and merged in Adobe Photoshop. Whole saccule area was measured in Image-J. We then measured the area of the central zone (delineated visually as the area surrounded by the more stereocilia-dense outer margin) and calculated the area of the marginal zone for each individual midshipman by subtracting the central zone from the whole saccule area. We estimated total number of HCs in individual saccules using the following equation described by Popper and Hoxter (1984):

$$\textit{Total HCs} = (\sum HC_c)(A_c/\sum A_c \textit{ regions}) + (\sum HC_m)(A_m/\sum A_m \textit{ regions})$$, where

$\sum HC_c$ **and** $\sum HC_m$ **=** the sum of HC bundles counted in all central and marginal regions,

A_c **and** A_m **=** the total area of the central and marginal zones measured in Image-J (in μm^2), and

$\sum A_c$ ***regions*** **and** $\sum A_m$ ***regions*** **=** the summed area (in μm^2) of the central and marginal regions operationally defined as the 100µm X 100µm square areas allotted for each region in Image-J (i.e., 30,000 μm^2 and 40,000 μm^2, respectively).

We then plotted total estimated HCs relative to SL to visualize HC quantities as a function of type I male and female size.

2.4 Results

2.4.1 Morphometrics:

We sampled a total of 36 adult plainfin midshipman: 13 reproductive type I males with a size range of 11.2 - 26.0 cm standard length (SL) (mean SL = 16.1 ± 5.5 cm SD, mean body mass (BM) = 79.2 ± 109.2 g SD, mean body condition (Fulton's K) = 1.2 ± 0.3 SD, and mean

gonadosomatic index (GSI) = 2.2 ± 1.5 SD), 12 nonreproductive type I males with a size range of 10.4-30.5 cm SL (mean SL = 13.0 ± 6.2 cm SD, mean BM = 61.7 ± 80.3 g SD, mean K = 1.1 ± 0.2 SD, and mean GSI = 0.5 ± 1.7 SD), and 11 reproductive females with a size range of 10.0 – 20.0 cm SL (mean SL = 14.3 ± 2.8 cm SD, mean BM = 36.8 ± 24.3 g SD, mean K = 1.1 ± SD, and mean GSI = 13.5 ± 8.6 SD). There was no difference in SL between nonreproductive and reproductive type I males (t-test, $t(23) = 0.12$, $p = 0.91$) or between reproductive type I males and reproductive females (t-test, $t(22) = 0.97$, $p = 0.34$). Reproductive and nonreproductive type I males did not differ in BM (t-test, $t(23) = 0.45$, $p = 0.66$) or K (t-test, $t(23) = 1.11$, $p = 0.27$). In addition, reproductive type I males and reproductive females did not differ in BM (t-test, $t(22) = 1.26$, $p = 0.22$) or K (t-test, $t(22) = 0.49$, $p = 0.63$). The GSI of type I males was larger in summer reproductive males than in winter nonreproductive males (t-test, $t(22) = 2.07$, $p < 0.05$). The timing of collection and the mean GSI of reproductive females sampled was comparable to those reported in previous studies (Coffin et al., 2012).

2.4.2 Differences in end organ hair cell densities between reproductive and nonreproductive type I males:

Bundle density was significantly greater in the saccules of reproductive type I males (mean HC density = 141.9 ± 28.9 SD/10,000 μm^2) compared to nonreproductive type I males (mean HC density = 119.9 ± 34.9 SD/10,000 μm^2) (two-way ANOVA, $F_{(1, 158)} = 37.5$, $p < 0.05$), with four of the seven saccule regions having HC densities that were greater in reproductive type I males than in nonreproductive type I males (*a priori* t-tests, $p < 0.05$). These were regions 3 and 6 (marginal epithelia zones) and regions 2 and 4 (central epithelia zones) (Fig. 2.1). We also found that HC density differed depending on saccule region (two-way ANOVA, $F_{(6, 158)} = 24.3$, $p < 0.05$). There were no differences in the HC densities between nonreproductive and reproductive

type I males sampled in the utricle (two-way ANOVA, $F_{(1, 41)} = 0.01$, $p = 0.92$) or in the lagena (two-way ANOVA, $F_{(1, 38)} = 1.1$, $p = 0.31$). HC density differed depending on the region in the utricle (two-way ANOVA, $F_{(2, 41)} = 19.7$, $p < 0.05$), but not in the lagena (two-way ANOVA, $F_{(2, 38)} = 3.2$, $p = 0.053$) (Fig. 2.2).

2.4.3 Differences in saccular support cell densities between reproductive and nonreproductive type I males:

Reproductive type I males had fewer apical SCs (mean SC density = 92.4 ± 14.6 SD/2,500 μm^2) compared to nonreproductive type I males (mean SC density = 103.8 ± 13.1 SD/2,500 μm^2) (two-way ANOVA, $F_{(1,26)} = 4.8$, $p < 0.05$). SC density did not differ in the central vs. marginal region (two-way ANOVA, $F_{(1, 26)} = 0.1$, $p = 0.78$) (Fig. 2.3). In order to compare the ratio of SCs to HCs, we examined in a separate analysis HC densities in the same 50µm x 50µm square areas and again found greater HC density in reproductive type I males (mean HC density = 39.4 ± 7.4 SD/2,500 μm^2) compared to nonreproductive type I males (mean HC density = 28.3 ± 12.4 SD/2,500 μm^2) (two-way ANOVA, $F_{(1,26)} = 26.7$, $p < 0.05$). Also, the analyzed marginal region (region 3, mean HC density = 38.5 ± SD/2,500 μm^2) had greater HC density compared to the central region (region 2, mean HC density = 30.1 ± SD/2,500 μm^2) (two-way ANOVA, $F_{(1, 26)} = 12.2$; $p < 0.05$), which is contrary to findings for SCs. Lastly, we analyzed differences in total saccule apical cells (calculated by adding HCs + apical SCs in the same 50µm x 50µm square areas) to determine whether there were seasonal changes in the total number of cells (HC+SC) and indirectly determine whether there was a seasonal change in the ratio of HCs to SCs. There was no significant difference in total apical cell density when comparing reproductive vs. nonreproductive type I males (two-way ANOVA, $F_{(1, 26)} = 0.003$, $p = 0.96$) and there was no difference in total apical cell density depending on region (two-way ANOVA, $F_{(1, 26)} = 2.2$, $p =$

0.15). In sum, our results indicate that the ratio of saccular HCs to apical SCs increases during the breeding season, but total apical cell density does not change (Fig. 2.3).

2.4.4 Differences in saccular hair cell density between reproductive type I males and females:

Reproductive females had significantly greater saccular HC density (mean HC density = 159.6 bundles ± 24.6 SD/10,000μm^2) compared to reproductive type I males (mean HC density = 141.9 bundles ± 28.9 SD/10,000μm^2), $F_{(1, 133)} = 20.9$, $p < 0.05$ (Fig. 2.4). After comparing each specific region across sex, we found that females had significantly greater HC density than type I males in regions 4 and 5, both central regions (*a priori* t-tests, $p < 0.05$) (Fig. 2.4). We again found that HC density differs by region, (two-way ANOVA, $F_{(6,133)} = 19.1$, $p < 0.05$).

2.4.5 Relationship between saccular hair cell density, reproductive condition, and size:

We ran separate multiple regression analyses with reproductive condition and SL as predictor variables to determine whether SL is a potential confound in the above comparisons. In the analysis for nonreproductive and reproductive type I males, reproductive condition significantly correlated with HC density ($F_{(2,25)} = 9.5$, $R^2 = 0.4$, $p < 0.0$; $B = -186.6$, $p < 0.05$) while SL did not ($B = -4.9$, $p = 0.23$). We found similar results in the multiple regression analysis between reproductive type I males and females ($F_{(2, 23)} = 6.4$, $R^2 = 0.3$, $p < 0.05$). Again, SL did not correlate with HC density ($B = -6.7$, $p = 0.15$), while reproductive condition did ($B = 56.0$, $p < 0.05$). Total HC density also did not correlate with size (SL) in any of the three reproductive conditions (i.e., reproductive type I males, reproductive females, and nonreproductive type I males) when analyzed in separate Pearson's correlations ($p > 0.05$) (Fig. 2.5).

Lastly, we found that estimated total saccular HC quantity increased as a function of SL in adult type I males of both reproductive conditions and adult females. The largest type I male (SL = 30.5 cm) had an estimated approximately 91,835 HCs while the smallest type I male (SL = 10.4 cm) had an estimated approximately 20,091 HCs (Fig. 2.6).

2.5 Discussion

The primary aim of this study was to determine if type I male midshipman exhibit seasonal and reproductive-state dependent changes in HC density in the three putative auditory end organs (saccule, lagena, and utricle). We show that saccular HC density was greater in reproductive type I males than in nonreproductive type I males, and that the increase in saccular HC density was concurrent with a decrease in saccular SC density with no overall change in total saccular cell density (i.e., HC + SC). In contrast, we did not observe any seasonal changes in HC density in the utricle or lagena between nonreproductive and reproductive type I males, similar to previous results found in female midshipman (Coffin et al., 2012). In addition, we also show that saccular HC density was greater in reproductive females compared to reproductive type I males, which suggests a sexually dimorphic difference in HC density between the two sexual phenotypes. In this discussion, we interpret our results as they relate to possible adaptations for the enhanced reception of acoustic signals during midshipman social behavior.

Our results indicate that HC density in the saccule is 1.18 times greater in reproductive type I males than in nonreproductive type I males. The increase in HC density in reproductive type I males occurred in four out of the seven epithelial regions sampled from the saccules in nonreproductive and reproductive type I males. This seasonal increase in saccular HC density occurred throughout the sensory epithelia in both the central and marginal epithelial regions of the saccule. Consistent with these findings, Coffin et al. (2012) showed that reproductive females

exhibited a 13% increase in saccular HC density that occurred in both the central and marginal regions of the saccular epithelia. In addition, the seasonal increase in HC density of reproductive females was concurrent with an increase in the recorded magnitude of evoked saccular potentials and a corresponding decrease in saccular thresholds (i.e., increase in sensitivity) recorded from the rostral, middle and caudal regions of saccule. This HC density increase in the central and marginal regions of the saccular epithelia is thought to, in part, contribute to the overall seasonal increase in female saccular sensitivity (Coffin et al., 2012). A similar correlation of increased HC density with enhanced saccular sensitivity likely exists in type I males as Rohmann and Bass (2011) showed that type I males also exhibit seasonal increases in HC sensitivity across the saccular epithelia during the reproductive season. Other teleost fishes including the cichlid oscar (*Astronotus ocelatus*), the goldfish (*Carassius auratus*), the European hake (*Merluccius merluccius*), the zebrafish (*Danio rerio*), and the closely related oyster toadfish (*Opansus tau*) and the Lusitanian toadfish (*Halobatrachus didactylus*) are known to add HCs throughout the entire epithelium during development and into the adult life history stage (Sokolowski and Popper, 1987; Popper and Hoxter, 1990; Lombarte and Popper, 1994; Lanford et al., 1996; Higgs et al., 2002; Chaves et al., 2017). This HC addition and change in saccular HC density during development is likely one of the main mechanisms that contributes to ontogenetic increases in auditory sensitivity (Corwin, 1983; Kenyon, 1996; Vasconcelos et al., 2015; Wang et al., 2015). Future studies that investigate midshipman inner ear development will be instrumental in understanding how HC addition and changes in HC density affect midshipman auditory sensitivity during ontogeny and across the adult reproductive cycle.

We found saccular–specific HC density increases in reproductive type I males similar to those reported for reproductive female midshipman (Coffin et al., 2012). The 13% increase in

HC density in female midshipman occurred in 5 out of the 7 saccular epithelial regions sampled in reproductive females compared to nonreproductive females. In addition, Coffin et al. (2012) reported a negative relationship between saccular HC density and body size (SL) in both nonreproductive and reproductive females such that as SL increased HC density decreased. This negative relationship between body size and HC density has also been observed in other teleosts such as the oscar, the European hake and the Lusitanian toadfish (Popper and Hoxter, 1984; Lombarte and Popper, 1994; Chaves et al., 2017). Our results did not indicate a negative relationship between saccular HC density and SL in type I males or females, but such results were expected because we sampled individuals within a limited adult size range that were sexually mature. Because there were no differences in body size (SL) between nonreproductive and reproductive type I males ($p = 0.91$) nor between reproductive type I males and reproductive females ($p = 0.34$), we are confident that our reported results of saccular HC densities differences between nonreproductive and reproductive males and between reproductive males and females were attributed to seasonal and sexually dimorphic differences, respectively, rather than to ontogenetic differences in saccule HC density (i.e., related to age or body size).

One likely explanation for the reported negative relationship between HC density and body size reported in female midshipman and other teleosts is the likelihood that ontogenetic HC addition is probably slower relative to epithelial growth and expansion, which likely results in the reported decrease in HC density with body size (Popper and Hoxter, 1984; Lombarte and Popper, 1994; Lombarte and Popper, 2004; Chaves et al., 2017). In contrast, ontogenetic HC addition in zebrafish was reported to result in increased HC density with body size during ontogeny from 1.2 to 3.7 cm total length (Wang et al., 2015). Whether seasonal changes in HC density also occur in the saccule of type II male midshipman, a divergent male sexual phenotype

that uses an alternative reproductive strategy to 'sneak' or satellite spawn, remains unknown. Future studies that investigate the relative HC densities of the auditory end organs in type II males across their reproductive cycle will be informative in determining whether seasonal HC density increases in the midshipman inner ear are a conserved trait and utilized by the three midshipman sexual phenotypes (females and type I and II males) during the reproductive season.

Although HC density is often negatively correlated with body size, the total number of HCs within the auditory end organs is known to increase with body size due to continual sensory epithelia growth and development (Popper and Hoxter, 1984; Lombarte and Popper, 1994; Chaves et al., 2017). In our study, the total estimated mean number of saccular HCs for type I males and females with a body size of 20-23.5 cm SL was 43,086 HCs, which was similar to the closely related Lusitanian toadfish with a comparable body size of 21-23 cm SL that had an estimated mean saccular number of 31,616 HCs (Chaves et al., 2017). Likewise, the estimated number of saccular HCs in the European hake of a similar total length range was less than 100,000 (Lombarte and Popper, 1994). In contrast, the estimated mean number of saccular HCs in the oscar with a body size of 19 cm SL was 252,385 HCs (Popper and Hoxter, 1984) and was approximately 6 times greater than that in the midshipman. While the area of the saccular macula in the oscar is generally larger than in the midshipman (e.g., a 19 cm SL oscar had saccular area of 6,370,145 um^2 compared to a mean saccular area of 4,351,552 um^2 in type I male and female midshipman of 20-23.5 cm SL), the 1.5 fold difference in the macular area of the saccule between the two species likely does not alone account for such differences in estimated HC numbers between the species. Taken together, these species-specific differences in HC addition in teleost end organs emphasize the need for future studies to examine how inner ear sensory

morphology and development vary in teleost species with different ecologies and life history strategies.

The observed increase in saccular HC density in reproductive type I males was concurrent with a decrease in saccular SC density while total cell density (HC + SC) on the apical surface of the saccular epithelia did not differ from that in nonreproductive type I males. We observed an 11% decrease in SCs in reproductive compared to nonreproductive type I males which led to an increase in HC to SC ratio in reproductive type I males while total apical cell (HCs + SCs) density in the saccule did not change. The change in HC to SC ratio in reproductive type I males suggests that SCs may transdifferentiate into HCs in both the central and marginal regions of the saccule prior to the summer breeding season, perhaps during the spring pre-nesting season when midshipman gonads undergo seasonal recrudescence and development (Sisneros et al., 2004). Nuclei of HC precursors are known to migrate to the apical surface of the sensory epithelium prior to differentiating into HCs during mitotic cell division and during direct transdifferentiation of SCs into HCs in both fish and birds (Raphael, 1992; Oesterle and Stone, 2008; Brignull et al., 2009). Previous work in 6 month-1 year old oscars found that new HCs arise from an actively dividing population of neuroepithelial cells where nuclei migrate to the apical lumen prior to division and differentiation into HCs and SCs (Presson and Popper, 1990). Because we used the HC marker phalloidin, we were only able to quantify SCs on the apical surface of the saccular epithelium and could not determine the location of nuclei. Quantifying SC cell density in the apical layer of the saccular epithelium likely provides important information regarding the changes that occur in the saccular epithelium because SCs always maintain an apical process (Presson and Popper, 1990). However, SCs at the apical layer may be compressed and not properly detected with the resolution available using confocal microscopy. Future studies

that examine HC proliferation and cell death using specific SC and HC markers for mitotic cell division and cell death at all epithelium layers in conjunction with scanning electron microscopy will provide valuable insight into the mechanisms responsible for the reproductive-state dependent changes in saccular HC density in type I male and female midshipman.

We found that reproductive type I males had a significantly lower saccular HC density than reproductive females during the summer breeding season. These differences in saccular HC density between type I males and females would suggest that there might be differences in saccular auditory sensitivity between type I males and females during the midshipman breeding summer. However, previous studies that examined the auditory evoked potentials of HCs in the saccule of females (Sisneros, 2009; Coffin et al., 2012) and type I males (Rohmann and Bass, 2011) revealed no such differences in auditory sensitivity at the level of the HCs between type I males and females. Auditory physiology studies that compare the sensitivity of the saccular afferents and higher order neurons in the auditory CNS of reproductive type I males and females have yet to be performed. The observed higher saccular HC density in females may lead to a higher convergence ratio of saccular HCs to saccular afferents which could effectively increase auditory sensitivity at the level of the auditory afferents as previously shown by Corwin (1983) in the thornback ray (*Raja clavata*) where the HC to ganglion cell convergence ratio was shown to increase during ontogeny resulting in increased sensitivity of the auditory afferents. Future studies that examine and compare the afferent connectivity of the saccular HCs in reproductive type I males and females would provide needed insight into whether there are sexually dimorphic differences in midshipman HC afferent connectivity and auditory sensitivity during the breeding season.

