

The Relevance of the Inner Ear and Lateral Line System for Sound Localization in  
Fish

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## **Dedication**

This thesis is dedicated to my family and friends who have provided me with support and encouragement throughout my education.

## **Abstract**

A long standing question in the field of neuroethology is how do fish localize sound? Fish are capable of detecting acoustic information through both an auditory system, the otolithic inner ear, and a mechanosensory system, the lateral line. Both systems are sensory hair cell based and exhibit directional sensitivity responses. Although the fundamental “auditory” anatomy is well characterized in fish, the physiological mechanism of how each system specifically aids in sound source detection is unknown. Using interaural time delays (ITDs) between sensory organs is the well characterized mechanism for terrestrial species sound localization; however, due to the rapid speed of sound underwater and the close proximity of otolithic organs in fish, using ITDs to localize sound is problematic. Alternatively, the conserved lateral line system in fish contains sensory organs located around the entire body, posing a potential sensory system capable of ITDs sufficient for sound localization. The inner ear also plays a dual role functioning in both the auditory and vestibular system, which further questions its potential for acute sound localization during self-induced movements, such as swimming and ventilation. Therefore, the lateral line system may provide additional information about the direction and distance of a sound source that the inner ear in fish cannot.

## **Table of Contents**

List of Tables.....	v
List of Figures.....	vi
Chapter 1: The Perception of Sound Localization through Fish Auditory Systems .....	1
Chapter 2: Bilateral Electrode Implants Help Determine the Relevance of the Anterior Lateral Line for Sound Localization in Toadfish ( <i>Opsanus tau</i> ).....	11
Chapter 3: Future Directions for Determining How Fish Localize Sound.....	37
References.....	39

## **List of Tables**

Table 1. Phase-locking strength and directionality categories.....	27
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## List of Figures

Figure 1. Spontaneous mean neural firing activity of characterized toadfish afferent fibers (N=24).....	28
Figure 2. Neural activity from a bilateral electrode implant.....	29
Figure 3. The effect of distance on evoked spike rate. ....	30
Figure 4. The location of recorded neuromasts innervated by the anterior lateral line nerve. .....	31
Figure 5. Polar plots of neuromast tuning curves .....	32
Figure 6. The effect of distance on the average coefficient of synchronization (R).....	33
Figure 7. Phase-locking comparison between male and female toadfish.....	34
Figure 8. Average time delays between neuromasts of the left and right anterior lateral line system. .....	35
Figure 9. Time delays between afferent fibers of the anterior lateral line nerve .....	36

## **Chapter 1: The Perception of Sound Localization through Fish Auditory Systems**

Reduced visibility under water increases the importance of other sensory systems, such as hearing, for survival of aquatic species. Basic auditory functions (e.g. acoustic feature discrimination, sound localization, frequency analysis, and auditory scene analysis) found throughout even advanced vertebrates evolved early in vertebrate history and were first seen in fish (Fay and Popper, 2000). These conserved functions are specific to sensory hair cells found in auditory structures across most primitive vertebrates. However, these sensory hair cells are located within both auditory and mechanosensory organs of fish. The primary auditory organs of the inner ear in fish play a dual role in the vestibular system, therefore, self-generated movement may obstruct sound localization capabilities of the inner ear. However, the mechanosensory lateral line has an adaptive filter to cancel out self-generated movement, and therefore, may provide sound source direction cues unidentified by the inner ear (Montgomery and Bodznick, 1994). Comparing the differences in acoustic detection by the inner ear and lateral line system in fish will provide knowledge of their relevance for sound localization.

### **Fish Hearing**

Localizing sound as a hearing mechanism remains a crucial, yet complex, necessity for species survival and is likely a major factor in the evolution of the vertebrate auditory system (Popper and Fay, 1997). Sound localization refers to the ability to identify the origin of a sound source in both direction and distance, and aids in communication, predator-prey interactions, and mating behavior for aquatic vertebrates.

Terrestrial vertebrates use binaural cues, such as interaural time and intensity differences, to localize sound (Schnupp and Carr, 2009). However, since water is denser than air, sound travels about 4-5 times faster under water (Au and Hastings, 2008), which may make interaural time differences negligible between the sensory organs of the inner ears in aquatic species (van Bergeijk, 1964). To determine the mechanism of sound localization through aquatic species auditory systems, it is necessary to evaluate what properties of sounds they are responsive to.

In an aquatic environment, fish are exposed to two physical characteristics of sound propagation: pressure waves and particle displacements. Pressure waves are characterized by a cyclic compression and rarefaction of water propagating from an acoustic source in the far-field. Particle displacements are defined as near-field motion of individual molecules (Popper and Fay, 1993; Rogers and Cox, 1988). Although terrestrial species auditory systems respond mainly to pressure waves, the consensus is that all fish primarily respond to the particle displacement characteristic of sound (Fay and Popper, 1999; Montgomery et al., 2006; Platt and Popper, 1981; Popper and Fay, 1993; Rogers and Cox, 1988; reviewed in Vasconcelos et al., 2016), due to the lack of compressibility and rapid speed of sound underwater. Fishes otolithic ear and lateral line system are known to respond to particle displacement, therefore, rather than utilizing an outer and inner ear structure for auditory reception like terrestrial species, fish and some aquatic amphibians use a system including both an inner ear and lateral line (Fay, 1984; Montgomery et al., 1995; Platt and Popper, 1981; Popper and Tavolga, 1981; Zeddes et al., 2010; Zeddes et al., 2012).

## *Inner Ear*

The teleost inner ear consists of three semicircular canals and three otolithic endorgans (saccule, utricle, and lagena). Each endorgan is composed of a dense, calcium composed otolith and a sensory epithelium lined with sensory hair cell bundles. The saccule is considered the primary auditory endorgan in fish (Popper and Fay, 1993; Popper and Fay, 2011); however, there is great diversity of otolithic endorgan size and shape, as well as orientation of the sensory epithelia and hair cell ciliary bundles (Ladich and Schulz-Mirbach, 2016; Popper, 1977) found throughout fish species. In general, otolithic endorgans play a dual role in both the auditory and vestibular system, since any self-generated movement impacts auditory sensitivity, suggesting potential obstacles for acute sound localization during movement (Ladich and Popper, 2004; Platt, 1983; Popper and Lu, 2000; Popper et al., 1982; Schellart and Popper, 1992).

Although the otolithic inner ear seems problematic for acute sound source detection, it is a hair cell based sensory system with directionally sensitive hair cell bundles. Additionally, these sensory hair cells have a variation of orientations innervated by afferent and efferent neurons and, therefore, indicate a direction detecting role (Hudspeth and Corey, 1977; Popper, 1977; Popper and Lu, 2000). When sound travels through a fishes body, the shift of the otoliths in relation to the sensory epithelia bends the hair cells bundles (Flock, 1971; reviewed in Vasconcelos et al., 2016). When water flows parallel to hair cells, a depolarization results in an excitation response, and the comparison of responses between hair cell bundles of differing orientations will elicit sound source direction information.