The reproductive-state dependent increase in saccular HC density in type I males likely enhances auditory sensitivity during the breeding season and such an increase in auditory sensitivity could potentially be beneficial for the detection of social acoustic signals. Type I males are known to produce three types of social acoustic signals that include grunts, growls, and advertisement calls (Bass et al., 1999). Much of the life history and social behavior of male and female midshipman rely on the production and detection of social acoustic signals, especially during the summer breeding season. Thus, reproductive state-dependent changes in HC density in reproductive type I males and females may, in part, contribute to the enhanced detection of midshipman acoustic signals. Based on saccule potential recordings, Rohmann and Bass (2011) showed that reproductive type I males are more sensitive than nonreproductive males to frequencies from 145-385 Hz, which is in the range of frequencies that contain the dominant acoustic energy associated within each of the three known midshipman vocalizations (i.e, grunts, growls, and hums) (Rohmann and Bass, 2011). Thus, this reproductive-state dependent change in auditory sensitivity may enable reproductive type I males to better detect and assess other territorial males in order to avoid or invite physical interactions while choosing nest locations in the intertidal nesting environment. Whether reproductive-dependent changes in saccular HC density also occur in type II males, which likely rely on eavesdropping for the selection of cuckoldry sites for satellite or sneaking spawning, remains to be determined.

2.6 Figures

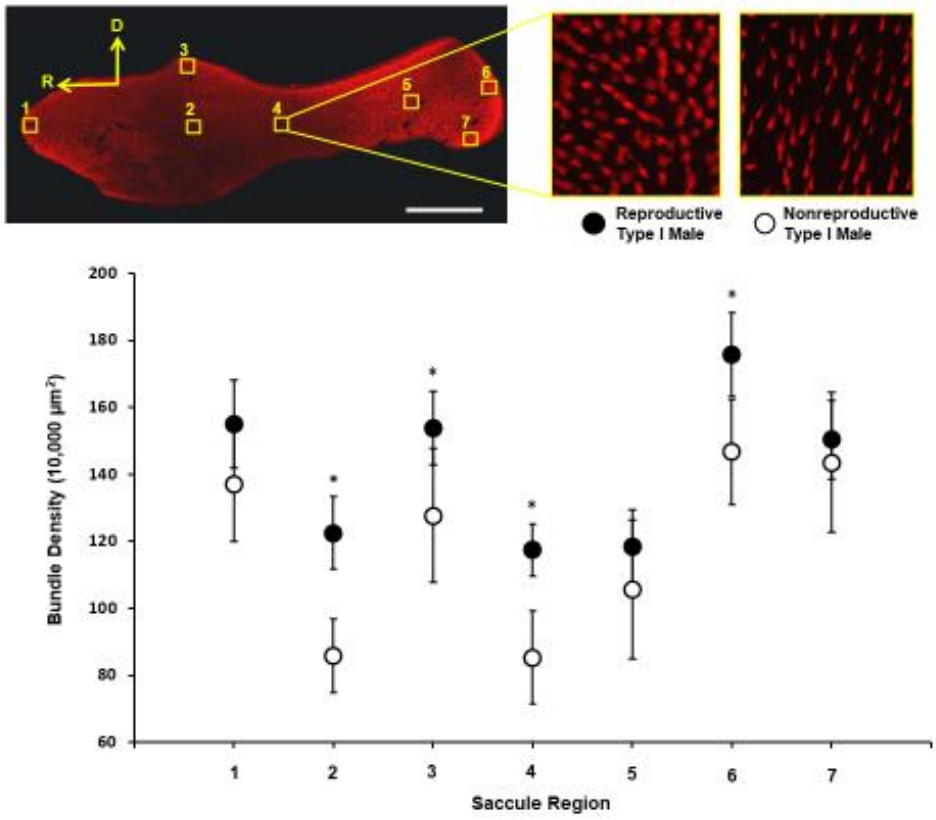

Figure 2.1. Saccular hair cell (HC) density in nonreproductive and reproductive type I males. Top panel, left: Hair cells were quantified in 4 marginal regions (1, 3, 6, and 7) and 3 central regions (2, 4, and 5). For HC counts, each region had a defined 100µm x 100µm square area and for SC counts, each region was a 50µm x 50µm square area. D = dorsal, R = rostral. Top panel, middle, right: A representative comparison of saccular HC densities in epithelia region 4 of the saccule between a reproductive type I male (middle) and a nonreproductive type I male (right). White scale bar = 500µm. Bottom panel: Bundle density (HC/10,000µm^2) was significantly greater in saccules of reproductive type I males compared to nonreproductive type I males. Data are plotted as means ±95% CI; * = $p < 0.05$. Filled symbols represent reproductive type I males while open symbols represent nonreproductive type I males.

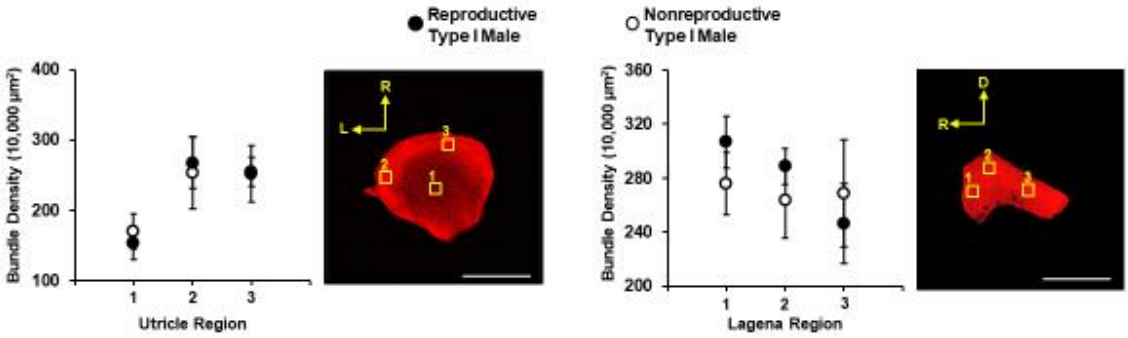

Figure 2.2. Utricular and lagenar hair cell (HC) density in nonreproductive and reproductive type I males. There was no difference in bundle density (HC/10,000µm^2) between reproductive and nonreproductive type I males in the utricle (left figure) or lagena (right). Data are plotted as means ±95% CI; $p > 0.05$. White scale bar = 500µm. Filled symbols represent reproductive type I males while open symbols represent nonreproductive type I males.

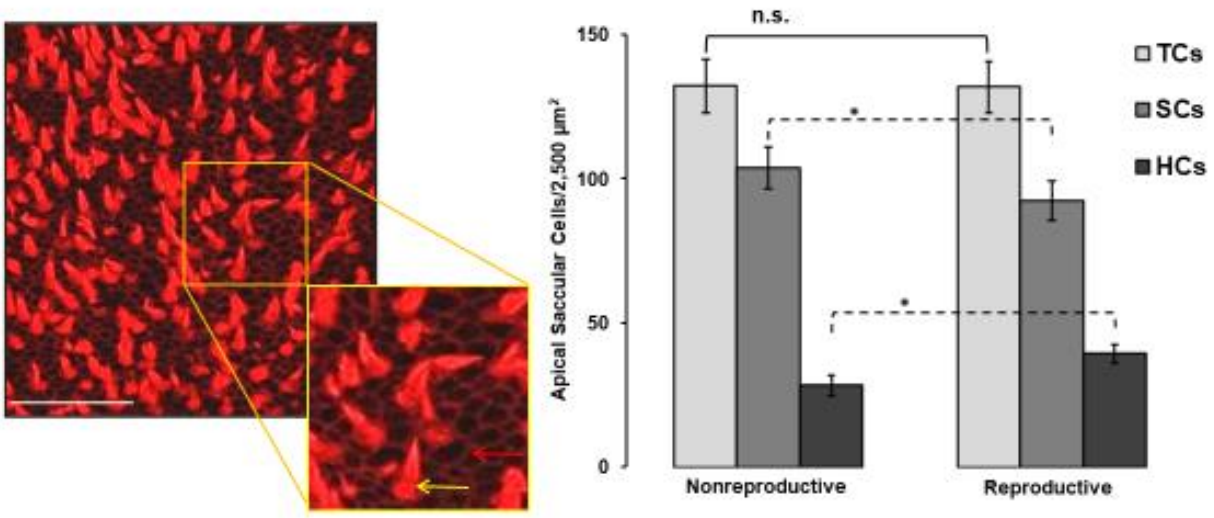

Figure 2.3. Saccular support cell (SC) density in nonreproductive and reproductive type I males**.** Left panel: SCs (red arrow, inset) were detectable in the same plane as HCs (yellow arrow) and were defined as cell outlines lacking stereocilia bundles. White scale bar = 50µm. Right panel: Data for the analyzed marginal region (region 3) and central region (region 2) were combined for the figure. There were differences in SC and HC density, but not for total cells (SC + HC) (cells/2,500µm^2). On average, reproductive type I male midshipman had greater HC density and lower SC density compared to nonreproductive type I males. Data are plotted as means ±95% CI; n.s. = not statistically significant ($p > 0.05$); * = $p < 0.05$. TCs = total cells (SC + HC), SCs = support cells, HCs = hair cells.

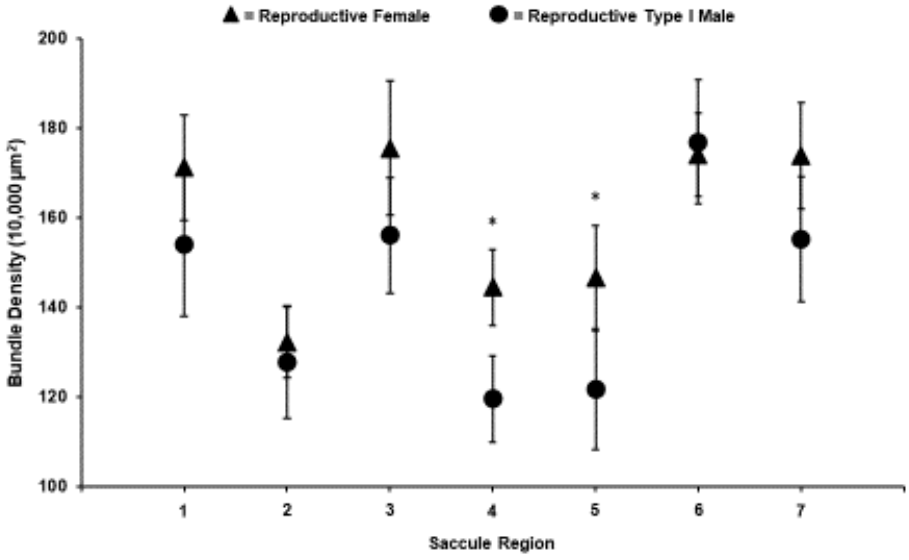

Figure 2.4. Saccular hair cell (HC) density in reproductive type I males and females. Bundle density (HC/10,000 μm^2) was greater in saccules of reproductive females compared to reproductive type I males. Data are plotted as means ±95% CI; * = $p < 0.05$.

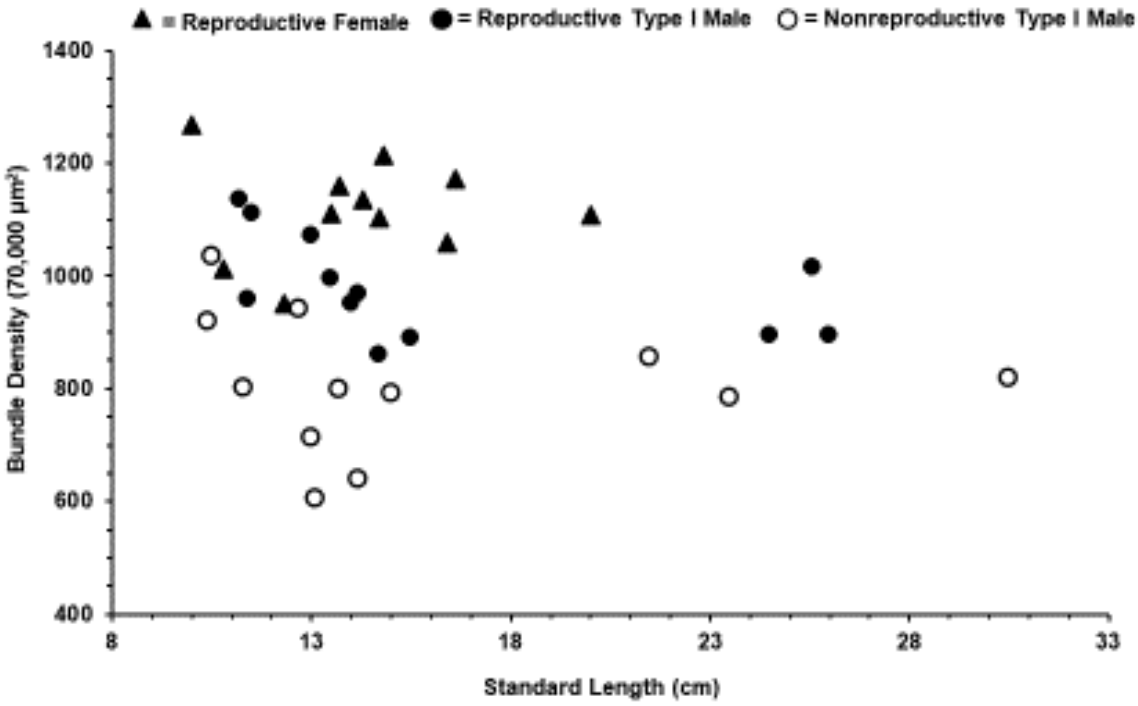

Figure 2.5. Saccular HC density plotted over standard length. HC density did not correlate with SL in nonreproductive type I males, reproductive type I males, or reproductive females; $p > .05$. Data are plotted as combined counts of all 7 regions in the saccule per individual within each phenotype.

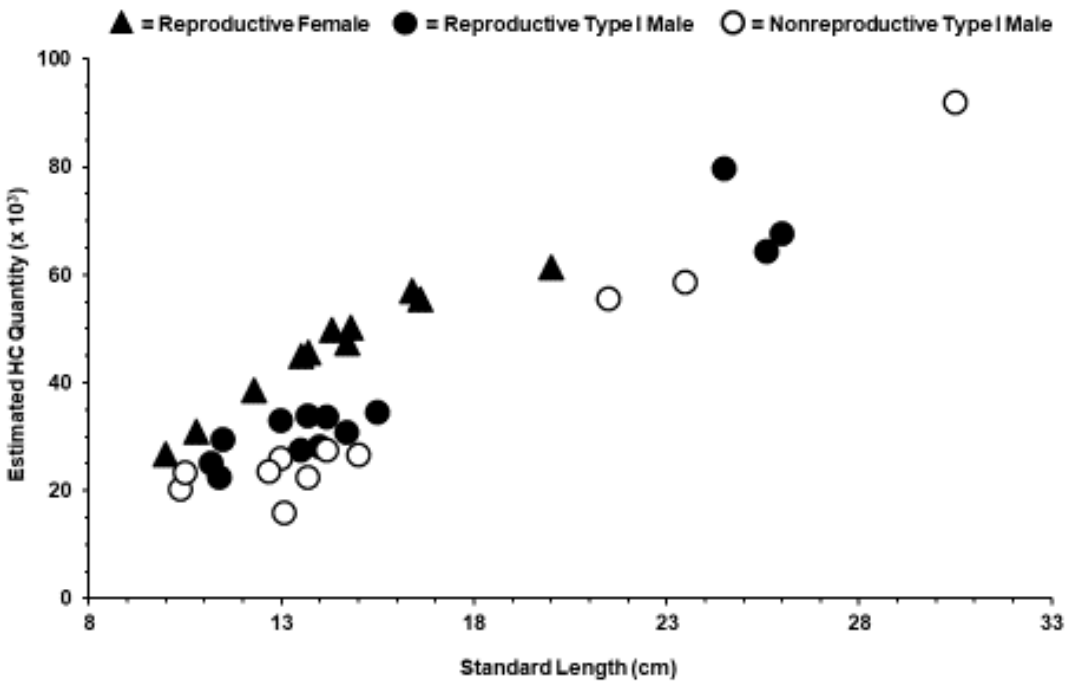

Figure 2.6. HC quantities in the saccule were estimated using the method described in Popper and Hoxter, 1984 (see methods section 2.2.6 for details) and plotted as a function of SL.

Chapter 3. Ontogeny of inner ear saccular development in the plainfin midshipman (*Porichthys notatus*)

3.1 Summary

The auditory system of the plainfin midshipman (*Porichthys notatus*) is an important sensory system used to detect and encode biologically relevant acoustic stimuli important for survival and reproduction including social acoustic signals used for intraspecific communication. Previous work showed that hair cell (HC) density in the midshipman saccule increased seasonally with reproductive state and was concurrent with enhanced auditory saccular sensitivity in both females and type I males. Although reproductive state dependent changes in HC density have been well characterized in the adult midshipman saccule, less is known about how the saccule changes during ontogeny. Here, we examined the ontogenetic development of the saccule in four relative sizes of midshipman (larvae, small juveniles, large juveniles and nonreproductive adults) to determine if the density, total number, and orientation patterns of saccular HCs change during ontogeny. In addition, we also examined whether the density and total number of HCs in the saccule differ from that of the utricle and lagena in nonreproductive adults. We found that HC density varied by saccular epithelial region and across developmental stage with mean saccular HC density decreasing from larvae (mean = 73 HCs/2,500 um^2) to nonreproductive adults (mean = 43 HCs/2,500 um^2). The ontogenetic reduction in HC density was concurrent with an ontogenetic increase in macula area. The orientation pattern of saccular HCs was similar to the standard pattern previously described in other teleost fishes, and this pattern of HC orientation was retained during ontogeny. Lastly, the estimated number of saccular HCs increased with developmental stage from the smallest larvae (2,336 HCs) to the largest

nonreproductive adult (145,717 HCs), and in nonreproductive adults estimated HC numbers were highest in the saccule (mean = 28,479 ± 4,809 SD HCs), intermediate in the utricle (mean = 11,008 ± 1,619 SD HCs) and lowest in the lagena (mean = 4,560 ± 769 SD HCs). In contrast HC density was highest in the lagena (mean = 74 ± 7 SD/2,500 um^2), intermediate in the utricle (mean = 58 ± 6 SD/2,500 um^2), and lowest in the saccule (mean = 46 ± 5 SD/2,500 um^2).

3.2 Introduction

The teleost inner ear is composed of three semicircular canals and three putative auditory end organs: the saccule, lagena, and utricle. Each auditory end organ contains a single, dense calcium carbonate otolith that rests on a sensory bed of hair cells (sensory macula) and responds to linear acceleration and functions as an inertial accelerometer. These otolithic end organs continue to grow (increase in size) in fish throughout early development and into the adult life history stage. As the inner ear sensory epithelia expand, hair cells (HCs) are continuously added, and the total number of HCs in the macula increases. This increase in total HCs generally coincides with a reduction in HC density because the rate of HC addition is often less than the rate of macular epithelial growth (Popper and Hoxter, 1984; Lombarte and Popper, 1994; Lombarte and Popper, 2004; Chaves et al., 2017; but see Lu and DeSmidt 2013, and Wang et al., 2015). In addition, as HCs are continuously added to sensory epithelia in the auditory end organs as fish age, changes in the size and shape of the macula may also occur (Corwin 1983, Popper and Hoxter 1984, Lombarte and Popper 1994).

Although development of the inner ear throughout the lifespan in fishes is well documented (Popper and Hoxter, 1984; Sokolowski and Popper, 1987; Popper and Hoxter, 1990; Vasconcelos et al., 2016), less is known about how ontogenetic changes in the inner ear affect auditory sensitivity in fishes. In the thornback ray (*Raja clavata*), the total number of HCs in the

macula neglecta, a non-otolithic detector of sound in elasmobranchs, increases as animals age (post-hatch) and was concurrent with increased afferent sensitivity of the macula neglecta (Corwin, 1983). In contrast, Higgs et al. (2002) measured auditory evoked potentials (AEPs) and surmised that the zebrafish (*Danio rerio*) experience no concomitant change in auditory sensitivity in association with the ontogenetic increase in total HC number in the saccule, the main organ of hearing in the zebrafish and most teleost fishes. However, Wang et al. (2015) showed a concurrent decrease in zebrafish AEP thresholds (i.e., increased auditory sensitivity) with ontogenetic increases in saccular HC density and numbers. Finally, Vasconcelos et al. (2015) showed that the Lusitanian toadfish (*Halobatrachus didaclytus*) exhibited developmental stage-dependent increases in auditory saccular sensitivity based on saccular potential recordings as toadfish transitioned from juveniles into adults, which is likely, in part, due to the observed ontogenetic increases in total HC number in the toadfish saccule (Chaves et al., 2017).

Previous work with the plainfin midshipman fish (*Porichthys notatus*), which belongs to the same family (Batrachoididae) as toadfish, has yielded conflicting results in terms of how peripheral auditory sensitivity changes during ontogeny. The plainfin midshipman has become a good model species to investigate mechanisms of acoustic communication and sound source localization (Bass and McKibben, 2003; Sisneros 2009; Sisneros and Rogers 2016). Previously, behavioral experiments measuring acoustic startle-like responses throughout development found that the auditory system of plainfin midshipman is functionally mature at the larval stage at 1.4 cm of length (Alderks and Sisneros, 2013). Alderks and Sisneros (2011) showed based on saccular potential recordings that midshipman exhibit no change in auditory sensitivity during development but instead show an ontogenetic retention of auditory sensitivity from small juveniles to adults. In contrast, Sisneros and Bass (2005) demonstrated, based on saccular

afferent recordings, that auditory sensitivity increased with size/age from small juveniles to adults. These results suggest that midshipman do exhibit ontogenetic increases in auditory sensitivity at least at the level of auditory afferents post synaptic to the hair cells. Whether these results are also related to morphological changes in the saccule throughout ontogeny remains to be determined.

The primary aim of this study was to characterize the ontogenetic development of the saccule in the plainfin midshipman (*P. notatus*). We examined how the density, number, and orientation patterns of HCs in the saccule change from larvae to adults. In addition, we assessed how the saccular epithelium changes in size and shape during development. A secondary aim was to examine the density and number of HCs in the utricle and lagena of adults to compare with the adult saccule. We discuss the potential functional significance of our findings and how they may relate to ontogenetic changes in the sensitivity of the auditory system in the plainfin midshipman.

3.3 Methods

3.3.1 Animal collection and care. All large juvenile (n = 11) and non-reproductive adult (n = 30) midshipman were collected via otter trawl in Monterey Bay near Moss Landing, CA during the month of January in 2018 and 2020. Large juveniles were distinguished from adults based on standard length (SL), which were less than 10.5 cm for males and less than 8.5 cm for females but larger than 3 cm for both males and females. Small juveniles (n = 20) (< 3 cm SL) were raised from larvae incubated in captivity. Midshipman larvae were collected from Hood Canal near Brinnon, WA and Tomales Bay near Marshall, CA in June and July 2019. Larvae were defined as any fish still nourishing on yolk (n = 11). Some larvae were held in tanks until all yolk was absorbed and they became free-swimming juveniles; up to three months after collection. The size ranges used in this study were similar to those used in previous midshipman studies

(Brantley et al., 1993; Bass et al., 1996; Grober et al., 1994; Foran and Bass, 1998; Sisneros, 2007; Alderks and Sisneros, 2011). All midshipman were maintained in 50 gallon recirculating saltwater tanks at approximately 25 parts per thousand salinity and an average temperature of 13.5°C. Winter-collected adult and large juvenile midshipman were held in tanks for 4.5 weeks or less after capture. Adults/large juveniles were maintained with an 8 hour light/16 hour dark photoperiod and embryos/small juveniles were maintained with a 12 hour light/12 hour dark cycle to simulate the photoperiod during the time of year in which the animals were collected. Adults and free-swimming juveniles were fed de-shelled raw shrimp 2-3 times per week. All animal care protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

3.3.2 Tissue collection. Prior to tissue collection all large juvenile and adult animals were anesthetized in a 10% benzocaine saltwater bath, transcardially perfused with teleost ringer solution followed by with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB) solution, and then all auditory end organs (saccule, lagena, and utricle) were removed from the otic capsule via a dorsal craniotomy. Auditory end organs were then immersed and post-fixed with 4% paraformaldehyde dissolved in 0.1 M PB for one hour. Larvae and small juveniles were not perfused but were sacrificed via benzocaine overdose. Auditory end organs were then immediately removed from the otic capsule following a dorsal craniotomy and immersion fixed with 4% paraformaldehyde dissolved in 0.1 M PB for two hours. Saccules, lagenae, and utricles of all developmental stages were washed in 0.1 M PB following immersion fixation. Sensory epithelia were isolated by removing the otolith and otolith membrane using forceps. End organ epithelia were kept in 0.1 M PB with 0.03% sodium azide in 4°C for 2 months or less prior to staining and imaging for the majority of tissue, with the exception of three fish collected in

January 2018 for which tissue was stored in the same conditions for 26 months. Following end organ removal, all fish were weighed and measured for standard length (SL) and then the gonads were dissected and weighed. Adult fish were sexed based on the presence of ovaries or testes. Juvenile fish were sexed based on the presence (female) or absence (male) of oocytes in the gonadal tissue. There was no way to distinguish between type I and type II males in juveniles as the weight of testes in this developmental stage were undetectable. Visual observation of oocytes could not be detected in larvae and therefore sex could not be determined at this stage of development.

3.3.3 End organ staining. The sensory epithelium of each end organ was washed in a 0.1 M phosphate buffered saline (PBS) solution and then stained with phalloidin (Invitrogen cat. no. R415) at a dilution of 1:40 for 1 hour. Epithelia were washed again in 0.1M PBS, placed on coverslips with the apical side against the glass and mounted to slides in Fluoromount-G mounting media (Southern Biotech cat. no. 0100-01). Slides were sealed with nail polish and were stored away from light in 4°C until imaging.

3.3.4 Fluorescence imaging. All epithelia were imaged on a Leica SP5 confocal microscope with an inverted stand (Leica Microsystems, Buffalo Grove, IL). The phalloidin used for staining was conjugated to rhodamine, so the laser was set to 560/570-620 excitation/emission spectral filter. Images used for HC counts and HC orientation were at 40 x 1.25 NA oil immersion objective for all developmental stages. Images analyzed for area measurements were performed using the 10 x 0.4 NA dry objective for adults and large juveniles, and the 20 x 0.7 NA dry objective for small juveniles and larvae. All images were captured in z-stacks at 0.5 um increments using Leica Application Suite Advanced Fluorescence software (Leica).

3.3.5 Hair cell counts/ HC orientation. End organ HCs were counted using Image-J software. Saccule HC counts were collected from 50um x 50um squares in seven discrete regions for nonreproductive adults and juveniles. The seven discrete regions chosen were similar to those used previously in adult female and type I male midshipman (Lozier and Sisneros, 2019) and they represent epithelia regions in both the marginal zone of the macula (peripheral regions 1, 3, 6, and 7) and the central zone of the macula (inner regions 2, 4, and 5). Both marginal and central zones of end organ maculae have been described in previous studies in other species (Corwin, 1981; Popper and Hoxter, 1984) and in adult midshipman (Coffin et al., 2012; Lozier and Sisneros, 2019). In addition, HC density was also examined in the rostral zone (anterior regions 1, 2, 3, and 4) and caudal zone (posterior regions 5, 6, and 7) of the saccular macula. In larvae, HC counts were collected from only six 50um x 50um regions because epithelial region 7 was not present in the saccule of larvae (see Fig. 3.1). Six of the saccular epithelia from fish collected in January 2018 were stained and used for counts in a previous study (Lozier and Sisneros, 2019). We used these same images for this study, but HCs were re-counted in this experiment. Hair cell counts were collected from three 50um x 50um squares in utricles and four 50um x 50um squares in lagenae. In our analysis, HCs were defined as separate stereocilia bundles. Bundles that were only partially within the counting box were included in the analysis. HC orientation in saccules was determined by imaging each HC at the level of the cuticular plate. The kinocilium does not contain f-actin, and therefore is not stained by phalloidin. The kinocilium appears as a black circle on the cuticular plate and the plane of maximum polarity of the HC stereocilia were determined visually (see Fig. 3.6, left panel).

3.3.6 Statistical analyses. To compare HC densities between each developmental group, we conducted a two-way mixed ANOVA with developmental stage (4 levels: larvae, small

juveniles, large juveniles, and adults) as the between-subjects factor and saccule region (6 levels for each region, excluding region 7 because this region is not present in larvae) as the within-subjects factor. To examine differences in HC bundle density between regions, we also ran separate two-way ANOVAs for each developmental stage with the two independent variables being marginal/central zones and caudal/rostral zones.

To examine the relationship between saccular HC density and saccular macula, we pooled all of the developmental stages together and plotted HC density as a function of macula area. Similarly, we plotted HC density as a function of SL. We excluded saccular regions 2 and 7 from these analyses because counts could not be made in many individuals for these regions due to tissue damage, and region 7 is not present in larval saccules. Individuals lacking counts in regions 1, 3, 4, 5, or 6 were excluded from analysis. The relationships between SL/macula area and saccular HC density were nonlinear, so we ran Spearman correlations.

Differences in saccular macula shape throughout ontogeny were examined by measuring the area of rostral, medial, and caudal maculae (see Fig. 3.5, left panel). Areas were log-transformed to meet the assumption of equality of variances and were compared in a two-way mixed ANOVA. Size (area) of each zone were compared between developmental groups with Bonferroni post-hoc tests.

To test for differences in saccular HC density between females and males, we conducted a two-way ANOVA comparing summed density counts in regions 1-6 in small juveniles, large juveniles, and adults of both sexes. Larvae were not included because oocytes are undetectable at this stage and therefore larvae could not be differentiated by sex. To size-match the individuals, the five largest males were excluded from this analysis. Region 7 was excluded because

measurements were lacking in many individuals due to tissue damage. Any individuals lacking counts in region 1-6 were excluded from this analysis.

To determine whether there was a significant difference in HC density and estimated HC quantities between the three end organs (saccule, utricle, and lagena) we conducted one-way ANOVAs with Bonferroni post-hoc tests in size-matched adult midshipman. For HC density comparisons, saccular HC counts were combined from two marginal and one central region (i.e., regions 1, 4, and 6), and from two striolar and one non-striolar region in utricles and lagenae (regions 1, 2, and 3 in utricles; and 1, 3, and 4 in lagenae). Like marginal regions in the saccule, the striolar regions in these end organs contain greater bundle density than the non-striolar area (Coffin et al., 2012). Estimated HC numbers were log-transformed to meet the assumption of equality of variances.

3.3.7 Estimated hair cell numbers. Saccule, lagena, and utricle macula areas were measured using the tracing tool in Image-J. HC numbers were estimated in all three end organs using the following equation described in Popper and Hoxter (1984):

Total estimated HCs = (∑HCc)(Ac/∑Ac macula regions) + (∑HCm)(Am/∑Am macula regions), where

∑HCc/HCm = Total counted HC bundles in central/marginal macula

Ac/Am = Area of the central/marginal macula (in um2), and

∑Ac/Am macula regions = the summed area of all 50um x 50um boxes in each zone of the macula.

Note that for the utricle and lagena, central macula = non-striolar, and marginal macula = striolar.

Because statistical evidence indicated that HC bundle density differed between caudal and rostral zones in saccules of larvae and small juveniles, and because we could not reliably delineate between the marginal and central zones visually in Image-J, estimated saccule HC numbers in these developmental stages were calculated using the following equation (Lombarte and Popper, 1994; Vasconcelos et al., 2016):

Total estimated HCs = (∑HCr)(Ar/∑Ar macula regions) + (∑HCc)(Ac/∑Ac macula regions), where

∑HCr/HCc = Total counted HC bundles in rostral/caudal macula

Ar/Ac = Area of the rostral/caudal macula (in um^2), and

∑Ar/Ac macula regions = the summed area of all 50um x 50um boxes in each zone of the macula.

Note that because no counting regions were located within the medial zone (see Fig. 3.1), the area of the medial zone was evenly divided between the rostral and caudal zones for these estimates.

3.4 Results

3.4.1 Morphometrics

We sampled a total of 72 plainfin midshipman: 11 larvae, 20 small juveniles, 11 large juveniles and 30 non-reproductive adults. The larvae (sex could not be determined; n = 11) had a mean standard length (SL) of 1.7 ± 0.2 cm SD (range = 1.5 to 2.1 cm) and body mass (BM) of

0.13 ± 0.01 g SD. For small juveniles, males (n = 12) had a mean SL of 2.5 ± 0.2 cm SD (range = 2.3 to 2.8 cm) and a mean BM of 0.18 ± 0.04 g SD while females (n = 8) had a mean SL of 2.6 ± 0.2 cm SD (range = 2.2 to 2.9 cm) and a mean BM of 0.17 ± 0.04 g SD. For large juveniles, males (n = 9) had a mean SL of 8.4 ± 1.3 cm SD (range = 6.6 to 10.4 cm) and a mean BM of 5.65 ± 3.08 g SD while females (n = 2) had a mean SL of 7.0 ± 0.2 cm SL (range = 6.8 to 7.1 cm), a mean BM of 2.91 ± 0.16 g SD, and a mean gonadosomatic index (GSI) of 3.1 ± 3.4 SD. For non-reproductive adults, type I males (n = 15) had a mean SL of 14.9 ± 5.8 cm SD (range = 10.6 to 30.5 cm), a mean BM of 52.0 ± 74.0 g SD, and a mean GSI of 0.5 ± 1.0 SD while females (n = 15) had a mean SL of 10.8 ± 1.0 cm SD (range = 8.9 to 12.5 cm), a mean BM of 12.21 ± 4.16 cm SD, and a mean GSI of 0.9 ± 0.2 SD. Note we could not calculate GSI in larvae, small juveniles, or large male juveniles because gonads were too small to weigh.

3.4.2 Hair cell bundle density of end organs

Hair cell bundle density of the saccule varied by epithelial region, developmental stage (larvae, small juveniles, large juveniles, and adults), and by epithelial region across developmental stage (two-way mixed ANOVA with main effects of epithelial region ($F_{(5,260)}$ = 65.15, $p < 0.05$) and developmental stage ($F_{(3,52)}$ = 133.73, $p < 0.05$); there was also a significant interaction of epithelial region and developmental stage ($F_{(15,260)}$ = 9.25, $p < 0.05$)) (Fig. 3.1). In larvae, HC bundle density varied across the six epithelial regions sampled with region 5 in the central zone having the greatest HC density (86 + 13 HCs SD/2,500 um^2) and region 2 in the central zone having the lowest HC density (64 + 9 HCs SD/2,500 um^2). Although there was no difference in HC bundle density observed between the marginal zone (peripheral regions 1, 3, and 6; mean = 77 ± 9 HCs SD/2,500 um^2) and the central zone (inner regions 2, 4, and 5; mean = 77 ± 8 HCs SD/2,500 um^2), HC bundle density was greater in the caudal zone (posterior

regions 5 and 6; mean = 81 ± 10 HCs SD/2,500 um^2) compared to the rostral zone (anterior regions 1, 2, 3, and 4; mean = 72 ± 6 HCs SD/2,500 um^2) ($F_{(1,49)} = 6.3$, $p < 0.05$).