To behaviorally study acoustic detection and localization in fish, Eaton et al. (1995) conditioned goldfish to an acoustic stimulus by stimulating Mauthner cells in the hindbrain. Stimulation of Mauthner cells initiates a startle reaction (c-start) in fish. A c-start response is a fast escape reflex characterized by the fish's body displaying a c-shape or 180 degree directional change. If the fish successfully detected the location of the sound, they oriented away from the acoustic stimulus using a c-start response. This study demonstrated that fish did determine sound source direction through particle motion inputs from the otolithic end organs (Eaton et al., 1995). However, a consensus for the exact mechanism allowing fish to detect the location of a sound source through the inner ear has not been reached.

### *Lateral Line*

In addition to the inner ear, fish have a lateral line system that also consists of hair cell based sensory organs, located subdermally and on the surface of the skin, that exhibit directional sensitivity (Flock and Wersäll, 1962) and bend in response to incoming mechanical signals. Therefore, both systems have the ability to assist fish in localizing biologically relevant sound (reviewed in Mensinger, 2016). The lateral line responds to water movement and vibrational stimulus in the aquatic environment, similarly to the sense of touch in humans, and remains conserved in all fish species. It is known to be solely sensitive to the particle displacement component of sound (hydrodynamic flow) and insensitive to pressure (Carton and Montgomery, 2002; Coombs and Montgomery, 1999; Montgomery et al., 1997; Montgomery et al., 2000), therefore, having the potential to assist in sound localization at near distances.

Compared to the fixed, close proximity of contralateral otolithic end organs, the lateral line neuromasts are located along the entire length of the body. The lateral line contains a series of interconnected canals found in the bones, scales, and epidermis of fish. Inside these canals, the sensory cells compose gelatinous cupulas enclosing sensory hairs that detect water movement in the surroundings. The sensory cells, termed neuromasts, share structural similarities to ampullary sensory cells in the inner ear. Two types of neuromasts exist: canal and superficial. Superficial neuromasts determine water velocity on the skin surface while canal neuromasts detect water acceleration within subdermal canals (Kroese and Schellart, 1992). Canal neuromast abundance lies in the head region while superficial neuromasts commonly exist along the fish's body (Allis Jr, 1889). Additionally, on each differentially oriented neuromast lies a single axis of best physiological sensitivity because of hair cell bundle orientations, suggesting a directional detection role (Rouse and Pickles, 1991).

Each hair cell bundle contains one long kinocilium and a cluster of shorter stereocilia. (Flock and Wersäll, 1962; Rouse and Pickles, 1991). These hair cells share structural similarities to those in the vestibular and inner ear auditory systems and therefore, the lateral line shares a similar sensory perception. The bending of hair cells in the direction of its orientation leads to a depolarization and increase in neural firing rates, while activation in the opposite direction leads to a hyperpolarized response and decrease in action potential firing (Flock, 1971; Flock and Wersäll, 1962), posing a potential direction detecting mechanism similar to the inner ear.

Overall, the lateral line is found to respond to water movement and vibrational stimulus in the aquatic environment and remains conserved in all fish species. The lateral line is most sensitive to low-frequency vibrations (~10 Hz – 100 Hz), such as water currents (Montgomery et al., 1997), and responds to near-field particle motion (Mirjany et al., 2011). It also mediates schooling behavior (Partridge and Pitcher, 1980; Pitcher et al., 1976), rheotaxis (Montgomery et al., 1997) and predator avoidance (Janssen and Corcoran, 1993; Mensinger and Palmer, 2004). Although, the role of the lateral line in sound detection has long been debated (reviewed in Braun et al., 2002), several studies have suggested that the fish's mechanosensory lateral line also plays a role in sound localization (Higgs and Radford, 2013; Mirjany and Faber, 2011; Mirjany et al., 2011; Radford and Mensinger, 2014; Weeg and Bass, 2002).

One method of behaviorally examining sound localization in fish involves the stimulation of the Mauthner cell system, which integrates vibrational input from both sensory systems (octavolateralis) to mediate an escape response directly away from an acoustic stimulus (Mirjany and Faber, 2011; Mirjany et al., 2011). Using a 200 Hz stimulus from an underwater speaker, Mirjany et al. (2011) elicited an auditory evoked escape response in goldfish. To determine the impact of the lateral line system on this reaction, the entire system was pharmacologically blocked, which resulted in fish escaping in random directions, providing stronger evidence for the importance of the lateral line system, rather than the inner ear, for sound localization. However, when only the posterior lateral line system was transected, proper escape directionality was seen, suggesting the anterior lateral line plays a larger role in sound source detection. Further,

Mirjany and Faber (2011) found monosynaptic connects between afferents of the anterior lateral line and the Mauthner cells, therefore, providing extended support for the anterior lateral line's role in acoustic source detection and predatory escape responses.

Although valuable information was found through behavioral experiments, electrophysiology experiments have become a predominant research method for studying hearing abilities of fish (Bhandiwad and Sisneros, 2016; Higgs and Radford, 2013; Radford and Mensinger, 2014). Higgs and Radford (2013) used auditory evoked potentials to determine that goldfish hearing sensitivity to low frequency acoustic signals decreased after the chemical ablation of canal lateral line neuromasts. These results suggest that the lateral line system, specifically canal neuromasts, play a role in fish capabilities to detect common low frequency acoustic signals (e.g. mating calls) for effective sound localization.

### Toadfish

Batrachoidid fishes, such as oyster toadfish (*Opsanus tau*) and plainfin midshipman fish (*Porichthys notatus*), represent some of the most well studied fish in terms of sound vocalization and reception (Bass and McKibben, 2003). Since toadfish display territorial behavior, both males and females produce grunts for defensive acoustic communication (Gray and Winn, 1961). They produce these vocalizations through the contraction and relaxation of sonic muscles that induce vibrations on their swim bladder, which normally serves as a buoyancy organ, but operates in some species as a sound production organ (Blaxter, 1981). Apart from both sexes producing grunts, only sexually mature male toadfish produce a bimodal vocalization, called a boat whistle, as a mating

call (Gray and Winn, 1961). Boat whistles consist of a brief, irregular grunt followed by regular pulsing and range in frequency from 90-250 Hz (Edds-Walton et al., 2002), depending on the season and geographical location (Watkins, 1968), and last ~ 500 ms (Tavolga, 1958). The sonic muscles responsible for the boat whistle production are recorded as the highest contracting frequency muscles found in vertebrates and serve as a study system for muscle function (Rome et al., 1996). These sonic muscles also help toadfish modulate call frequency and produce louder than average sounds, up to 140 dB re: 1 $\mu$ Pa (Tavolga, 1971).

During late spring and early summer, males move to shallow water (1 – 3 m) where they occupy nesting sites and begin calling to females around dawn and dusk (Fine, 1978). Attracted females deposit their eggs at nesting sites for the male to fertilize (Fine et al., 1977). The male then guards the developing embryos for up to three weeks. Past observations found multiple clutches from multiple females, as males continue to call throughout the mating season. For mating success, the females need to successfully locate these mating calls in densely populated nesting sites with multiple males calling simultaneously. Although it has been observed that females successfully locate male boat whistles and exhibit mate preference, the contribution of the inner ear and lateral line system for localizing the calls remains unknown.