In small juveniles, HC bundle density varied across the seven epithelial regions sampled with region 7 in the marginal zone having the greatest HC density (88 + 8 HCs SD/2,500 um^2) and region 2 in the central zone having the lowest HC density (59 + 10 HCs SD/2,500 um^2). Hair cell bundle density was greater in the marginal zone (regions 1, 3, 6, and 7; mean = 82 ± 5 HCs SD/2,500 um^2) compared to the central zone (regions 2, 4, and 5; mean = 70 ± 6 HCs SD/2,500 um^2) ($F_{(1,117)} = 48.2$, $p < 0.05$). Also, HC density was greater in the caudal zone (regions 5, 6, and 7; mean = 80 ± 6 HCs SD/2,500 um^2) compared to the rostral zone (regions 1, 2, 3, and 4; mean = 72 ± 5 SD/2,500 um^2) ($F_{(1, 117)} = 17.2$, $p < 0.05$).

In large juveniles, region 3 in the marginal zone had the greatest HC density (58 + 9 HCs SD/2,500 um^2) and region 2 in the central zone had the lowest HC density (31 + 6 HCs SD/2,500 um^2). Hair cell bundle density was greater in the marginal zone (mean = 55 ± 4 SD/2,500 um^2) compared to the central zone (mean = 37 ± 5 SD/2,500 um^2) ($F_{(1,72)} = 102.8$, $p < 0.05$), but there was no difference in HC density between the caudal zone (mean = 47 ± 5 SD/2,500 um^2) and the rostral zone (mean = 45 ± 4 SD/2,500 um^2) ($F_{(1,72)} = 0.7$, $p = 0.42$).

In nonreproductive adults, region 3 in the marginal zone had the greatest HC density (56 ± 10 HCs SD/2,500 um^2) and region 2 in the central zone had the lowest HC density (29 ± 7 HC SD/2,500 um^2). Hair cell bundle density was greater in the marginal zone (mean = 54 ± 5 SD/2,500 um^2) compared to the central zone (mean = 31 ± 5 SD/2,500 um^2) ($F_{(1,194)} = 348$, $p < 0.05$), but there was no difference in HC density between the caudal zone (mean = 43 ± 5 SD/2,500 um^2) and rostral zone (mean = 42 ± 5 SD/2,500 um^2) ($F_{(1,194)} = 1.7$, $p = 0.2$). In sum, HC bundle density was greater in the marginal zone compared to the central zone in the saccule

of small juveniles, large juveniles, and non-reproductive adults, while HC bundle density was greater in the caudal zone compared to the rostral zone only in larvae and small juveniles.

Mean HC bundle density of the saccule decreased with developmental stage from larvae and small juveniles to large juveniles and nonreproductive adults. The mean HC bundle density of larvae (mean HC density = 76 ± 8 SD/2,500 um2) and small juveniles (mean HC density = 73 ± 6 SD/2,500 um^2) was greater than the mean HC density of large juveniles (mean HC density = 45 ± 5 SD/2,500 um^2) and nonreproductive adults (mean HC density = 43 ± 6 SD/2,500 um2) ($p < 0.05$). There was no difference in mean HC bundle density between larvae and small juveniles, nor between large juveniles and nonreproductive adults ($p = 1.0$) (Fig. 3.2). In addition, we found no evidence that HC density varied between the sexes across the stages of development where the sexes could be identified (two-way ANOVA with a main of effect of developmental stage (excluding larvae), ($F_{(2,40)} = 128.6$, $p < 0.05$); no effect of sex, ($F_{(1,40)} = 0.2$, $p = 0.7$), and no interaction between developmental stage and sex,($F_{(2,40)} = 2.3$, $p = 0.12$), Thus there was no difference in mean HC density between females (322 ± 41 HCs SD/15,000 um^2) and males (326 ± 30 HCs SD/15,000 um^2). In sum, saccular HC bundle density is greater in larvae and small juveniles than in large juveniles and non-reproductive adults, and there was no difference in saccular HC densities between females and males.

HC bundle density of the saccule was observed to decrease with a concurrent increase in saccular macula area during saccular development. Saccular HC bundle density decreased nonlinearly as a function of macula area (saccular HC bundle density = -51.03ln * (macula area) + 275.16) and was negatively correlated with macula area ($rs = -0.80$, $p < 0.05$) (Fig. 3.3). In addition, saccular HC bundle density was also observed to covary with body size during ontogeny such that HC bundle density decreased nonlinearly as a function of SL (HC bundle

density = -86.74ln * (SL) + 441.63, $R^2 = 0.82$) (Fig. 3.4), and was negatively correlated with SL ($rs = -0.79$, $p < 0.05$). In nonreproductive adults, mean HC bundle density was found to vary among the inner ear auditory end organs (saccule, lagena, and utricle). Greatest HC bundle densities were observed in the lagena (mean = 74 ± 7 SD/2,500 um^2) followed by intermediate HC densities in the utricle (mean = 58 ± 6 SD/2,500 um^2) and lowest HC densities in the saccule (mean = 46 ± 5 SD/2,500 um^2) (one-way ANOVA ($F_{(2,43)} = 92.8$, $p < 0.05$)) (Fig. 3.8).

3.4.3 Saccular macula shape and hair cell orientation

The size (area) of the saccular macula increased with developmental stage from larvae to nonreproductive adults. Larvae had the smallest macular area (mean = 0.08 ± 0.02 mm^2 SD) followed by small juveniles (mean = 0.17 ± 0.02 mm^2 SD) and then large juveniles (mean = 1.22 ± 0.45 mm^2 SD), while greatest macular area was observed in adults (mean = 2.64 ± 1.7 mm^2 SD) ($F_{(3,62)} = 434.0$, $p < 0.05$). Saccular macula size based on positional area (rostral, medial, and caudal) decreased in order from the rostral, medial, and caudal area in all developmental stages ($p < 0.05$), but the relative size of the caudal area in larvae and small juveniles is smaller than in large juveniles and adults (see the slopes of lines in Fig. 3.5).

The pattern of HC orientation in the midshipman saccule is most similar to the "standard pattern" described in other teleost fishes (Popper and Schilt, 2008), and this standard pattern of HC orientation was generally retained throughout saccular development. In the rostral zone, HCs were oriented in a horizontal plane of maximum stereocilia depolarization. The border of the rostral and medial macula was defined by an abrupt 90-degree change in orientation as the stereocilia in the medial macula are oriented vertically in the z-axis. In the caudal zone, the stereocilia gradually transitioned from a vertical orientation in the medial area of the macula to a horizontal orientation at the peripheral caudal end (Fig. 3.6). There were no obvious differences

in saccule shape or HC orientation between large juveniles and adults (bottom diagram in figure 3.6; right panel represents both developmental stages). In addition, there were also no differences in HC orientation patterns between females and males (based on developmental groups where sex was known), therefore the sketches in figure 3.6 represent data taken from both sexes.

3.4.4 Estimated hair cell numbers in the saccule, lagena, and utricle

The estimated number of HCs in the saccule increased with developmental stage from larvae to nonreproductive adults. The estimated number of HCs in the saccule increased logarithmically during ontogeny as a nonlinear function of body size (SL) (estimated number of HCs = 1.24 * (SL)1.3, $R^2 = 0.93$). The smallest larva (SL = 1.5 cm) had an estimated 2,336 HCs and the largest nonreproductive adult (SL = 30.5 cm) had the highest estimated number of HCs (145,717 HCs) (Fig. 3.7). The estimated number of HCs in the utricle and lagena also increased with developmental stage from large juveniles to nonreproductive adults. The estimated HC number in the utricle and lagena increased as a nonlinear function of body size in large juvenile and adult midshipman (Estimated number of utricle HCs = 1.18 * (SL)0.92, $R^2 = 0.55$; Estimated number of lagena HCs = 0.57 * (SL)0.86, $R^2 = 0.12$). The estimated HC numbers varied among inner end organs in nonreproductive adults ($F_{(2,44)} = 502$, $p < 0.05$), with the saccule having the highest estimated number of HCs (mean = 28,479 ± 4,809 SD) followed by the utricle (mean = 11,008 ± 1,619 SD) and then the lagena (mean = 4,560 ± 769 SD), which had the lowest estimated number of HCs ($p < 0.05$) (Fig. 3.9).

3.5 Discussion

The primary aim of this study was to characterize the ontogenetic saccular development in the plainfin midshipman and determine if the density, total number, and orientation patterns of HCs

in the saccule change during ontogeny from larvae to nonreproductive adults. A secondary aim was to determine if the density and total number of HCs in the saccule differed from that of the utricle and lagena in nonreproductive adults. We found that saccular HC density varied by epithelial region and across developmental stage with mean saccular HC density decreasing from larvae to nonreproductive adults. The ontogenetic reduction in HC density was concurrent with an increase in macula area. The orientation pattern of saccular HCs was similar to the standard pattern previously described in other teleost fishes, and this pattern of HC orientation was retained during ontogeny. Lastly, the estimated number of saccular HCs increased with developmental stage from larvae to nonreproductive adults, and in nonreproductive adults estimated HC numbers were highest in the saccule, intermediate in the utricle, and lowest in the lagena. In contrast HC density was highest in the lagena, intermediate in the utricle, and lowest in the saccule. In this discussion, we interpret our results as they relate to the physiology of the auditory inner ear end organs.

The observed decrease in saccular HC density with midshipman developmental stage from larvae to nonreproductive adults was similar to that observed in other teleost fishes including the oscar cichlid (*Astronotus ocellatus*), European Hake (*Merluccis merluccius*), and the Lusitanian toadfish (*Halobatrachus didactylus*) (Popper and Hoxter, 1984; Lombarte and Popper, 1994; Lombarte and Popper, 2004; Chaves et al., 2016), with the exception of zebrafish (*Danio rerio*) where HC density increased from small juveniles to young adults and then decreased in older adults (Wang et al., 2015). The mean saccular HC density in midshipman was greater in larvae (mean = 76 ± 8 SD/2,500 um^2) and small juveniles (mean = 73 ± 6 SD/2,500 um^2) than in large juveniles (mean = 45 ± 5 SD/2,500 um^2) and nonreproductive adults (mean = 43 ± 6 SD/2,500 um^2) with no difference in HC density between larvae and small juveniles, nor

between large juveniles and nonreproductive adults. This decrease in saccular HC density was likely related to the observed concurrent increase in saccular macula area during development. The area of the saccular macula increased nonlinearly from larvae (mean = 0.08 ± 0.02 mm^2 SD) to small juveniles (mean = 0.17 ± 0.02 mm^2 SD) to large juveniles (mean = 1.22 ± 0.45 mm^2 SD) and then to adults (mean = 2.64 ± 1.7 mm^2 SD). Thus, this non-linear increase in saccular area during ontogeny coupled with the addition of new HCs in the growing macula likely contributes in part to the observed decrease in mean saccular HC density during development.

We examined HC density in different regions of the saccular macula to determine if HCs were potentially added uniformly throughout the macula or instead added differentially at the periphery (i.e., marginal zones) or in the anterior or posterior areas of the macula (i.e., rostral and caudal zones). We found that HC density varied by saccular epithelial region and across developmental stage from larvae to nonreproductive adults. In larvae, there was no difference in HC density between the marginal and central zones of the saccular macula, however HC density was found to be greater in the caudal zone compared to the rostral zone. In contrast, HC density was greater in the marginal zone compared to the central zone in small juveniles, large juveniles, and nonreproductive adults. In addition, HC density was found to be greater in the caudal zone compared to the rostral zone in small juveniles while there was no difference in HC density between rostral and caudal zones in large juveniles and nonreproductive adults. Our observed results of greater HC density in the saccular marginal zones compared to central zones in juveniles and adults is similar to that reported for the oscar (Popper and Hoxter, 1984). It is tempting to suggest that HCs may be added at a greater rate to the marginal zones where HC density is higher, but Popper and Hoxter (1990) showed that newly proliferating HCs in the oscar occurred post-embryonically throughout the entire saccular epithelium in young and older adult

stages. In other teleost fishes including the hake and goldfish there is no evidence of saccular HC proliferation occurring in the marginal zones, but rather new HCs are also added post-embryonically throughout the saccular epithelium (Lombarte and Popper, 1994; Lanford et al., 1996). Thus, future studies that examine saccular HC proliferation in midshipman will be instrumental in determining how newly proliferating HCs are added and dispersed in the midshipman saccular epithelium during ontogeny.

Previously Lozier and Sisneros (2019) showed a sexually dimorphic difference in saccular HC density between females and type I males during the summer reproductive season with females having greater saccular HC densities than type I males. In addition, reproductive type I males were also shown to have greater saccular HC densities than nonreproductive type I males (Lozier and Sisneros, 2019). In our current study we did not observe any sexually dimorphic differences in saccular HC densities during development in juveniles or in nonreproductive adults. The observed reproductive state dependent changes in saccular HC density exhibited by type I males and females (Coffin et al. 2012, Lozier and Sisneros, 2019) are likely mediated by seasonal increases in circulating gonadal steroids that occur during the seasonal recrudescence of the gonads prior to the breeding season in both sexes (Sisneros et al., 2004). Future studies will be needed to determine the specific mechanisms responsible for HC proliferation that occur during development and in the breeding season, as well as to determine how reproductive state related increases in gonadal steroids (i.e., testosterone and estrogen) in adults may promote seasonal increases in saccular HC density in both males and females.

We found that the HC orientation patterns in the midshipman saccule were similar to the "standard" pattern described by Popper (1981) and this observed pattern was generally retained in midshipman throughout ontogeny. We observed in juveniles and nonreproductive adults

horizontally oriented HCs in the rostral zone, vertically oriented HCs in the medial part of the macula, and vertically oriented HCs that gradually shifted to a horizontal orientation in the caudal zone that "followed" the margin of the macula (see Fig. 3.6). This particular HC orientation pattern falls under the standard pattern that is generally found in species that lack an otophysic connection (Popper, 1978; Popper, 1981; Popper and Schilt, 2008). In addition, the HC orientation pattern and overall shape of the midshipman saccular macula also resembled that found in the closely related Gulf toadfish (*Opsanus beta*), Oyster toadfish (*Opsanus tau*), and Lusitanian toadfish (Popper, 1981; Edds-Walton and Popper, 1995; Chaves et al., 2016). Although the overall shape of the saccular macula was retained during ontogeny and was similar to that observed during saccular development in the Lusitanian toadfish (Chaves et al., 2016), we did observe that the relative size of the rostral and caudal areas grew in greater proportion than the medial area of the saccular macula. While the overall saccular macula area increased 31x from 0.08 mm^2 in larvae to 2.65 mm^2 in adults, the rostral and caudal areas of the macula increased 35x compared to a 25x increase in the medial area of the saccule from larvae to adults (Fig. 3.5). Interestingly, we observed an ontogenetic change in the orientation of HCs at the marginal end of the caudal macula in larvae (red arrowhead, Fig. 3.6) that changed from vertically oriented HCs to horizontally oriented HCs that followed the macula margin in juveniles and adults. Recently Colleye et al. (2019) showed that female midshipman which possess sexually dimorphic rostral swim bladder extensions have enhanced auditory sensitivity to sound pressure and higher frequencies > 305 Hz. The swim bladder horn-like extensions decrease the distance between the swim bladder and saccule to effectively enhance the detection of local particle motion generated by pressure-induced vibrations of the swim bladder when exposure to sound. Thus, the swim bladder in females and type II males, which also possess

rostral swim bladder extensions (Mohr et al. 2017), is thought to serve as an acoustic organ that enables the indirect detection of sound pressure stimuli needed for conspecific localization and social signal detection of conspecifics. The observed ontogenetic enlargement of the caudal and rostral areas of the saccular macula, which contain primarily horizontally oriented HCs, may play an important role in the detection of sound pressure-induced vibrations of swim bladder in juveniles and adults, especially in females and type II males. It still remains unclear whether type I males, which do not possess the rostral horn-like swim bladder extension, are able to detect the pressure induced vibrations of the swim bladder. Future work that investigates the local particle motion and directional movement of the sagitta (saccular otolith) in response to the sound pressure-wave induced vibrations of the midshipman swim bladder will be informative in determining the importance and function of the horizontally oriented HCs in the caudal and rostral areas of the saccule during development.

Our results indicate that the estimated number of HCs in the midshipman saccule increases nonlinearly as the saccular macular epithelium grows during ontogeny. The estimated number of saccular HCs increased from 2,336 HCs in the smallest larva of 1.5 cm SL to 145,717 HCs in the largest nonreproductive adult of 30.5 cm SL (Fig. 3.7). In comparison, we found that estimated saccular HC numbers were approximately 2x higher than that reported for the closely related Lusitanian toadfish (Chaves et al., 2016). Larval Lusitanian toadfish that ranged from 1.3 to 1.4 cm SL had an estimated 1,247 saccular HCs while adult toadfish that ranged from 20 to 23 cm SL had an estimated 31,616 saccular HCs (Chaves et al., 2016). Based on our analysis, a nonreproductive adult midshipman with a body size of 21.5 cm SL would have an estimated 75,914 saccular HCs or approximately 2.4 x more saccular HCs than an adult Lusitanian toadfish of equivalent size. This difference in estimated saccular HC number between the midshipman

and toadfish is likely due in part to the larger saccular macula area of the midshipman that was 1.5x larger in larvae and 2.7x larger in adults. Midshipman larvae (SL range = 1.5 – 2.1 cm) had an average macular area of 0.09 mm^2 and adults (SL range = 8.9 – 30.5 cm) had an average macular area of 6.49 mm^2 while Lusitanian toadfish larvae (SL range = 1.3 – 2.0 cm) had an average macular area of 0.06 mm^2 and 2.4 mm^2 in adults (SL range = 20 – 23 cm). In comparison to other teleost fishes such as the European hake, adults with an approximate total length (TL) of 21 cm had an estimated saccular macular area of 3.3 mm^2 and total estimated HC number of approximately 67,000 saccular HCs (Lombarte and Popper, 1994). In contrast, the adult oscar cichlid with a body size of 19 cm SL had an estimated 252,385 saccular HCs (Popper and Hoxter, 1984) while the adult zebrafish with a body size that range from 3.9 to 4.0 cm TL only had approximately 3,200 saccular HCs (Wang et al., 2015). Thus, the number of HCs that are estimated to accumulate in the teleost saccule during ontogeny varies greatly across the small number of species that have been examined and it remains unclear how these gross differences in saccular HC number correlate with a given species' auditory saccular sensitivity.