The same three endorgans of other teleosts (saccule, utricle, and lagena) compose the inner ear system of the toadfish. The otoliths activate hair cells that line the sensory epithelium and greater movement of this epithelium by particle motion causes deflection of hair cells apical structures. From there, a maximum excitation occurs towards the

single kinocilium along the central axis of best sensitivity. In all other axes, a decreased response arises and often no response when particle motion flows perpendicularly to the central axis. These hair cells respond to particle motion at multiple angles in the vertical plane and to movement in the horizontal plane dependent on the angle of the endorgan, therefore, potentially supplying directional information of the sound source. However, even with the knowledge that the otolithic organs, specifically the saccule and utricle, detect acoustic stimuli (Maruska and Mensinger, 2015), their overall influence on sound localization capabilities remains unknown. Vasconcelos *et al.* (2012) physically removed otoliths unilaterally or bilaterally to determine their role in body posture and movement (vestibular system). They found that the utricle, and potentially the saccule, functions both as an auditory and vestibular endorgan in the Lusitanian toadfish. With the otoliths partially functioning in the vestibular system, forms of self-generated movement lessen the auditory ability of the sensory cells responding to movement, which raises problems during courtship behavior in toadfish.

With all the potential physiological problems that arise with the inner ear anatomy of toadfish, the lateral line system as a sound localization pathway began receiving further interest. Toadfish respond at a maximum sensitivity to a 100 Hz acoustic stimulus, which lies in the response range of the lateral line (Radford and Mensinger, 2014). Additionally, Radford and Mensinger (2014) found that individual neuromasts exhibit directional sensitivity and their primary afferent fibers strongly phase-lock to toadfish boat whistle calls, which could be a physiological mechanism for acute sound localization. An increase in stimulus intensity also increased action potential firing rate.

Since toadfish produce boat whistle calls up to 140 dB, this high intensity acoustic stimulus expectantly increases the firing rate of female toadfish neurons while localizing sound, suggesting that spike rate and phase-locking potentially provide directional information about the location of sound. In summary, this study determined that the anterior lateral line responds to toadfish vocalizations and that neuromasts potentially determine source direction of near-field particle displacements produced by vocalizing fish.

Documentation of toadfish neuroanatomy throughout the century leads one step closer to putting together the pieces of their neurophysiology. The short distance between toadfish auditory organs makes binaural cues problematic and therefore, makes them a suitable animal model for lateral line sound localization studies. Consideration of toadfish as a model organism for understanding basic physiological mechanism remains further aided because of their abundance, ease of capture, and aquarium hardiness. Overall, looking into a conserved system throughout most fish species, the lateral line, benefits the field of neuroethology by providing insight into a multimodal sensory system for the phenomenon of sound localization in fish.

## **Chapter 2: Bilateral Electrode Implants Help Determine the Relevance of the Anterior Lateral Line for Sound Localization in Toadfish (*Opsanus tau*)**

### **Bilateral electrode implants help determine the relevance of the anterior lateral line for sound localization in toadfish (*Opsanus tau*)**

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#### **Abstract**

Male oyster toadfish (*Opsanus tau*) acoustically attract females to nesting sites using a boat whistle call. The rapid speed of sound underwater combined with the close proximity of the otolithic organs makes interaural time differences an unlikely mechanism to localize sound. To determine the role that the mechanosensory lateral line may play in sound localization, microwire electrodes were bilaterally implanted into the anterior lateral line nerve to record neural response to a 60 Hz vibrational stimuli. Highest spike rates and strongest phase-locking occurred at near distances and decreased as the vibrational sphere was moved further from the fish. Additionally, directionally sensitive and omnidirectional units were observed with varying degrees of phase-locking. Since the lateral line system responds to near field water displacements, its ability to localize sound would be limited to short distances (< 2 wavelengths). Therefore, multimodal sensory input processing through both the inner ear (far field) and lateral line (near field) may allow for effective sound localization in fish.

## **Introduction**

Batrachoid fish (*Opsanus sp* and *Porichthys sp*) have been used as biological models for investigating muscle physiology (Elemans et al., 2014; Harwood et al., 2011), excretory function (Barimo et al., 2007) and vestibular physiology (Rabbitt et al., 1995). However, sound generation and reception is an integral part of their natural history. Both male and female oyster toadfish produce broadband grunts by means of rapid contraction of sonic muscles surrounding the swim bladder and sexually mature male toadfish produce a bimodal vocalization, termed a boat whistle, which is used to acoustically attract females to nesting sites (Fine et al., 1977; Gray and Winn, 1961; Maruska and Mensinger, 2009). Although the production and reception of sound has resulted in investigations both on sonic muscle and auditory physiology (Edds-Walton et al., 2013; Harwood et al., 2011; Mensinger, 2014), the physiological mechanisms by which female fish locate the males remain largely unknown, although it is suggested that directional cues are available through particle motion elicited by low frequency sounds (Zeddes et al., 2010; Zeddes et al., 2012).

The saccule is considered the primary auditory endorgan in fish (Popper and Fay, 1993; Popper and Fay, 2011); however, both the saccule and utricle are sensitive to linear accelerations, acoustic particle motion, display directional sensitivity, and function predominantly as low frequency (60 to 1000 Hz) detectors (Boyle et al., 2001; Fay, 1984; Fay and Edds-Walton, 2000; Lu et al., 2004; Mensinger, 2006). The mechanism by which otoliths contribute to sound localization remains unclear. While terrestrial vertebrates use interaural time delays (ITDs) to localize sound in the azimuth (Schnupp

and Carr, 2009), the small distances between otolith pairs and the relatively rapid underwater speed of sound makes using time disparities challenging for teleosts. For example, the maximum ITD for fish with an interaural distance of approximately 3 cm would be less than 20  $\mu$ s, which is at or near the threshold ITD of the most sensitive terrestrial vertebrates (Grothe et al., 2010).

Fish also possess a hair cell based mechanosensory lateral line that functions in schooling behavior (Partridge and Pitcher, 1980), rheotaxis (Montgomery et al., 1997), hydrodynamic imaging (Weissert and Von Campenhausen, 1981) and predator/prey interactions (Montgomery et al., 1995). Although, the role of the lateral line in sound detection has long been debated (see Braun et al., 2002 for review), several studies have suggested that the fish's mechanosensory lateral line may play a role in sound localization (Higgs and Radford, 2013; Mirjany and Faber, 2011; Mirjany et al., 2011; Radford and Mensinger, 2014; Weeg and Bass, 2002). The lateral line is a near field particle displacement detector that potentially contributes to hearing sensitivity and sound localization (Mirjany et al., 2011). In fish, delay lines, such as found in owls (Carr and Konishi, 1988), have not been discovered, but interneuromast distance, combined with afferent nerve length and conduction velocities, may be sufficient for aquatic sound localization through the use of sound delays. For example, neuromasts of the anterior lateral line can be separated around 10 cm and neuromasts between the anterior and posterior lateral line are capable of exceeding 25 cm. If a 25 cm standard length fish receives an underwater sound directly in front of it, the foremost anterior lateral line neuromast will respond 16  $\mu$ s prior to arriving at posterior lateral line neuromasts. If you

factor in toadfish cranial nerve diameters [1 to 12  $\mu\text{m}$ , (Mensinger and Highstein, 1999)], conduction speeds associated with myelinated nerves of these diameters (10 to 50 m/s), and afferent lengths to second order neurons (up to 5 cm length for anterior and 20 cm for posterior; Mensinger unpublished), delays to the central nervous system would range from approximately 400  $\mu\text{s}$  to 2 ms. These time delays between sensory organs of the lateral line are within the time frame used by other vertebrates for acute sound localization.