We also found that the estimated number of HCs in adults varied among the three midshipman auditory end organs with the saccule having the greatest number of HCs but also having the lowest sampled HC density. In nonreproductive adults, the mean HC number in the saccule (mean = 28,479 HCs) was 2.6x greater than that in the utricle (mean = 11,008 HCs) and 6.2x greater than in the lagena (mean = 4,560 HCs). In contrast, the HC density of the lagena (mean = 74 HCs/2,500 um^2) was 1.3x more dense the utricle (mean = 58 HCs/2,500 um^2) and 1.6x more dense than the saccule (mean = 46 HCs/2,500 um^2). These end organ differences in HC number and density are likely related to the end organ size of the macular area with the saccule having the largest macular area in general (note, we did not compare the macular areas of

the utricle, lagena, and saccule, but the differences in end organ size visually are obvious). Although the adult utricle has a significantly lower number of HCs than the saccule, a recent study by Rogers and Sisneros (2020) showed that the auditory sensitivity of the utricle based on HC auditory evoked potentials was very similar to the saccular sensitivity of type I males at frequencies < 305; however, at frequencies >305 Hz the utricle was even more sensitive than the saccule (Colleye et al. 2019). Given that the otoliths of the saccule (sagitta) and utricle (lapillus) are both primarily composed of calcium carbonate in the form of aragonite (Thorrold and Hare, 2002) and would share similar densities but different overall otolith masses, it remains unclear how differences in otolith composition (aragonite vs valerite), ototlith mass, and end organ HC density effect auditory sensitivity based on HC auditory evoked potentials.

Our results show that the utricle has a greater HC density than the saccule, which is concurrent with the utricle having a similar auditory sensitivity to the saccule at lower frequencies (<305 Hz) and higher sensitivity at higher frequencies (>305 Hz) in type I males (Rogers and Sisneros, 2020). Furthermore, the saccule in adult Lusitanian toadfish is known to have a greater HC density (based on mean total HC number/mean saccular macular area: 31,616 HCs/2.39 mm^2 = 13,228 HC/mm^2), but less estimated total number of HCs, than the saccule of adult midshipman (28,479 HCs/2.64 mm^2 = 10,787 HC/mm^2); however, Vasconcelos et al. (2015) showed that the saccular sensitivity of the adult Lusitanian toadfish is 10 dB re:1uPa more sensitive than the adult saccular sensitivity of the midshipman to frequencies from 105 to 585 Hz. Interestingly, the midshipman lagena has the highest HC density but it also has the lowest otolith mass (smallest of the three end organs) and contains an otolith (asteriscus) that is predominantly composed of lighter vaterite (Campana, 1999; Reimer et al., 2016). Not surprisingly, the lagena is significantly less sensitive than the saccule and utricle based on HC

auditory evoked potentials (Colleye et al., 2019; Vetter et al. 2019; Rogers and Sisneros, 2020). Such differences in HC density and otolith mass of the saccule during ontogeny may also in part explain the observed ontogenetic retention of auditory saccular sensitivity in the midshipman (Alderks and Sisneros, 2011). Previously, Alderks and Sisneros (2011) showed that the auditory threshold tuning curves based on auditory evoked saccular potentials did not differ among small juveniles, large juveniles and adults from 75 to 785 Hz. We show in the current study that the mean saccular HC density was higher in small juveniles (mean = 73 HCs/2500 um^2) compared to large juveniles (mean = 45 HCs/2500 um^2) and nonreproductive adults (mean = 43 HCs/2500 um^2). Future work that examines ontogenetic changes in mass of the sagitta concurrently with changes in saccular HC density will be instrumental determining if there is a relationship between changes in otolith mass and HC density that affects auditory HC sensitivity of the saccule during ontogeny.

3.6 Figures

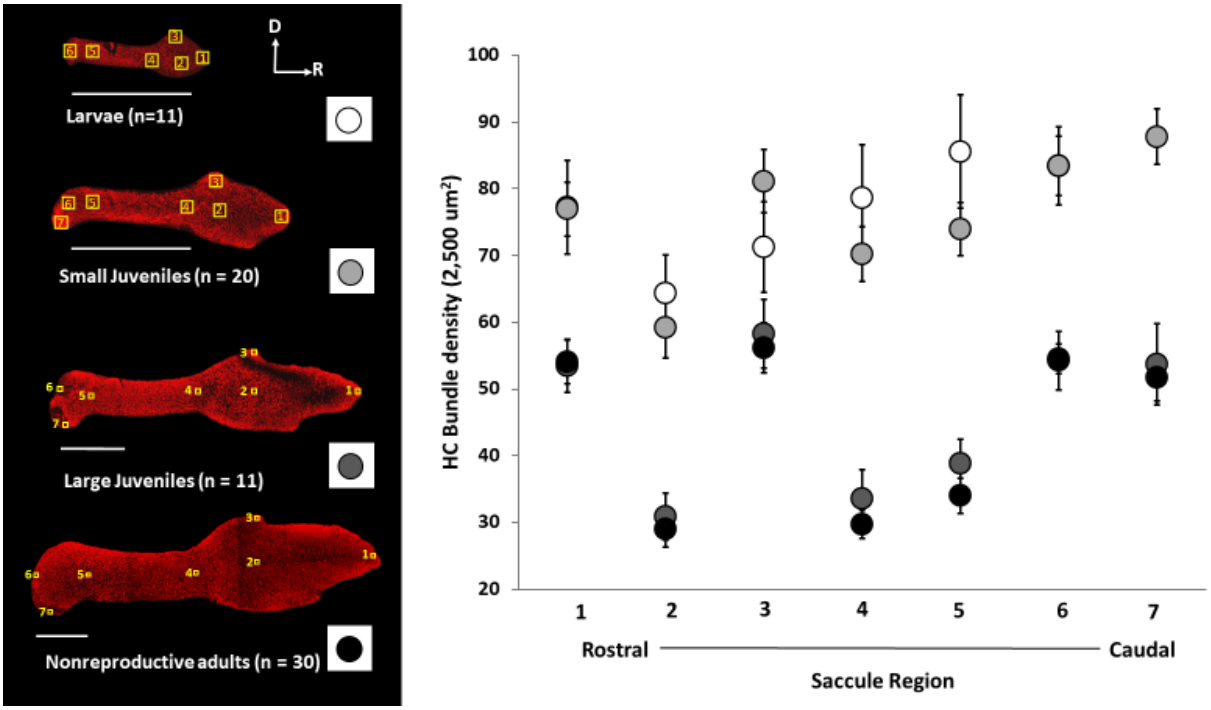

Figure 3.1. Saccular HC density by region in larvae, small juveniles, large juveniles, and nonreproductive adult midshipman. Left panel: Representative saccular maculae from larval, small juvenile, large juvenile, and nonreproductive adult plainfin midshipman. Hair cell bundles were labeled with phalloidin conjugated to rhodamine which is why the shape of the saccular macula appears red. Hair cell bundles were counted in 6 discrete 50 x 50 um regions in larvae and 7 discrete regions of the same dimension in small juveniles, large juveniles, and nonreproductive adults. Counts were not be made in region 7 in larvae because this region had not yet developed in the saccular macula. All boxes are drawn to scale. D = dorsal, R = rostral. White scale bar = 500 um. Right panel: Average hair cell bundle density in each region for each developmental stage. Each number on the x-axis corresponds to the number of each region shown in the left panel. White circles = larvae, light gray = small juveniles, dark gray = large

juveniles, and black = nonreproductive adults. For statistical differences see results section. Error bars represent ± 95% CI.

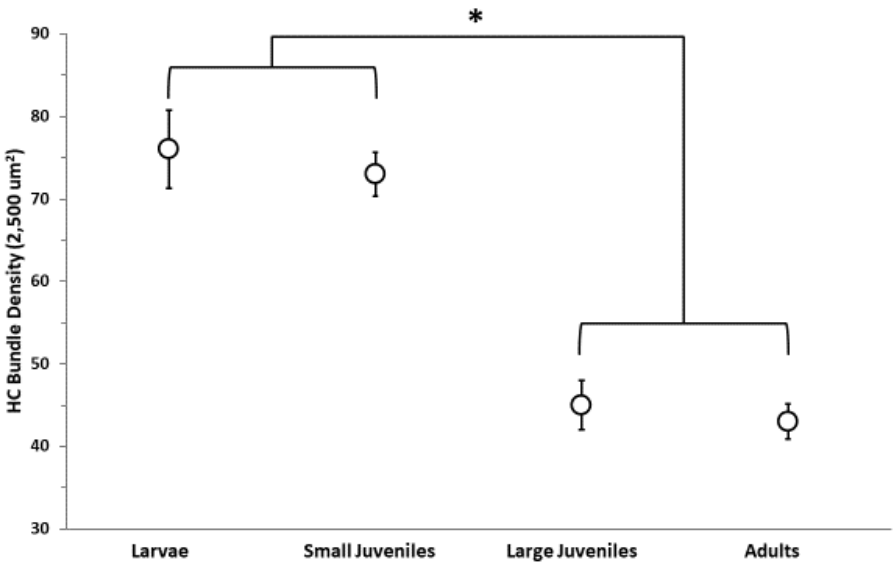

Figure 3.2. Mean HC density comparisons between larvae, small juveniles, large juveniles, and nonreproductive adult midshipman. Larvae and small juveniles had greater bundle density than large juveniles and adults. The asterisk denotes a statistical difference ($p < 0.05$).

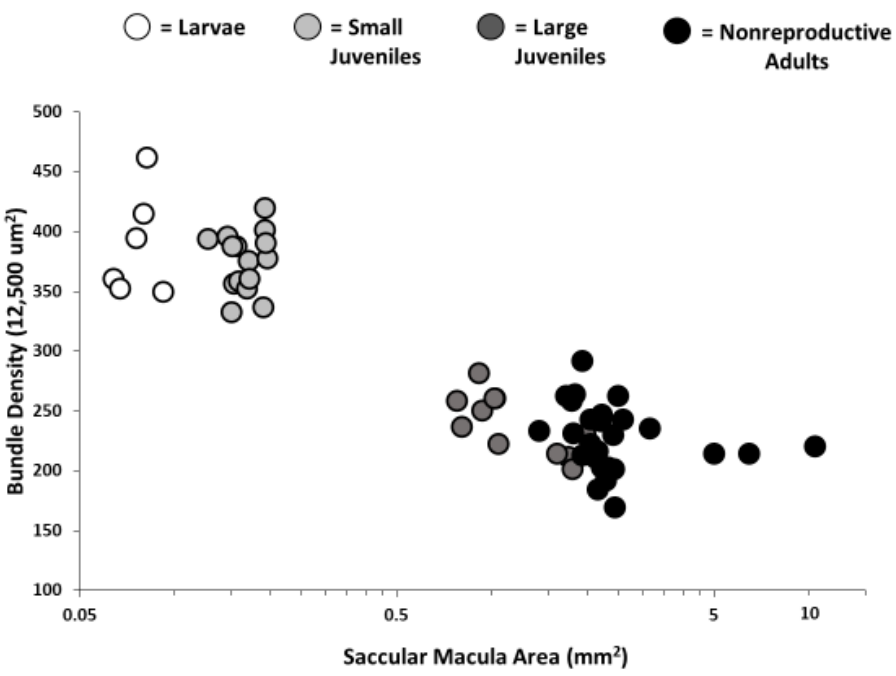

Figure 3.3. Saccular HC bundle density as a function of saccular macula area. Bundle density decreases as a logarithmic function of saccular macula area. Black circles = males; white circles = females; gray circles = larvae. There was no sexual dimorphic difference in bundle density. Note that bundle density was pooled over 5 regions (regions 2 and 7 were excluded, see text), so the y-axis is summed bundle density. Note that larvae could not be differentiated by sex.

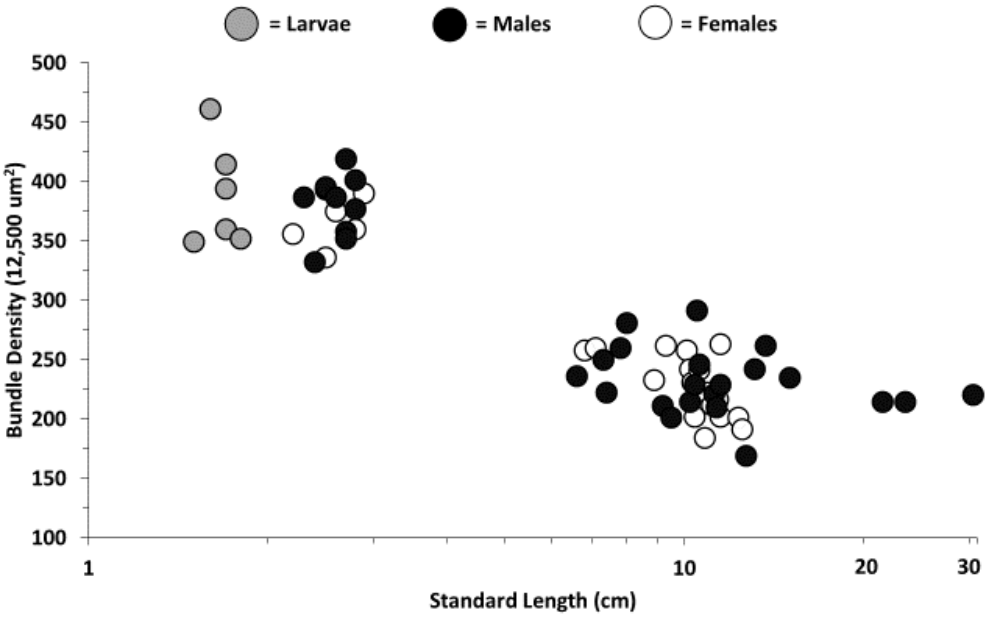

Figure 3.4. Saccular HC bundle density as a function of standard length. HC bundle density decreases as a logarithmic function of standard length. Black circles = males; white circles = females; gray circles = larvae. There was no sexual dimorphic difference in HC bundle density. Note that bundle density was pooled over 5 regions (regions 2 and 7 were excluded, see text), so the y-axis is summed bundle density. Note that larvae could not be differentiated by sex.

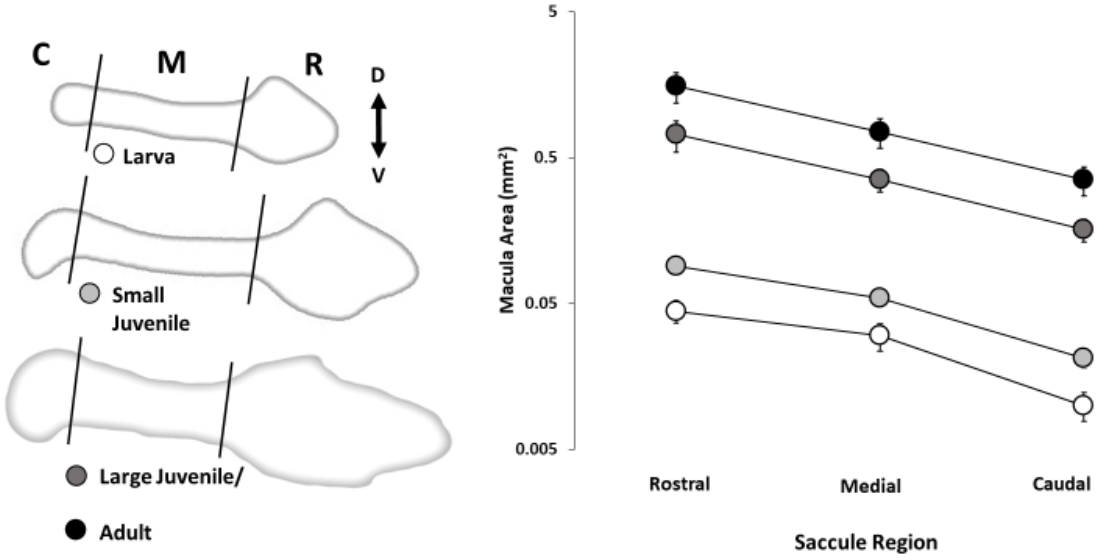

Figure 3.5. Comparisons of saccule area and shape. Left panel: To determine differences in saccular shape, the macula was separated into Caudal (C), Medial (M), and rostral (R) areas. Borders were defined by the abrupt change from horizontal to vertical orientation of HCs between the rostral and medial macula and where HCs begin to transition from vertical orientation to horizontal between the medial and caudal macula. Sketches are not drawn to scale. V = ventral, D = dorsal. Right panel: Log-transformed saccule area was significantly different between all developmental stages with adults having the largest area and larvae having the smallest area.