Sound localization in fishes have recently been reviewed (Sisneros and Rogers, 2016) with compelling evidence for the use of particle motion for midshipman fish to locate distant sound sources. However, the lateral line's ability to detect near field particle motion suggests that it could function in short distance sound localization. As male toadfish can be found in shallow water and soft bottoms, which can quickly attenuate sound, final mate selection may hinge on accurate sound localization in the near field. The present study demonstrates that the anterior lateral line has the potential to use ITD's for determining sound source location in freely swimming toadfish.

## **Materials and Methods**

### *Animal Husbandry*

Adult toadfish ( $26 \pm 3.8$  cm standard length) were collected near the Marine Biological Laboratory in Woods Hole, Massachusetts. They were kept in flow-through seawater tanks at temperatures between 18 - 22 °C from mid-May to mid-August and were fed *ad libitum*. All animal care and experimental procedures conformed to institutional animal care protocols.

### *Electrode Construction*

To record neural activity, microwire electrodes were bilaterally implanted into the left and right anterior lateral line nerves. Microwire electrodes consisting of a twin pair of insulated 20  $\mu\text{m}$  diameter, 10% platinum/iridium wire (Sigmund Cohn) were custom fabricated for each implant. Each microwire was fixed to silver-plated copper multistrand wire (25  $\mu\text{m}$  diameter, New England Wire) with conductive silver paint. The multistranded wire was attached to silver wire (320  $\mu\text{m}$ ) that terminated into a multipin underwater connector. The anterior portions of the microwires were threaded through a 1 cm length polymide tubing (180  $\mu\text{m}$  outer diameter) to maintain the recording sites in proximity. Any exposed wire/connectors were encased in medical devise adhesive (Loctite 3341; Henkel Loctite Corp., Rocky Hill, CT, USA) and cured with ultraviolet light. Implanted electrodes ranged in impedance between 0.5 and 1.5 M $\Omega$  (FHC impedance meter).

### *Dorsal Craniotomy*

Toadfish were anesthetized by immersion in 0.005% tricaine (MS-222; 3-aminobenzoic acid ethyl ester) in seawater and paralyzed with an intramuscular injection of 0.01% pancuronium bromide (600  $\mu\text{m}/\text{kg}$ ). The fish was then placed in a custom-designed, Plexiglas stereotactic tank on a vibration isolation table. An incision was made through the dorsal musculature overlying the sagittal crest and the muscle was retracted. Two small holes were made in the cranium on both sides of the sagittal crest and posterior to the transverse crest to expose the right and left anterior lateral line nerves proximal to their exit from the braincase.

### *Electrode Implantation*

Each pair of microwire electrodes were implanted into the dorsal branch of the right or left anterior lateral line nerves proximal to their exit from the braincase. Extracellular potentials were differentially amplified (Dagan, Minneapolis, MN, USA) and monitored on a portable computer using LabChart 7 software on Powerlab 4SP. After an afferent fiber was located, fish were left undisturbed for 30 minutes to ensure recording site stability. Cyanoacrylate gel was used to attach the electrodes to the skull and to seal the craniotomy. The muscle was returned to its original position and the incision triple sutured (fascia, muscle, and epidermis) for a waterproof seal. The waterproof electrode connector was mated to a flexible 1.5 m tether that terminated into the head stage of the differential amplifier. A small brush was run over the fish's head to determine the approximate locations of the neuromasts. The fish was allowed at least one hour to recover from surgery prior to testing.

### *Experimental Design*

Fish were moved to a Plexiglas experimental tank (50 cm x 85 cm), located on a vibration isolation table, with a water depth of 8 - 10 cm. Toadfish were free to move, but remained relatively inactive throughout experimental trials. A solid plastic sphere (15 mm diameter) was attached to a mini-shaker (Bruël and Kjaer, model 4810) by a 15 cm metal shaft and suspended vertically midway in the water column. An externally triggered function generator (Tektronix FG 501A; Beaverton, OR) was used to drive the mini-shaker at 60 Hz. The toadfish remained in position while the sphere was moved in a semicircle (0° directly left, 90° in front, 180° directly right) using the midline of the

premaxilla as the reference point. The vibrational stimulus was tested in 30° increments relative to the fish to test for directional sensitivity in anterior lateral line afferent fibers. Additionally, at each 30° position, seven distances (0.5 – 8.9 cm) were tested. Each stimulus trial was 25 seconds in duration with two five second stimulus periods bracketed by five seconds of spontaneous activity. Multi-unit recordings were amplified, filtered using a high pass setting of 100 Hz, and recorded onto a computer using Powerlab.

#### *Data Analysis*

Waveform analysis was performed on the data, using Spike2 software (Cambridge Electronic Design Ltd., version 7), to discriminate individual units in the extracellular recording. The spontaneous spike rate before and after the presentation of the vibrational stimulus was recorded for each neuron. Neural responses to the 60 Hz vibrational stimulus were quantified for evoked spike rate and vector strength. The synchronization coefficient ( $R$ ), which determines strength of phase-locking (Goldberg and Brown, 1969), was calculated for evoked spike rates. To describe the strength of phase-locking of the afferents, a previously published criterion (Lu and Fay, 1993; Lu and Fay, 1995) was applied to distinguish strongly phase-locked afferents ( $R \geq 0.5$ ) from weakly phase-locked afferents ( $R \leq 0.5$ ). However,  $R$  can be misinterpreted when the sample size ( $N$ ) is small. To correct this issue, the Rayleigh statistic ( $Z$ ) was used as a combined measure of the number of discharges and strength of phase locking (Lu and Fay, 1993; Lu and Fay, 1995).  $Z$  is defined as  $N * R^2$ , where  $N$  is the total number of spikes (Batschelet, 1981), and represents the response magnitude of the lateral line afferents. An afferent was significantly phase-locked to the stimulus if  $Z \geq 3.1$  ( $p <$

0.001). All phase-locking analysis was done in Matlab using the CircStat toolbox (Berens, 2009). Polar plots were constructed using R values at each stimulus location.

## Results

### *Spike Rate*

Bilateral chronic electrodes were successfully implanted into the anterior lateral line of six fish and a total of 24 units were recorded and fully characterized to the experimental parameters. All units were spontaneous active afferent fibers with an average spontaneous spike rate of  $14.9 \pm 0.3$  spikes s<sup>-1</sup> (range  $7.4 \pm 0.3 - 28.9 \pm 0.6$  spikes s<sup>-1</sup>) (Figure 1).

Figure 2 shows the neural activity recorded from two bilateral implants before, during, and after the vibrational stimulus. One afferent fiber shown is modulated to the vibrational stimulus by displaying an increase in spike rate during the 60 Hz stimulus, while the contralateral unit displays a fiber with no modulation. Modulated fibers showed a decrease in activity as the probe was moved away from the fish (Figure 3).

### *Directionality and Phase-Locking*

The approximate neuromast position for 22 of the 24 units was localized on the fish (Figure 4). The left electrodes were consistently implanted in afferent fibers innervating neuromasts on the left side of the head, between the orbit and eye and anterior margin of the operculum. Right side neuromasts were in similar locations, however, two extended to the suborbital portion of the lateral line and one was anterior to the orbit.