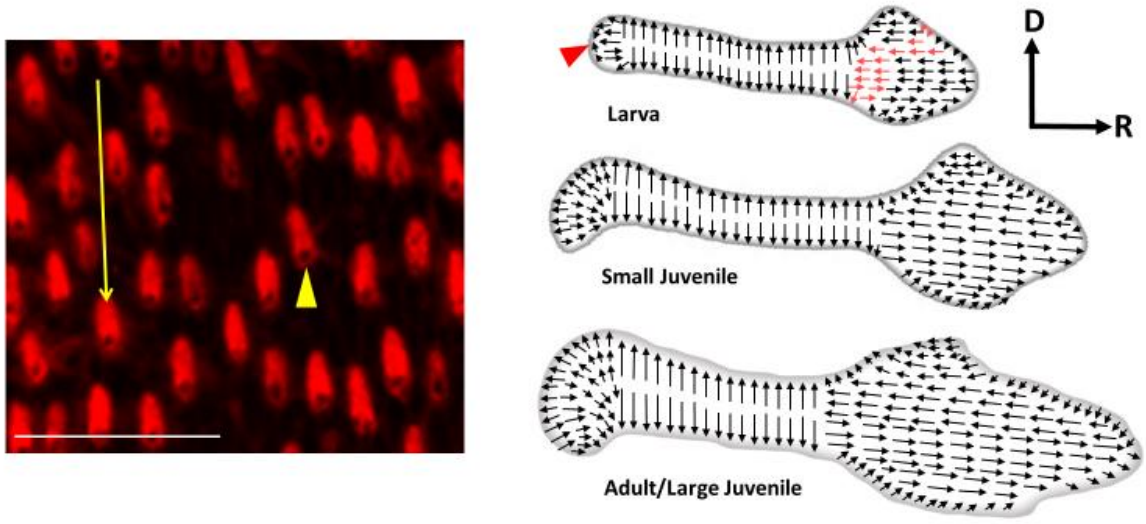

Figure 3.6. Hair cell orientation patterns. Left panel: Representative image of an adult's saccular macula. HC orientation was determined by visualizing the location of the kinocilium on the cuticular plate (yellow triangle). The yellow arrow denotes direction of maximum HC depolarization. Hair cell bundles were labeled with phalloidin conjugated to rhodamine which is why the cuticular plate appears red. The white scale bar = 25 um. Right panel: HC orientation patterns in larvae (n = 10), small juveniles (n = 12), and adult/large juvenile saccular maculae (n = 4). Sketches of the maculae are not drawn to scale. The red triangle at the caudal end of larval maculae denotes the difference in HC orientation in this developmental stage compared to the others. The red arrows in the larval sketch were not visualized and are instead estimated HC orientation patterns based on the other developmental stages. D = dorsal, R = rostral.

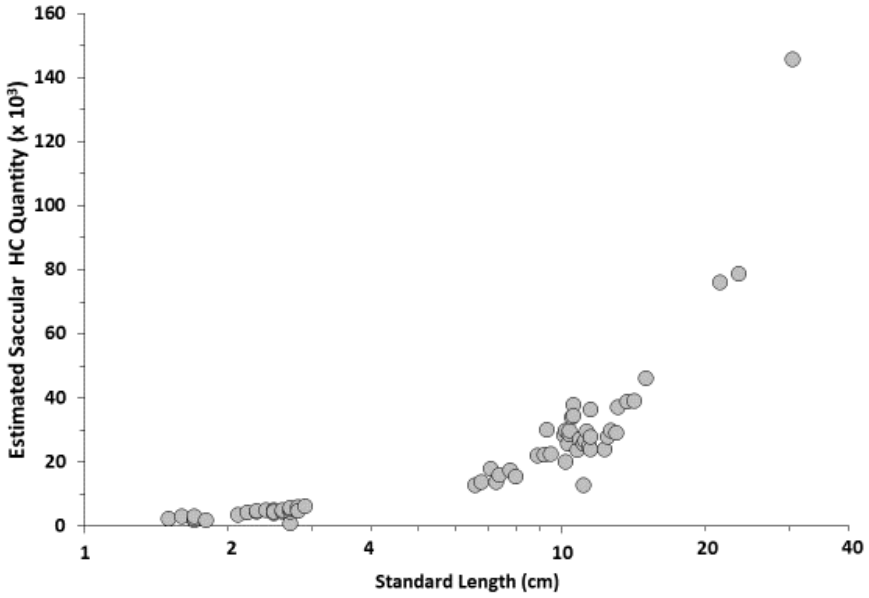

Figure 3.7. Estimated saccular hair cell numbers. The estimated total number of saccular HC increases as a power function of standard length during ontogeny from larvae to adults.

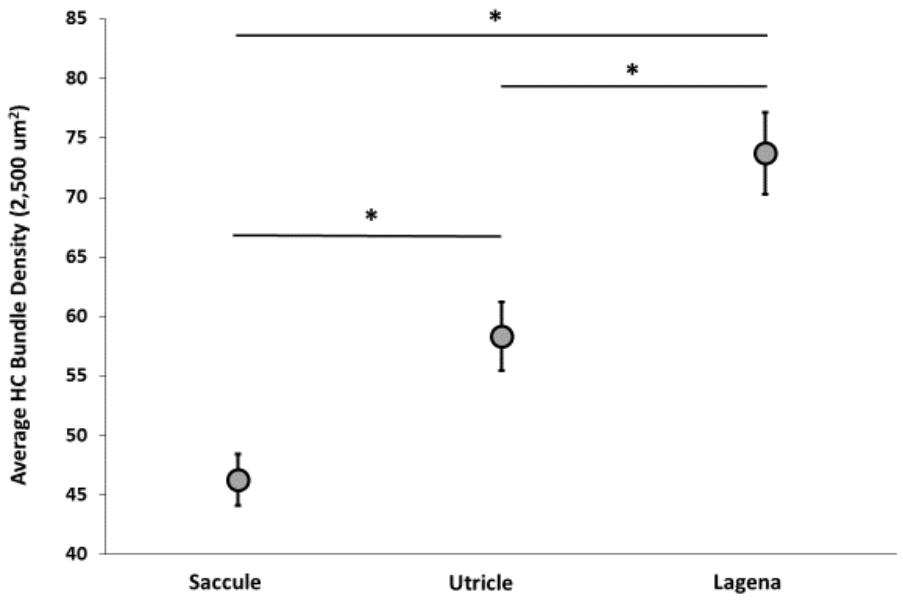

Figure 3.8. Mean hair cell bundle density in the saccule, utricle, and lagena of adult midshipman. Average HC bundle density was greatest in the lagena, intermediate in the utricle, and lowest in the saccule. Asterisks denote statistically significant differences in Bonferroni pair-wise comparisons ($p < 0.05$).

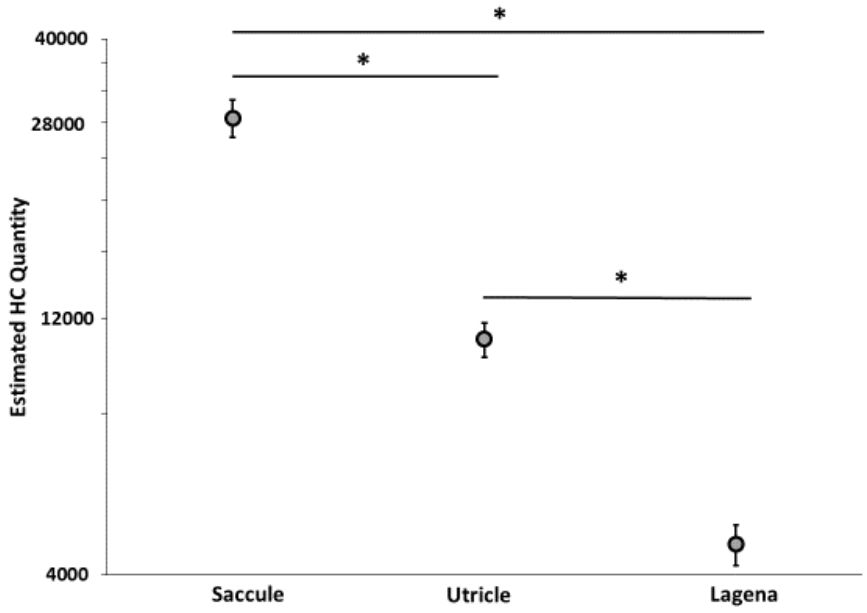

Figure 3.9. Estimated hair cell numbers of the saccule, utricle, and lagena in adult midshipman. Estimated HC numbers were highest in the saccule, intermediate in the utricle, and lowest in the lagena. Asterisks denote statistically significant differences in Bonferroni pair-wise comparisons ($p < 0.05$).

Chapter 4. A reproductive state-dependent change in swim bladder morphology may shift secondary adaptations in the vocal plainfin midshipman fish (*Porichthys notatus*)

4.1 Summary

The swim bladder has multiple adaptive functions in fish including aiding in sound reception and sound production. In the plainfin midshipman fish (*Poricthys notatus*), the swim bladder is known to be sexually dimorphic likely because it aids in sound detection for females. However, in vocal reproductive type I males, it functions in the production of long duration acoustic signals. Type I males vocalize by contracting their sonic muscle which attaches to the swim bladder, and they produce this signal only during the breeding season, which occurs during the summer months. The type I male's sonic muscle hypertrophies in preparation for the breeding season and then atrophies at the end of the breeding season. We hypothesized that the morphology of the swim bladder changes due to sonic muscle hypertrophy in the summer as type I males transition into the reproductive state. We tested this hypothesis by imaging intact swim bladders using computerized tomography (CT) scanning in type I male midshipman in the reproductive and nonreproductive states. We found that the proximity of the swim bladder rostral extensions to the inner ear decreases in reproductive type I males, which may be an adaptation to minimize stimulation of their own inner ears when vocalizing. This morphological change is likely due to the rostral extensions of the swim bladder being pushed medially by hypertrophied sonic muscle because the lateral distance between rostral extensions was shorter in reproductive males, and lateral distance negatively correlated with sonic muscle mass. We discuss a possible

functional change of the swim bladder as an accessory auditory organ into a sonic organ as type I males transition into the reproductive state.

4.2 Introduction

Most teleost fish contain an internal gas-filled swim bladder which, depending on the species, may have multiple functions including respiration, buoyancy regulation, vocalizing, and aiding in auditory reception of sound (for review see Alexander, 1966; Braun and Grande 2008; Fine and Parmentier, 2015). Therefore, the shape of the swim bladder often varies between species depending on its adaptative uses. A widely studied example are the various morphological changes in the swim bladder that have evolved in species where pressure detection of high frequency sound is important for survival. In otophysans, where detection of the auditory environment likely introduces a strong selective pressure, Weberian ossicles (bones that mechanically couple the swim bladder to the inner ear) have evolved to increase spectral sensitivity. Similarly, rostral extensions of the swim bladder or otic bullae have evolved in other species which also allows for pressure detection and increased spectral sensitivity (for review see Fay and Popper, 2000; Braun and Grande, 2008).

Swim bladder morphology is sexually dimorphic in at least one species of minnow (Conway et al., 2014), multiple species in the order ophidiiformes (including blennies and cusk eels) (Rose, 1961; Courtenay, 1971; Kéver et al., 2012), and in the order batrachoidiformes (toadfish and midshipman) (Mohr et al., 2017). In some species of ophidiiformes and batrachoidiformes, there is also a sexual dimorphism in swim-bladder associated sonic muscles used for vocalizing (Kéver et al., 2012; Bass and Marchaterre, 1989; Fine et al., 1990). In batrachoidiformes, males of multiple species have much larger vocal muscles than females because production of

courtship vocalizations is extremely important for their reproductive success (Bass and Marchaterre, 1989; Fine et al., 1990).

The plainfin midshipman fish (*Porichthys notatus*) is one such batrachoidid species that is known to have inter and intra-sexual dimorphisms of the sonic muscle (Bass and Marchaterre, 1989; Brantley et al., 1993). Midshipman rely on acoustic communication for courtship and agonistic encounters during the summer breeding season. Nest guarding males produce long duration continuous courtship vocalizations by contracting the sonic muscles that laterally encapsulate and intrinsically attach to the swim bladder. When the sonic muscles contract, they vibrate the gas-filled swim bladder, which produces a multi-harmonic call or "hum" with a fundamental frequency centered around 100 Hz (Brantley and Bass, 1994). Reproductive females utilize this vocalization for the localization of reproductive males to spawn (McKibben and Bass, 1998). A divergent male morph, referred to as type II, employs an alternative reproductive strategy and satellite spawns to steal fertilizations from the vocal nest-guarding type I males (Brantley and Bass, 1994). Therefore, vocalizations are not important in this male subtype for reproductive success. Sonic muscles undergo annual hypertrophy in type I males in preparation for the breeding season. Type I male sonic muscles begin to atrophy at the end of the breeding season and become more similar to the sonic muscles of females and type II males in winter (Sisneros et al., 2009).

Previous work has found an inter- and intra-sexual dimorphism in midshipman swim bladder morphology where reproductive females and type II males have horn-like elongated rostral extensions of the swim bladder in close proximity to the inner ear (Mohr et al., 2017). In females, this was found to increase physiological sensitivity in the saccule and lagena, two of the putative auditory end organs in teleosts (Colleye et al., 2019; Vetter and Sisneros, in press).

Because reproductive type I males lack these elongated rostral extensions, they are likely not reliant on the swim bladder as a sound detecting organ but rather as a sound producing organ (Mohr et al., 2017). The proximity of the swim bladder to the inner ear in nonreproductive type I males, when sonic muscles have atrophied, has not been examined.

While the morphology of the swim bladder is known to vary between species, throughout ontogeny, or between sexes, less is known about how the swim bladder may change within the adult life history stage due to reproduction or parental care. In the mouthbrooding African cichlid (*Astatotilapia burtoni*), the shape of the swim bladder changes to accommodate the shift in weight as parental females hold developing fry in their mouths. This alteration in shape is due to a change in proportion of gas volume in the anterior vs. posterior chambers of the swim bladder to maintain stable posture in the water column (Butler et al., 2017). There have been no studies examining possible reproductive state-dependent changes in the structure of the swim bladder related to a change in secondary adaptations to date.

We hypothesized that swim bladder morphology changes in type I male plainfin midshipman as they transition into the reproductive state. We posited that the swim bladder rostral extensions are pushed medially in the summer due to the previously documented sexually dimorphic sonic muscle hypertrophy in preparation for the breeding season. To test this hypothesis, we imaged intact swim bladders in winter-collected, nonreproductive type I males vs. summer-collected, reproductive type I males via computerized tomography (CT) scanning. We then measured the shortest distance of the swim bladder to the inner ear auditory end organs (lagena, saccule, and utricle), the swim bladder rostral extension length, the rostral extension shape, and the lateral distance between the rostral extensions. We found that the shortest distance of the swim bladder to the inner ear increases in reproductive type I males. This change may be due to the

hypertrophy of sonic muscles because the lateral distance between the rostral extensions was shorter in reproductive type I males, and the lateral distance negatively correlated with sonic muscle mass. How this change in swim bladder morphology may relate to a shift from an accessory auditory organ to a vocal organ is discussed.

4.3 Methods

4.3.1 Animal collection/care

Nonreproductive type I male midshipman (n = 9) were collected in Monterey Bay near Moss Landing, CA in January 2020 via bottom otter trawl. Size-matched reproductive type I males (n = 7) were collected by hand at low tide in Tomales Bay near Marshall, CA in June 2020. Summer-collected fish were kept at 12-hour light/12-hour dark photoperiod and winter-collected fish at 8-hour light/16-hour dark photoperiod to mimic the external environment at the time of collection. All fish were housed in 50-gallon recirculating water tanks at 25 ppt salt water and 13°C for seven days or less prior to sacrifice via benzocaine overdose and CT scanning. All experimental protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

4.3.2 CT scanning

The nonreproductive cohort was scanned on a SkyScan 1173 High Energy microCT with scans ranging in resolution from 35.1 - 35.5 um/pixel. Reproductive type I males were imaged on a NSI X5000 CT scanning system with scan resolution ranging from 57.7 – 62.9 um/pixel. Volumes for nonreproductive males were reconstructed on NRecon software (Micro Photonics Inc., Allentown, PA), while volumes for reproductive males were reconstructed using efX-CT software (North Star Imaging, Inc., USA).

4.3.3 Morphometric analyses

Following scans, fish were removed from the scanner and morphometrics including standard length (SL), swim bladder length, and mass of gonads and sonic muscle were measured. Gonadosomatic index (GSI) and sonic muscle somatic index (SMSI) were calculated by dividing gonad or sonic muscle mass, respectively, by body mass excluding gonad or sonic muscle mass, multiplied by 100. All reconstructed CT images were rendered as 3D volumes using Drishti version 2.6 software. Swim bladders were separated using the Drishti paint program, while otoliths were separated by altering the histogram threshold of the transfer function in Drishti. Measurements of the shortest distance of the swim bladder to otoliths were made using the point tool and path commands in Drishti. Dorsal snapshots of the 3D rendered swim bladders were analyzed for rostral extension length, 2D shape, and lateral distance between the rostral extensions via Image-J. Rostral extension length was operationalized as the rostral-most point of each swim bladder extension to the rostral-most point on the circumference of the width of the swim bladder. Rostral extension shape was analyzed by measuring the width of the caudal base of each swim bladder extension (where the swim bladder extension meets the rostral-most point of the circumference of the swim bladder width) and then calculating the aspect ratio of extension length/caudal base width. The lateral distance between the rostral extensions was measured as the distance between the rostral-most points (for visual representations of all measurements see figure 4.1). For all measurements (except for lateral distance between rostral extensions), both swim bladder extensions were measured and the average value was used for statistical analyses.

4.3.4 Statistical analyses

Potential differences in SL, GSI, and SMSI were compared between reproductive and nonreproductive type I male midshipman via independent samples t-tests. Absolute distances of the swim bladder rostral extensions to the auditory end organ otoliths were compared between reproductive and nonreproductive males via one-way MANOVA. These distances were normalized to SL and also analyzed via MANOVA. SL normalized swim bladder rostral extension length, absolute lateral distance between swim bladder rostral extension comparisons, and rostral extension aspect ratio (length/base width) between reproductive and nonreproductive type I males were compared via independent samples t-tests. Lastly, potential associations between SMSI and normalized rostral extension length and SMSI and lateral distance between each extension were analyzed with Pearson's correlations.

4.4 Results

4.4.1 Morphometrics

Gonadosomatic index (GSI) calculations validated that winter-collected males were in the nonreproductive state (mean = 0.08 ± 0.04 SD) and had significantly lower GSI than reproductive males (mean = 1.3 ± 0.64 SD) ($p < 0.05$). Similarly, sonic muscle somatic index (SMSI) was significantly lower in nonreproductive type I males (mean = 0.49 ± 0.13 SD) compared to reproductive males (mean = 1.61 ± 0.19 SD) ($p < 0.05$), indicating that reproductive males had much larger sonic muscles independent of fish size (see figure 4.2). There was no significant difference in standard length (SL) between reproductive type I males (mean = 13.5 ± 0.92 cm; range = 12.4 – 14.9 cm) and nonreproductive type I males (mean = 12.3 ± 1.23 cm, range = 11.0 – 14.5 cm) ($p = 0.052$).

4.4.2 Swim bladder distance to otoliths

One-way MANOVA revealed that the absolute distance from the swim bladder to the auditory inner ear was significantly greater in nonreproductive type I males compared to reproductive males ($F_{(3,12)} = 6.7$, $p < 0.05$). For separate end organ comparisons, the distance was greater from the swim bladder to the lagena, saccule, and utricle in reproductive type I males (mean distance to lagena = 5.7 ± 1.3 mm SD; mean distance to saccule = 6.3 ± 1.5 mm SD; and mean distance to utricle = 10.0 ± 1.6 mm SD) compared to nonreproductive males (mean distance to lagena = 3.8 ± 0.9 mm SD; mean distance to saccule = 3.7 ± 1.2 mm SD; and mean distance to utricle = 7.6 ± 1.6 mm SD) ($p < 0.05$). Distances were then normalized to SL and compared. Again, the normalized distances from the swim bladder to the inner ear were greater for reproductive type I males compared to nonreproductive males ($F_{(3,12)} = 7.4$, $p < 0.05$). In individual comparisons of end organs, normalized distance to the saccule was greater in reproductive type I males (mean = 0.047 ± 0.01 SD) compared to nonreproductive males (mean = 0.030 ± 0.01 SD) and lagena (reproductive type I male mean = 0.042 ± 0.01 SD vs. nonreproductive type I male mean = 0.031 ± 0.01 SD) ($p < 0.05$). There was no difference in normalized distances to the utricle (reproductive type I male mean = 0.074 ± 0.01 SD vs. nonreproductive type I male mean = 0.062 ± 0.01 SD) ($p > 0.05$) (see figure 4.3).

4.4.3 Swim bladder rostral extension length

There was no difference in SL normalized rostral extension length between nonreproductive type I males (mean = 0.06 ± 0.01 SD) and reproductive males (mean = 0.05 ± 0.01 SD) ($p > 0.05$). There was no significant correlation between SMSI and normalized rostral extension length ($r = -0.34$, $p > 0.05$) (see figure 4.4).

4.4.4 Swim bladder rostral extension shape

There was no difference in rostral extension aspect ratios between reproductive (mean = 1.76 ± 0.4 SD) and nonreproductive type I males (mean = 1.63 ± 0.4 SD) ($p > 0.05$). However, there was a significant difference in the lateral distances between the rostral extensions where reproductive type I males had on average shorter distances (mean = 2.5 ± 0.8 mm SD) compared to nonreproductive males (mean = 4.3 ± 1.1 mm SD) ($p < 0.05$). Lateral distance between swim bladder rostral extensions was negatively correlated with SMSI ($r = -0.63$, $p < 0.05$) (see figure 4.5).

4.5 Discussion

We found that the shortest distance of the swim bladder rostral extensions to the inner ear is greater in reproductive type I males than nonreproductive type I males. This increase in distance in reproductive type I males coincides with a shorter lateral distance between the rostral ends of the swim bladder extensions. This medial shift in the orientation of the rostral extensions is likely the mediating factor of reduced proximity of the swim bladder to the inner ear because neither the length nor the aspect ratio of the swim bladder rostral extensions changed. Sonic muscle mass negatively correlated with lateral distance between the rostral-most point of the rostral extensions and may have caused this medial shift due to hypertrophy in reproductive type I males. These changes may occur as the secondary function of the swim bladder shifts from an accessory auditory organ in nonreproductive type I males into a vocal organ as they transition into the reproductive state.

The increase in the distance of the swim bladder to the inner ear in reproductive type I males may be protective to avoid overstimulation of an individual's inner ears while producing the long duration hum. Type I males are known to vocalize at amplitudes over 150 dB re 1 uPa for over an hour at a time and high amplitude, long duration sounds can cause inner ear damage in fish

(McCauley et al., 2003). Swim bladder ablations in the blue gourami (*Trichogaster trichopterus*) and oyster toadfish (*Opsanus tau*), which lack an otophysic connection, showed that there was no pressure sensitivity in these species (Yan et al., 2000), so the apparent lack of an otophysic connection in reproductive type I males may be enough to reduce stimulation of their own inner ears when vocalizing.

In addition to increasing the distance to the inner ears, the medial shift of the swim bladder rostral extensions may also affect the directionality of sound radiation produced by the swim bladder as type I males vocalize. Indeed, Barimo and Fine (1998) found that the shape of the swim bladder and location of the intrinsic sonic muscles in the closely related oyster toadfish likely evolved to produce a tradeoff between maximizing omnidirectional radiation of the produced sound while minimizing excitation of an individual's inner ear. They propose that the lower intensity in the plane of maximum saccule excitation may be due to the indentation and bifurcation of the swim bladder at the rostral midline (Barimo and Fine, 1998). In contrast, the sonic muscle in midshipman seems to push the bifurcated rostral extensions medially based on our results. Future studies recording radial sound amplitude and particle motion vectors of vocalizing type I males will examine how the change in swim bladder morphology may affect the directional propagation of sound in vocalizing type I male plainfin midshipman and how the sound propagates in the plane of maximum excitation of the ears.

Brantley et al. (1993) induced sonic muscle hypertrophy in juvenile type I male midshipman via androgen implants. However, this did not lead to an apparent medial shift in swim bladder rostral extensions (see photographs in figure 1 of Brantley et al., 1993). This is likely because the change in juvenile type I male sonic muscle somatic index (SMSI) was only 2x greater following androgen implant (Brantley et al., 1993) as opposed to a 3x difference in SMSI between

reproductive and nonreproductive adult type I males in this study. Consistently, Sisneros et al. (2009) found a 3x greater SMSI in nesting type I males compared to nonreproductive type I males. Therefore, there may have been an apparent medial shift of swim bladder horns in androgen-implanted juveniles if muscle mass increased at the higher rate described in adults. Alternatively, the morphology of the swim bladder may differ in juvenile compared to adult type I male midshipman (i.e., longer swim bladder rostral extensions relative to fish size in juveniles) independent of effect from the intrinsically attached swim bladder. Future work examining possible sexually dimorphic swim bladder morphology throughout ontogeny in midshipman will shed further light on this question.

Swim bladder rostral extension proximity to the inner ear in nonreproductive type I males is intermediate when comparing distances in reproductive females, reproductive type II males, and reproductive type I males. Reproductive females had an average size (SL) normalized distance of 0.021 to the saccule (0.017 in type II males), 0.024 to the lagena (0.020 in type II males), and 0.042 to the utricle (0.043 in type II males) (Mohr et al., 2017) compared to 0.030, 0.031, and 0.060, respectively, in nonreproductive type I males in this study. Size normalized measurements for reproductive type I males in this study (0.047 saccule, 0.042 lagena, and 0.074 utricle) are slightly longer, but match closely with measurements for reproductive type I males in the previous study (0.040 saccule, 0.036 lagena, and 0.067 utricle). This suggests that females and type II males have slightly longer rostral swim bladder extensions even when type I male sonic muscle has atrophied. Indeed, the mean normalized swim bladder rostral extension length was 0.070 in reproductive females and 0.077 in type II males (Mohr et al., 2017) compared to 0.06 in nonreproductive type I males, suggesting a slight inter and intra-sexual dimorphism in extension length. Unfortunately, sonic muscle mass was not recorded in females or type II males

preventing a direct comparison, though previous results by Brantley et al. (1993) found that pre-reproductive type I males have 2x greater sonic muscle mass than juvenile females and type II males. Thus, nonreproductive type I males may have shorter rostral extension length than reproductive females and type II males due to slightly larger sonic muscles. Our results in comparison to those by Mohr et al. (2017) suggest that in the winter, nonreproductive type I males may be pressure sensitive, though likely not as sensitive as reproductive females or type II males. This may be due to weaker selection pressures on the morphology of the swim bladder in type I males as an accessory auditory organ because they do not rely on sound source localization for reproductive success as females and type II males likely do. The magnitude to which swim bladder ablations affect physiological sensitivity in nonreproductive or reproductive type I males remains to be tested.

Hair cell orientation patterns on the saccule (the primary auditory organ in teleosts) in type I male midshipman may be oriented to receive indirect stimulation from the gas-filled swim bladder. Indeed, hair cell orientation patterns in other teleost species have been found to be associated with otophysic connections. Popper has defined multiple hair cell orientation patterns including the "standard pattern" which is common in species lacking otophysic connections and the "dual pattern" which is similar to the general pattern except for an abrupt change in hair cell orientation pattern from vertical to horizontal at the caudal end of the macula. Many species with the dual pattern have an otophysic connection (Popper, 1977; Popper, 1981; for review see Popper and Schilt 2008). By definition, midshipman have a standard hair cell orientation pattern because the vertical orientation of hair cells in the medial saccular macula gradually shifts to a horizontal pattern as the macula curves at the caudal end (Coffin et al., 2012; Lozier and Sisneros, in prep). Functionally, however, the hair cells in the caudal end of the macula may be

similar to those in the dual pattern of hair cell orientation, depending on the innervation patterns of afferent fibers (i.e., if specific afferent fibers only innervate hair cells oriented horizontally in the caudal macula). The fact that there is no sexual dimorphism in hair cell orientation patterns in males and females further suggests, albeit it indirectly, that both type I males and females may be pressure sensitive, at least for part of the year in type I males.

Interestingly, type I males have been found to have a reproductive state-dependent increase in hair cell density throughout the saccule (Lozier and Sisneros, 2019). This increase in hair cell density likely contributes to enhanced auditory sensitivity as it was also found that reproductive type I males have lower auditory evoked hair cell potential thresholds than nonreproductive type I males (Rohmann and Bass, 2011). This change in hair cell density and increase in physiological sensitivity may be an adaptation to compensate for a decrease in auditory sensitivity due to the loss of pressure sensitivity in reproductive type I males. Future work examining the effects of swim bladder ablation on hair cell evoked potentials in reproductive vs. nonreproductive type I males will aid in the understanding the changes to auditory reception in type I males throughout the reproductive cycle.

This is to our knowledge the first recorded example of a change in swim bladder morphology in a male teleost due to reproductive state. Butler et al. (2017) found that the morphology of the swim bladder changes in the female African cichlid depending on parental state when hydrostatic position in the water column is affected by mouthbrooding. This change is rapid (on the order of minutes) due to a shift in the ratio of gas in the anterior vs. posterior chambers of the swim bladder, and quickly reverses again as the fry become free-swimming and are released from their mouth (Butler et al., 2017). This previous study was the first to find that the swim bladder changes in shape to balance a weight shift, however, the function of the swim bladder

(maintaining posture and position in the water column) remained the same. Our results suggest that the change in swim bladder shape in reproductive type I male plainfin midshipman occurs because the function of the swim bladder shifts to a sound producing organ when needed to produce courtship vocalization and back to a sound detecting organ during the non-breeding season.

4.6 Figures

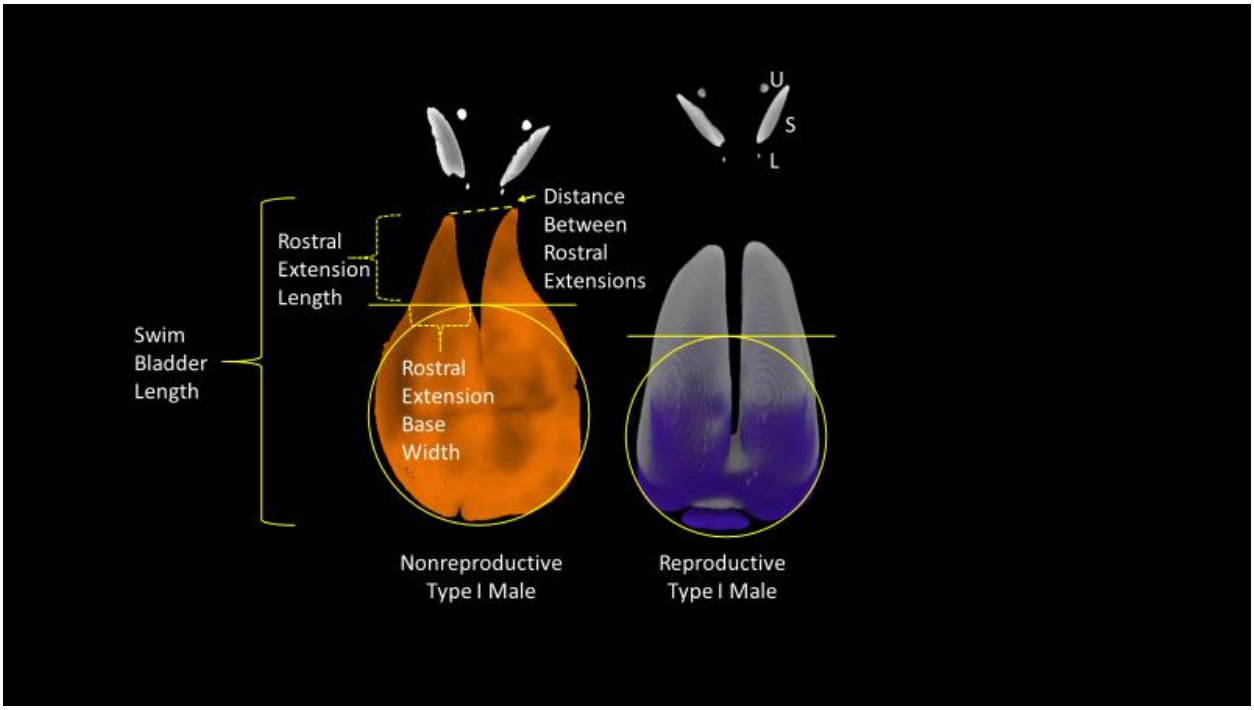

Figure 4.1. Representative dorsal 3-dimensional renderings of swim bladders of a nonreproductive type I male (orange, left, 13.8 cm SL) and reproductive type I male (violet, right, 14.4 cm SL) and inner ear otoliths. Measurements of rostral swim bladder extensions, rostral extension aspect ratio, and distance between rostral extensions were made on similar dorsal 3-D renderings of all individuals. Swim bladders have been pseudo-color filled using Drishti rendering software. 3-D renderings are of separate scans and have not been precisely matched by size. U = utricle otolith; S = saccule otolith; and L = lagena otolith. R = rostral; C = caudal.

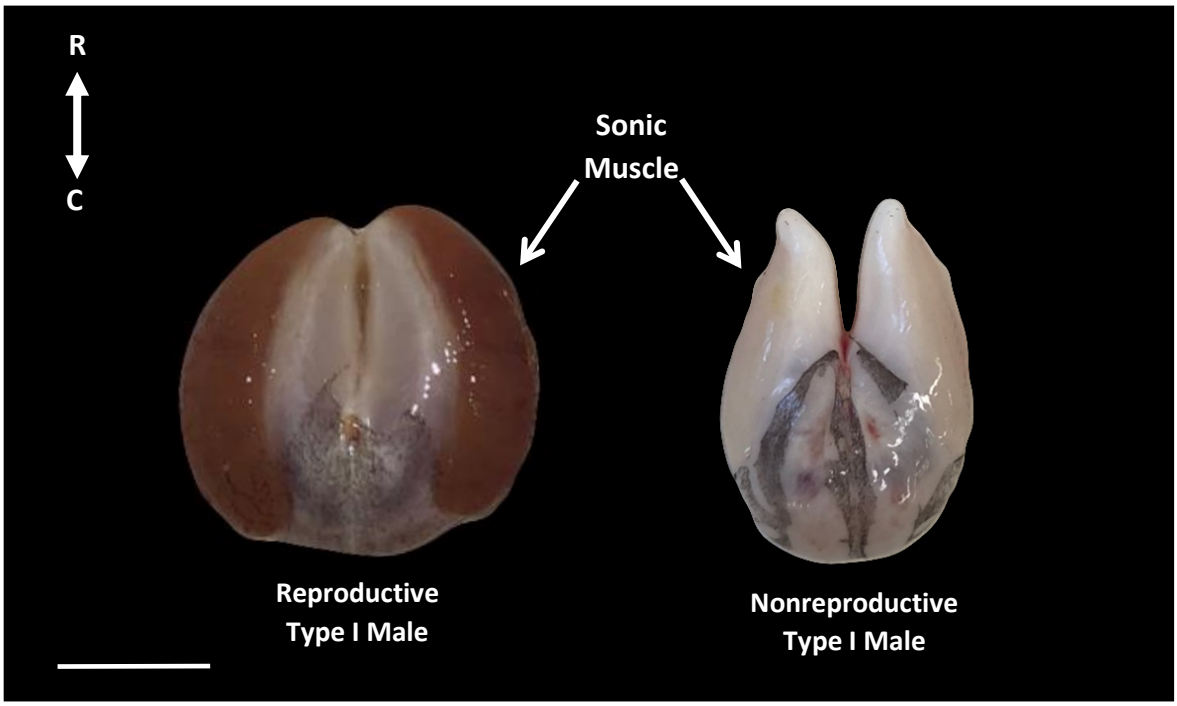

Figure 4.2. Representative swim bladders of a reproductive (left) and nonreproductive type I male plainfin midshipman. Rostral extensions of the swim bladder are oriented medially in reproductive type I males while they are oriented laterally in nonreproductive type I males. Note the pigmented, enlarged sonic muscle in reproductive compared to nonreproductive type I males. Scale bar = 1 cm. R = rostral; C = caudal.