Approximately half of the afferent fibers showed strong phase-locking ( $R > 0.5$ ) and directional sensitivity. The directional sensitivity was characterized as sharply tuned (one angle  $> R = 0.5$ ), intermediate (two angles  $> R = 0.5$ ), or broad ( $\geq 5$  angles  $> R = 0.5$ ) (Figure 5). Six other fibers showed directional sensitivity, but were weakly phase-locked, and seven fibers did not demonstrate phase-locking or directionality. Broadly tuned/omnidirectional units were found in both weak and strong phase-locked units from both the left and right anterior lateral line afferent fibers (Figure 5B). Afferent fibers that expressed no phase-locking or direction detection were also found on both sides of the lateral line (Figure 5C). However, neurons without clear directionality still responded robustly (31 - 51 spikes  $s^{-1}$ ) during the stimulus presentation. Overall, both sides of the anterior lateral line nerve exhibit both directional and omnidirectional units, as well as strong, weak, and little-to-no phase-locking fibers.

Phase-locking results were compared at distances ranging from 0.5 cm – 8.9 cm from the toadfish head. Phase-locking was strongest nearest the fish ( $Z > 3.1$ ;  $p < 0.01$ ) for both the left and right units (Figure 6). The weakest phase-locking occurred at the furthest distance from the fish ( $Z > 3.1$ ;  $p < 0.05$ ) for all units. Therefore, a similar descending trend of phase-locking strength near to far stimulus distances for afferent fibers on both the left and right side of the toadfish was found. Although phase-locking was overall strongest nearest the fish, the median  $R$  value for both the left ( $< 0.3$ ) and right ( $< 0.1$ ) units were  $< 0.5$ , which is categorized as weakly-phase locked.

Phase-locking strength and directionality results from this study can be organized into five main categories: strong phase-locking/omnidirectional (16.7%), weak phase-

locking/omnidirectional (20.8%), strong phase-locking/directional (25%), weak phase-locking/directional (12.5%), and little-to-no phase-locking (25%) (Table 1). When combining all units, the majority exhibited either strong phase-locking directionally (25%) or little-to-no phase-locking (25%). Overall, nine directionally sensitive units were observed (37.5%).

In comparing male and female toadfish, a noticeable difference of phase-locking was seen between sexes. Males (8 units) had a lower average strength of phase-locking ( $R = 0.060 \pm 0.005$ ) compared to females (16 units) ( $R = 0.290 \pm 0.022$ ) (Figure 7). However, both average R values are below 0.5, suggesting that there may be a wide variation of phase-locking strengths between individual afferent fibers in both male and female toadfish. Additionally, the presence of phase-locked anterior lateral line nerve fibers is higher in females (93.75%) compared to males (37.50%).

### ***Time Delay***

Neural spike rate recordings that displayed strongly phase-locked afferent neurons from both sides of the anterior lateral line nerve were used to determine time delays. Three fish exhibited strong phase-locking simultaneously in both the left and right anterior lateral line nerves at the nearest distance to the fish  $60^\circ$  to the left. The distances between these strongly phase-locked left and right neuromasts varied slightly, ranging from 6.6, 6.9, and 7.9 cm. After comparing the time difference between phasic firing of the left and right afferent fibers, the neuromasts spaced 6.9 cm apart had the longest time delay (~ 2 ms) (Figure 8). However, there was no statistically significant difference

between time delays at any distance tested ( $p > 0.05$ ). Overall, the average calculated time delays at all three distances were  $\leq 2$  ms.

## **Discussion**

The objective of this study was to determine if response characteristics during vibration stimulus to contralateral neuromasts could provide sufficient information that toadfish could use for directional location. This study was the first to bilaterally record from the anterior lateral line nerve in toadfish. Response variations were found between individual neurons, suggesting differential input can be integrated for source localization.

### ***Spike Rate***

All twenty-four units analyzed were spontaneous afferent fibers. These spontaneous fibers fired at an average of 15.5 spikes/sec, classifying them with a slow irregular rate (< 20 spikes/sec) found in past experiments (Maruska and Tricas, 2009). The effect of MS-222 as an anesthetic is known to decrease spontaneous spike rate; however, neural activity was seen to return to normal levels after a 90 minute recovery period (Palmer and Mensinger, 2004). Therefore, MS-222 as an anesthetic should not have influenced experimental results. A potential explanation for irregular recorded resting rates could stem from the lateral lines adaptive filter suppressing its response to the continuous experimental tank flow during the 90 minute recovery time, since the toadfish remained behaviorally inactive (Montgomery and Bodznick, 1994).

Although innervated neuromasts spontaneous spike rates, approximate locations, and distances from one another were determined, which type of neuromasts were innervated was undetermined. There are two types of neuromasts: superficial and canal.

Superficial neuromasts are located on the surface of the skin and canal neuromasts are located in subdermal canals (reviewed in Coombs and Montgomery, 1999). Superficial neuromasts have a smaller frequency sensitivity range, responding best to lower frequencies, compared to canal (reviewed in Montgomery et al., 1995; Montgomery et al., 2000; Voigt et al., 2000), so canal neuromasts may play a larger role in increasing hearing sensitivity and sound localization (Higgs and Radford, 2013). However, superficial neuromasts are still characterized by an axis of best sensitivity and therefore cannot be ruled out for aiding in sound origin detection. Additionally, lateral line neuromasts are arrayed in different orientations on different planes, which may allow for acoustic directional sensitivity (Marranzino et al., 2013). The plane (azimuth vs elevation) in which the neuromast were located was not recorded and may be responsible for variations and overlaps found between afferent units in this study.

The use of a mini-shaker allowed for pure-tone particle motion reception by the toadfish for measuring acoustically evoked potentials in the anterior lateral line system. A greater increase in spike rate when the vibrational sphere was nearest the fish supports the lateral line system as a near-field responder to particle motion, but lacks evidence for the anterior lateral line aiding specifically in sound direction detection. However, phase-locking is predicted to determine relevant frequencies for sound source direction detection rather than evoked spike rates (reviewed in Maruska and Tricas, 2009). Therefore, determining phase-locking potential of the anterior lateral line afferent fibers to an acoustic stimulus may provide insight on the relevance of the anterior lateral line for sound localization in fish.

### ***Phase-Locking***

If significant phase-locking differences were found between afferent fibers of the anterior lateral line at varying distances and angles, it would suggest directional responses to an acoustic stimuli. In the current study, phase-locking results did vary in both strength and directionality between afferent fibers, suggesting a potential mechanism for sound source detection. Teleost lateral line neuromasts are known to exhibit directional sensitivity by containing an axis of best sensitivity that may provide directional information to the fish about an incoming sound source (Coombs and Montgomery, 1999; Flock and Wersäll, 1962). Both the left and right anterior lateral line nerve exhibited units with strongly phase-locked directional properties, suggesting phase-locking as a mechanism for sound direction detection. However, only nine units were observed to be directionally sensitive, with others classifying as omnidirectional or not phase-locked. To truly compare whether the anterior lateral line can contribute to sound localization, time delays between neuromasts need to be determined.

Phase-locking afferents occur at the same phase of a sinusoidal stimulus, but with lower sound frequencies, action potentials do not occur during every cycle and may be slightly off in phase firing (Fay and Coombs, 1983). Therefore, our 60 Hz stimulus may not have been strong enough to elicit strong-phase locking in all of these afferent fibers. However, the lateral line has responded to frequencies below 80 Hz in past studies (Montgomery et al., 1988; Weeg and Bass, 2002). Another study found that high-frequency lateral line units are better detected rostrally on a fish while low-frequency units are located more caudally (Echteler, 1985), which would account for our variation

in results as well. Overall, using phase-locked afferents to determine binaural signal differences between sensory organs may lead to further insight on how fish localize sound.

### ***Time Delay***

Time delays between sensory neuromasts of the anterior lateral line system were  $\leq 2$  ms, which falls in the range of typical interaural time differences (400  $\mu$ m to 2 ms) for other vertebrates (reviewed in Mensinger, 2016). However, this prediction that the lateral line can encode sufficient time delays like other vertebrates for sound localization stemmed from calculations between the neuromasts of the anterior versus posterior lateral line system. The rapid speed of sound underwater suggests potential negligible delay times between sensory organs in solely the head region. In the current study, only a few coefficients of synchronization reached  $> 0.5$  (strong phase-locking), so revising the protocol to introduce a stimulus with a frequency and threshold to elicit stronger phase-locking in afferent fibers can lead to an extensive comparative analysis of time delays between different regions of the lateral line system. For example, a 100 Hz stimulus (found to be maximum sensitivity of the anterior lateral line; Radford and Mensinger, 2014) or natural toadfish vocalizations may be a more appropriate stimulus. Further determining time delays between regional components of the lateral line would suggest if this system encodes sufficient interaural time differences for successful sound source localization.

### ***Inner Ear vs Lateral Line***

The questions still beckons, what does the lateral line system tell that the inner ear does not? Both systems are hair-cell based, show directional sensitivity to pure tones and toadfish vocalizations, and become saturated during swimming and predatory strikes (Maruska and Mensinger, 2015; reviewed in Mensinger, 2016; Palmer et al., 2005). The differences emphasized thus far include the inner ears potential detection of pressure through accessory structures for far-field acoustic detection and its known role in the vestibular system. For successful toadfish mating, females need to localize male mating calls to deposit their eggs in nests (Gray and Winn, 1961). Pressure detection by the inner ear may provide preliminary data on the source of the boat whistles in the far-field, but near source particle displacement is known as the determining factor in mate choice (reviewed in Bhandiwad and Sisneros, 2016; McKibben and Bass, 1998). Therefore, it is unknown what contribution each system plays in sound source detection, but it is suggested that both components of the octavolateralis system can contribute to some degree in fish sound localization.

### ***Conclusion***

The success of bilaterally implanting chronic microwire electrodes into the toadfish anterior lateral line system paves the way for future sound localization studies (e.g. regional time delays). In this study, the anterior lateral line neuromasts displayed directional sensitivity to incoming acoustic stimuli, which suggests its potential for aiding in sound localization. Additionally, evoked spike rates and strength of phase-locked afferents increased at near distances to the toadfish, providing further support of the

anterior lateral line detecting near field particle displacements. The present study also demonstrates that the anterior lateral line system of the toadfish may encode sufficient time delays between sensory organs for determining sound localization cues. Future studies should focus on determining time delays between neuromasts of the anterior versus the posterior lateral line system to conclude whether fish localize sounds through interaural time differences similarly to terrestrial vertebrates.

### **Acknowledgements**

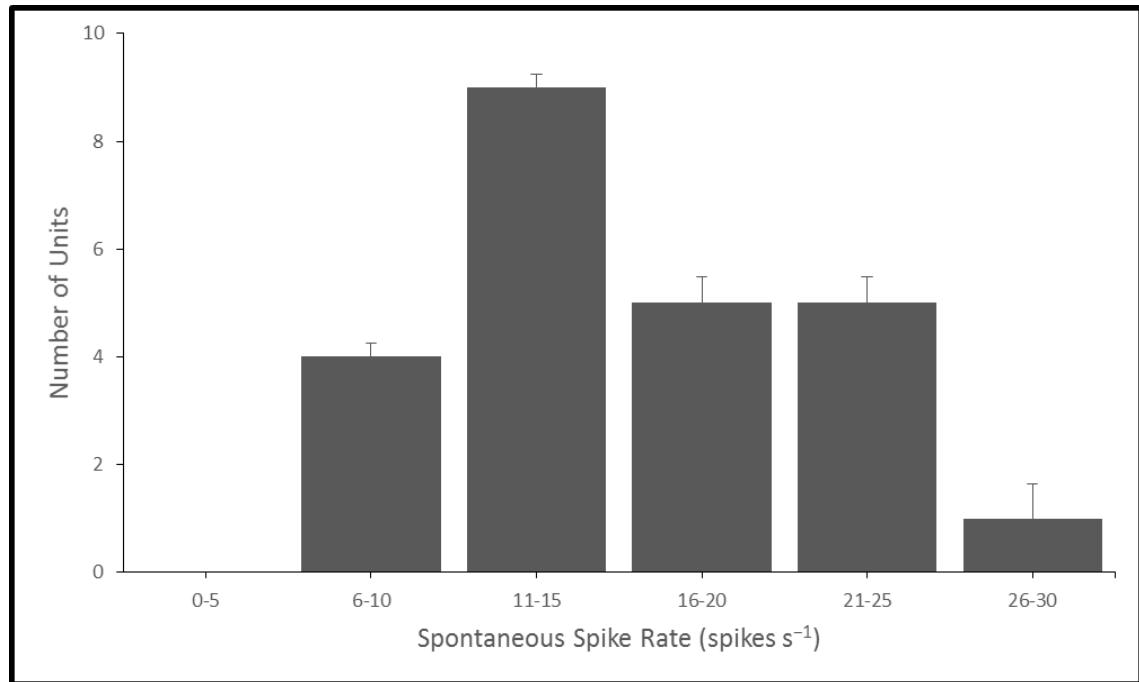
Funding Source: National Science Foundation IOS 1354745