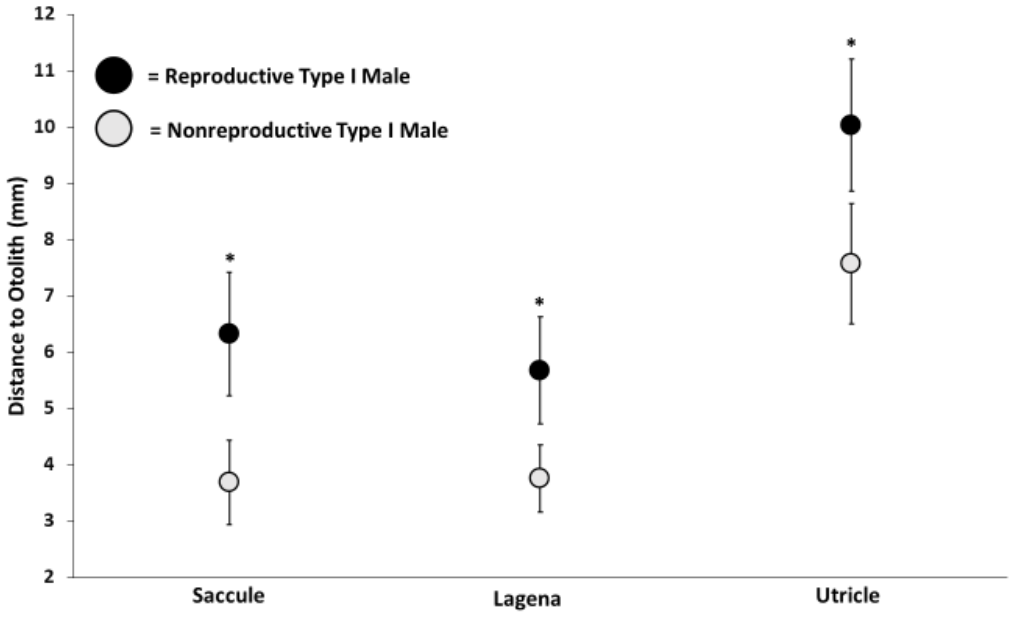

Figure 4.3. Shortest distance of the swim bladder to the otoliths of the three putative auditory end organs, saccule, lagena, and utricle. When comparing the distance to each inner ear end organ individually, the distance was significantly greater for reproductive type I males compared to nonreproductive type I males in the saccule, lagena, and utricle. Asterisks denote statistical significance ($p < 0.05$).

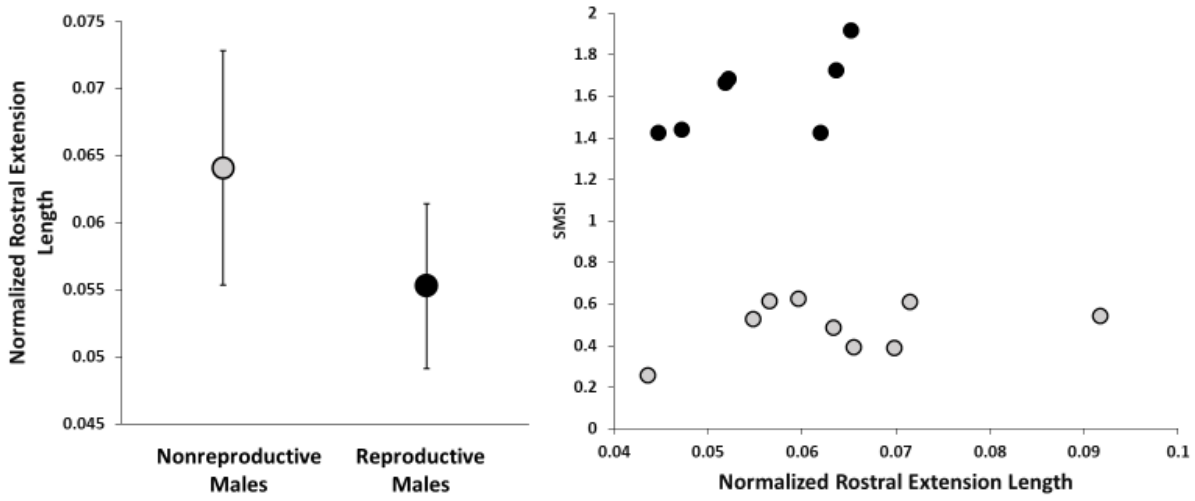

Figure 4.4. Swim bladder rostral extension length normalized to body size (SL) and sonic muscle somatic index (SMSI) as a function of normalized rostral extension length. Left panel: There was no difference in normalized rostral extension length in reproductive type I males compared to nonreproductive type I males ($p > 0.05$). Right panel: SMSI was not correlated with normalized rostral extension length ($p > 0.05$).

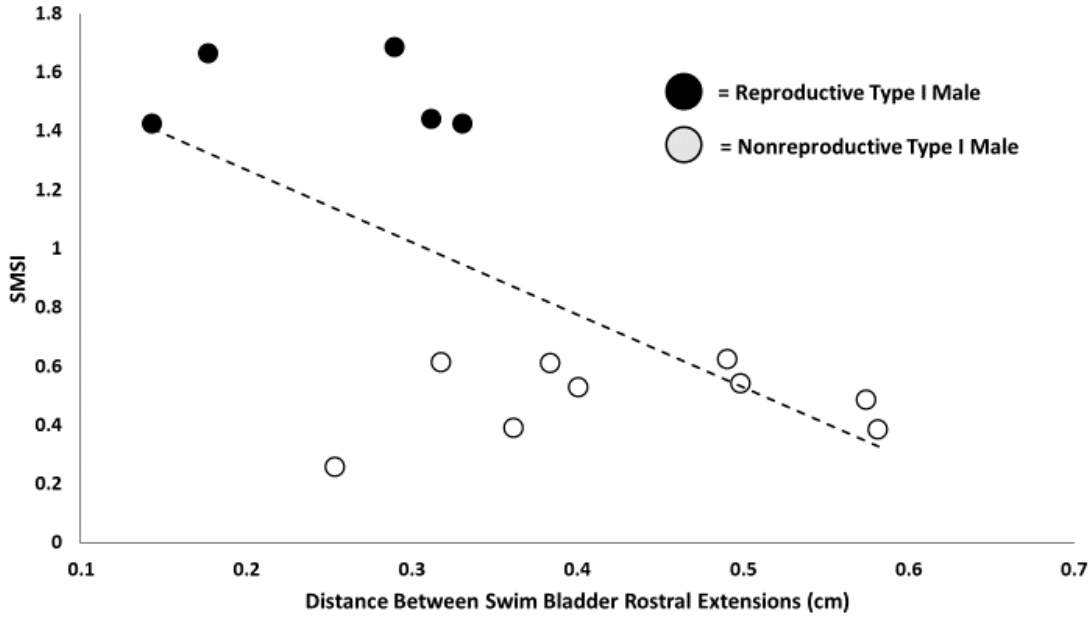

Figure 4.5. Sonic muscle somatic index (SMSI) as a function of absolute distance between swim bladder rostral extensions. There was a negative linear correlation between SMSI and distance between swim bladder rostral extensions ($p < 0.05$).

Chapter 5. Summary and Future Directions

This dissertation describes changes to the auditory end organs and swim bladder in plainfin midshipman fish related to auditory sensitivity. I used fluorescence confocal microscopy and computerized tomography scanning techniques to conduct this research. This final chapter summarizes these findings individually and then discusses possible future directions using the midshipman as a model organism.

5.1 Reproductive state-dependent changes in hair cell density in type I males

In chapter 2, I examined changes to the auditory inner ear end organs due to reproductive state in type I male plainfin midshipman. I found that saccular hair cell density increased in reproductive type I male midshipman, while there was no effect of reproductive state on hair cell density in the other two putative auditory end organs, the lagena and utricle. These changes in saccular hair cell density may be due to direct transdifferentiation of saccular support cells as the ratio of support cells to hair cells decreased in reproductive type I males, but the density of overall apical cells (hair cells + support cells) did not change. Also, there was a sexual dimorphism in hair cell density between reproductive females and reproductive type I males as females had greater hair cell density.

The finding that density of support cells changes due to reproductive state implies that transdifferentiation is a mechanism for increased hair cell density in reproductive type I males, but this interpretation is indirect. To more completely answer the question regarding the cellular mechanism of increased hair cell density, markers for cell division and cell death, such as BrdU and cleaved caspase 3 (the active form of the apoptosis protease), respectively, will be needed to more clearly elucidate cell turnover in the saccule. Additionally, measuring these changes both

as the fish transition into the reproductive state and as they transition back into the nonreproductive state using these markers will give a clearer picture of how the inner ear changes over time, as opposed to the two timepoints (nonreproductive vs. reproductive state) used in this study.

Another interesting question to pursue is why hair cell density changes in the saccule, but not the utricle or the lagena. The localization of aromatase, androgen receptor, and estrogen receptors have been described in great detail in the saccule (Forlano et al., 2015), suggesting that estradiol (E2) and testosterone (T) mediate these reproductive state-dependent changes, but the localization of these proteins is unknown in the utricle and lagena. Future immunohistochemistry studies may show different expression patterns of these proteins in these end organs which may help explain why these end organs do not experience reproductive state-dependent changes in hair cell density.

5.2 Ontogeny-dependent changes in saccule morphology

In chapter 3, I discussed changes to the saccule throughout ontogeny (larval, small juvenile, large juvenile, and adult midshipman developmental stages) including hair cell density, total hair cell number, hair cell orientation, saccular macula shape, and macula size. I found that midshipman, like most other studied teleosts, have an increase in saccular macula area throughout ontogeny and a reduction in hair cell density. However, because the macula increases in size throughout early ontogeny and into the adult life history stage, overall hair cell numbers increase throughout development. I also found that generally the shape of the macula is retained with some slight differences in the caudal area between developmental stages.

Another interesting finding is that hair cells were oriented in the "standard pattern" throughout ontogeny (Popper and Schilt, 2008). Functionally, however, this orientation may be more like the "dual pattern" which is found in many species with an otophysic connection due to horizontally oriented hair cells at the caudal end of the saccular macula. Investigating the innervation patterns of saccular afferents throughout ontogeny and the maximum planes of afferent excitation will give a clearer explanation for the functional importance of saccular hair cell orientation in midshipman. Additionally, modeling the direction and amplitude of otolith oscillations throughout ontogeny, both with and without input from the swim bladder, will give insight into how orientation of hair cells relates to indirect displacement of otoliths due to swim bladder resonance.

5.3 Reproductive state-dependent change in swim bladder morphology in reproductive type I males

In chapter 4, I described a change in morphology of the swim bladder in reproductive compared to nonreproductive type I males. The rostral extensions of the swim bladder were pushed medially and the distance to the inner ear otoliths were found to increase in reproductive type I males. The change in morphology of the swim bladder is likely due to the hypertrophy of sonic muscles as adult type I males transition into the reproductive state. This is the first recorded example of a change in swim bladder morphology due to reproductive state in the context of communication in any species of fish. I proposed that the change in morphology may be adaptive to prevent stimulation of their own ears when type I males produce their long duration, loud courtship vocalizations (Brantley and Bass, 1994). However, in the winter, when sonic muscles have atrophied, the swim bladder may function as an accessory auditory organ (which has been

demonstrated in reproductive females (Mohr et al., 2017)) in addition to its role in buoyancy regulation.

These results demonstrate a change in the morphology of the swim bladder, likely due to androgen-mediated hypertrophy of sonic muscles in reproductive type I males that pushes the rostral extensions medially. Future studies should examine the functional significance of this morphological change. Swim bladder ablation experiments in both reproductive and nonreproductive type I males will test the hypothesis that the swim bladder enhances auditory sensitivity in the nonreproductive state. Also, recording radial sound pressure amplitudes and particle motion vectors in vocalizing males will explain how the reproductive state-dependent change in swim bladder morphology might protect the inner ears of vocalizing males.

5.4 Future Directions

In summary, the results of this dissertation describe changes to the inner ear throughout ontogeny related to auditory sensitivity and changes to the inner ear and swim bladder in type I male midshipman depending on reproductive state related to acoustic communication. In the previous sections I suggest incremental next steps specific to these questions. In this section, I will discuss future directions in a broader context.

5.4.1 Why do midshipman have a functioning auditory system so early in development?

Physiological (Sisneros and Bass, 2005; Alderks and Sisneros, 2011) and behavioral (Alderks and Sisneros, 2013) studies have shown that midshipman are capable of hearing at the juvenile and larval stages. Why it might be adaptive for larvae to have a functioning auditory system is unknown. One possibility is that the auditory system of larvae is important for survival and used to evoke an acoustic startle-like response to the loud male advertisement calls which facilitates

the detachment of developed juveniles from the nest and prevents them from being cannibalized by other nesting males. Type I male midshipman are known to cannibalize larvae from nests (Sisneros et al., 2009). Additionally, juveniles may use features of the acoustic soundscape of the intertidal zone to move into the deeper seagrass beds for safety, a behavior which has been described in reef dwelling juvenile fish (Parmentier et al., 2015). Future studies examining the behavioral response of juveniles to playback of various underwater sounds from biologically relevant habitats and male vocalizations may provide information on the adaptive role of a functioning auditory system so early in development.

5.4.2 What are the molecular mechanisms of reproductive state-dependent changes in hair cell density?

One very exciting future avenue of research on this topic is investigating the molecular mechanisms underlying the change in saccular hair cell density in reproductive animals. One of the results in chapter 2 suggests that direct transdifferentiation may lead to this change in type I males. This suggests support cells differentiate into hair cells independent of support cell division. Consistent with this finding, there was no difference in levels of phosphorylated histone 3, an indicator of cell division, in the saccular macula of reproductive vs. nonreproductive females. Additionally, reproductive females had less cell death in the saccular macula than nonreproductive females, suggesting a protective effect on hair cells and/or support cells (Coffin et al., 2012). One possibility is that E2 plays a protective role on saccular macula cells as estrogen receptor β has been found to protect mice from sensorineural hearing loss (Meltser et al., 2008; Simonoska et al., 2009). To more clearly understand this mechanism, future investigations could study the effect of E2 and T implants in both nonreproductive type I male

and female plainfin midshipman on saccular hair cell density, support cell division, and support cell and hair cell death.

Another avenue of research could examine the effects of the neurotrophic factors and growth factors in reproductive state-dependent hair cell plasticity in midshipman. In the songbird vocal motor pathway, which similarly undergoes reproductive state-dependent changes in neuron number and size, brain derived neurotrophic factor (BDNF) has been found to mediate T-induced neural plasticity in the HVC, a nucleus in the vocal motor pathway (Rasika et al., 1999; Wissman and Brenowtiz, 2009; for review see Brenowitz, 2013). There is evidence that increased transcription of BDNF is due to an increase in endothelial cells in the HVC due to increased vascular endothelial growth factor (VEGF) signaling (Louissant et al., 2002). VEGF has also been found to increase support cell and hair cell regeneration in the inner ear of chickens following hair cell death due to antibiotic delivery (Wan et al., 2020). There may be a similar relationship between BDNF, VEGF, and gonadal sex hormones in the peripheral auditory system in midshipman. Future studies examining possible changes in the transcription of these ligands and the localization of their receptors (trkB for BDNF and VEGFRs for VEGF) in the inner ear may more clearly explain the molecular mechanism for hair cell plasticity in reproductive midshipman.

5.4.3 Is there a change in the innervation pattern or number of saccular afferents throughout ontogeny and reproductive state in midshipman?

Our findings that hair cell density decreases in midshipman throughout ontogeny may reconcile previous physiological findings in midshipman. Alderks and Sisneros (2011), found that small juveniles have greater magnitude evoked hair cell potentials while Sisneros and Bass (2005) found that adults have lower evoked thresholds (i.e., greater sensitivity) at the level of saccular

afferents. A future study examining the number of saccular afferents throughout ontogeny could explain these differences. Afferents were found to proliferate at a much lower rate than hair cells in auditory end organs in the thornback ray (Corwin, 1983) and the oscar cichlid (Popper and Hoxter, 1984), and there could be a similar process in midshipman. If saccular afferents increase at a lower rate than hair cells throughout ontogeny, this could explain the seemingly conflicting results from recordings at the level of the hair cells vs. the afferents. Greater hair cell density in juveniles may contribute to a larger evoked potential at the level of the hair cells because there are more hair cells in proximity to the recording electrode, while a higher convergence ratio of hair cells to ganglion cells could contribute to more sensitive saccular afferents in adult midshipman.

Similarly, an increase in the convergence ratio of hair cells to saccular afferents of reproductive adult type I male and female plainfin midshipman may explain the adaptive value of increased hair cell density during the breeding season. If there is no change in auditory afferent numbers, and the existing afferents make more synaptic connections with new hair cells, the amount of hair cells providing input to a single afferent will increase overall sensitivity or gain of the auditory afferent neurons. Thus, an overall increase in auditory afferent sensitivity may enhance the ability to detect vocal conspecifics in the breeding environment. Also, there may be a difference in the length of hair cell stereocilia that are added seasonally which could contribute to changes in higher frequency sensitivity. The saccular macula of goldfish was found to be somewhat tonotopically organized, with hair cells in the caudal end being more sensitive to low frequency sounds and those in the rostral end to high frequency sounds (Furukawa and Ishii, 1967). Later it was found that hair cell stereocilia decrease in height from the caudal to the rostral end of the goldfish saccular macula so that hair cell stereocilia height negatively

correlated with frequency of transduction (Platt and Popper, 1984). Interestingly, reproductive female midshipman had more hair cells with short stereocilia bundles than nonreproductive females, though whether these were morphologically distinct mature hair cells or simply immature hair cells is unknown (Coffin et al., 2012). Future studies quantifying saccular afferents, the number and size of synapses between saccular afferents and hair cells, and measuring the length of stereocilia in the hair cells will provide a morphological explanation, at least in part, to the seasonal sensitivity of saccular afferents and hair cells previously found in reproductive females (Sisneros and Bass, 2003).

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