## Tables

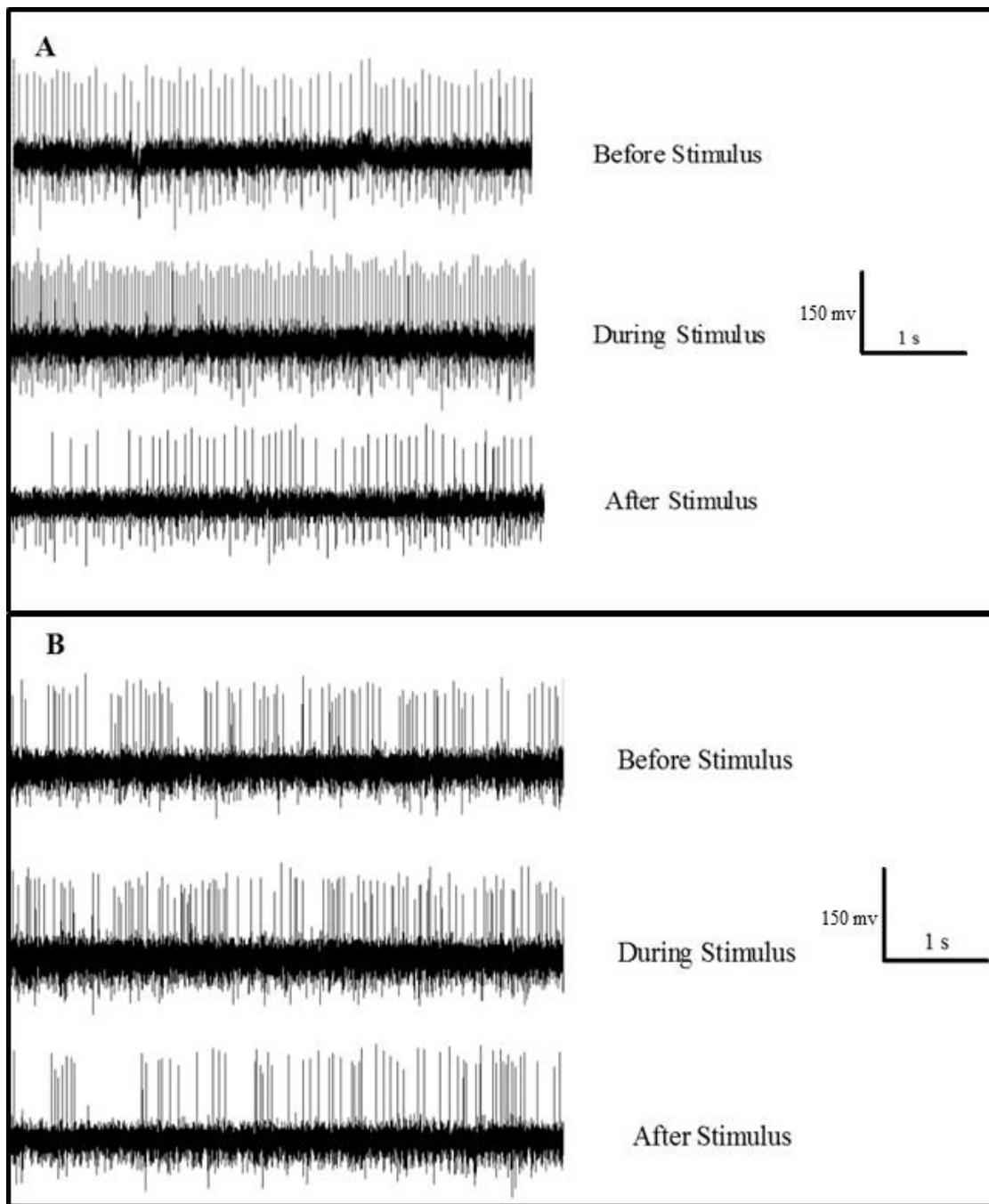
**Table 1. Phase-locking strength and directionality categories**

Category	Left Units (%)	Right Units (%)	Combined (%)
Strong Phase-Locking/ Omnidirectional	25.0	8.30	16.7
Weak Phase-Locking/ Omnidirectional	16.7	25.0	20.8
Strong Phase-Locking/ Directional	33.3	16.7	25.0
Weak Phase-Locking/ Directional	16.7	8.30	12.5
<u>Little-to-no Phase-Locking</u>	<u>8.30</u>	<u>41.7</u>	<u>25.0</u>

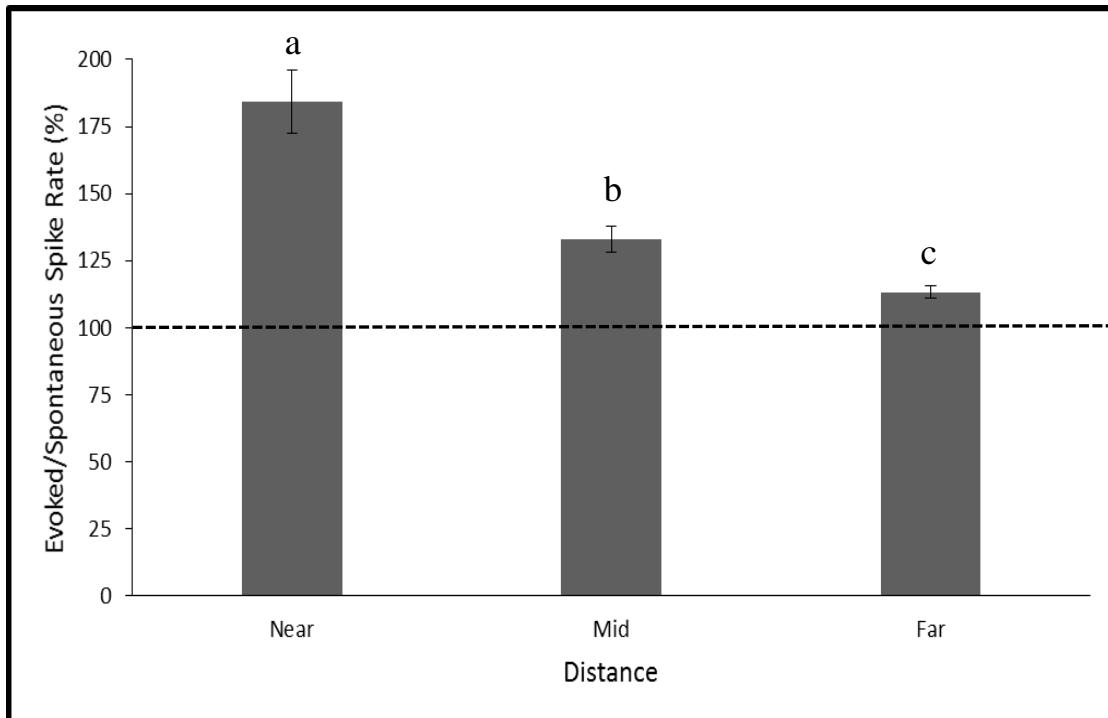
## Figures



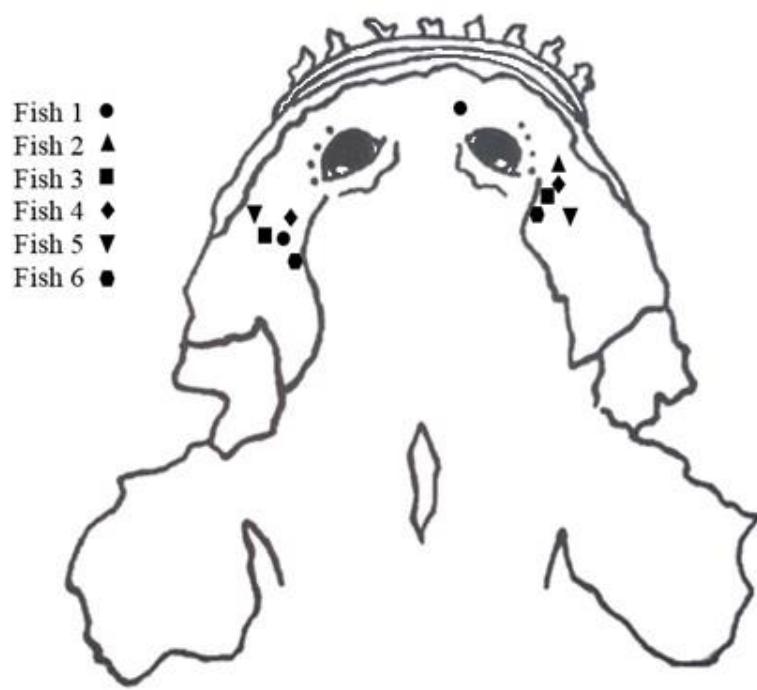
**Figure 1. Spontaneous mean neural firing activity of characterized toadfish afferent fibers (N=24).** Rates are binned in 5 spike s<sup>-1</sup> intervals. Error bars represent ± 1 standard error.



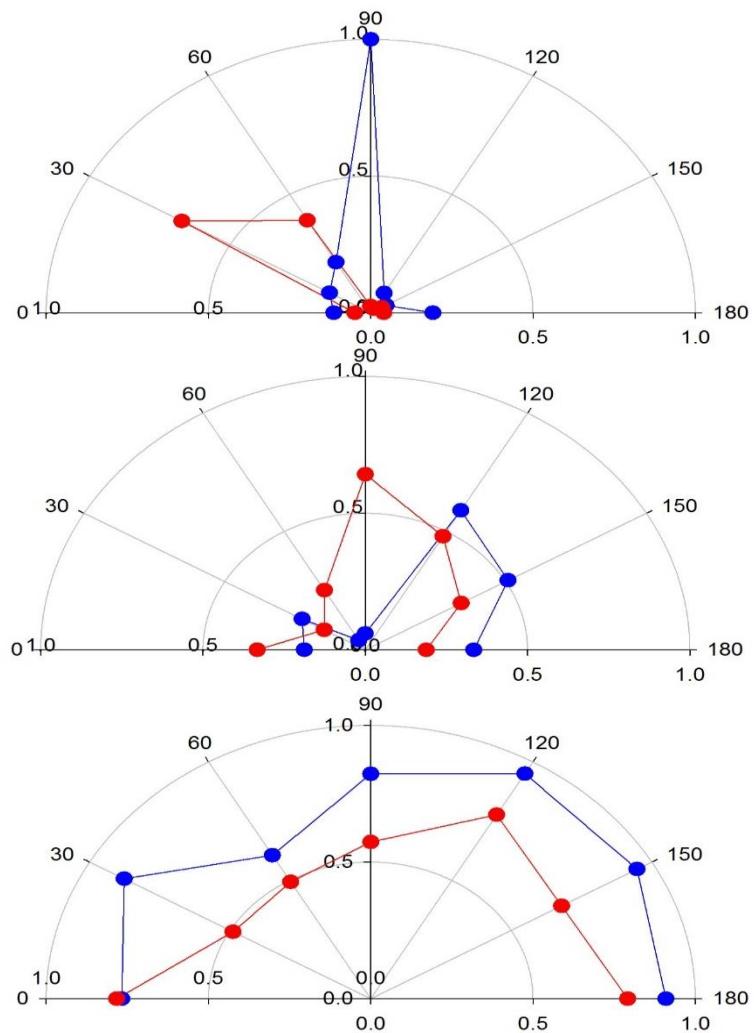
**Figure 2. Neural activity from a bilateral electrode implant.** Each panel (A, B) shows 5 seconds before, during, and after a 60 Hz stimulus in the same toadfish. Panel A shows the modulated recording from a neuromast on the right side while panel B shows the left neuromast uneffected by the stimulus.



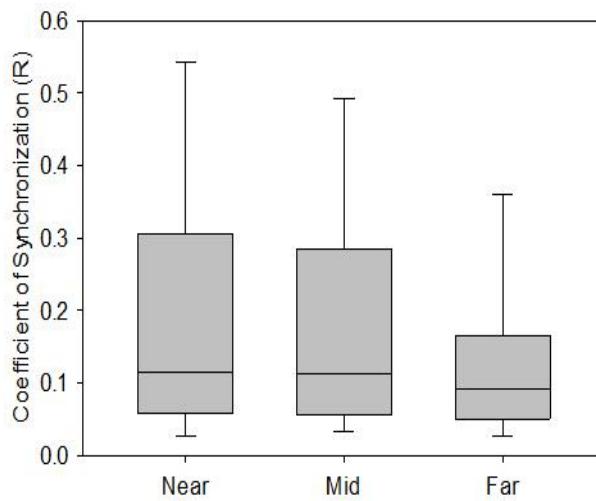
**Figure 3. The effect of distance on evoked spike rate.** Each bar represents the percent increase from the resting rate during a 60 Hz vibrational stimulus for all units, at all angles, near (0.5 cm), mid (4.7 cm), and far (8.9 cm) distances from the fish. The dotted line represents equal firing rates. Error bars represent  $\pm 1$  standard error. Lower case letters indicate significant differences between means (one-way ANOVA,  $p < 0.01$ ).



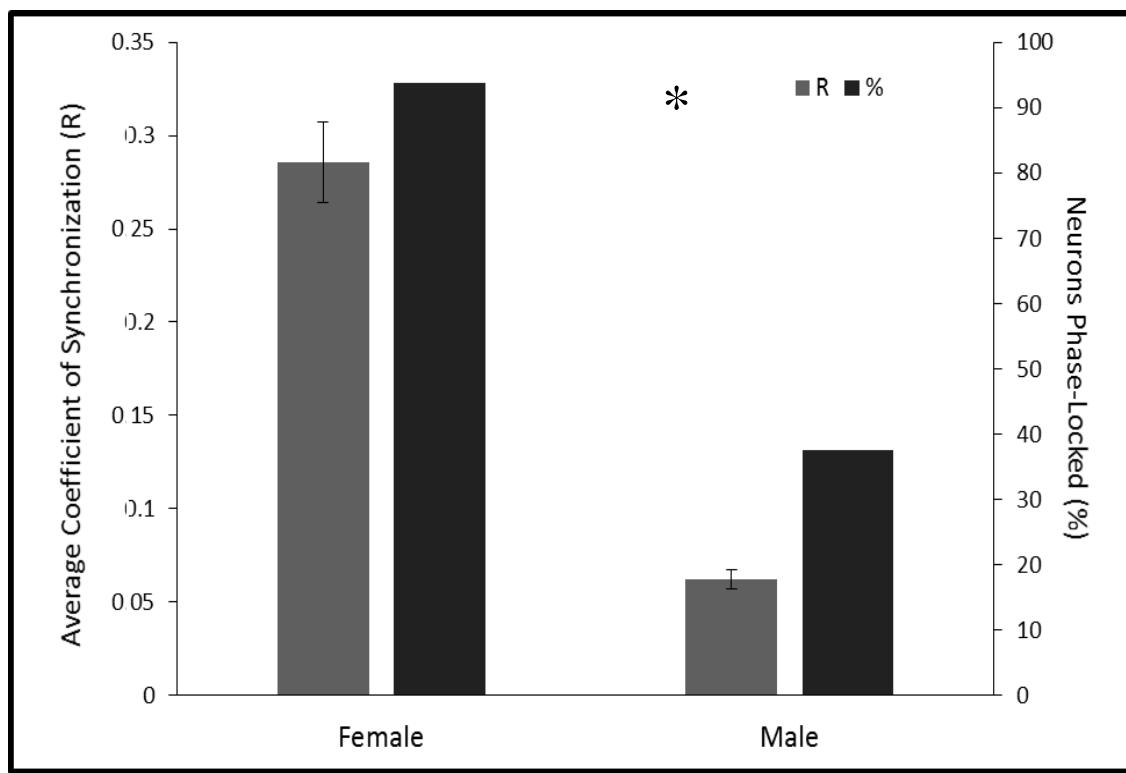
**Figure 4. The location of recorded neuromasts innervated by the anterior lateral line nerve.** Eleven neuromasts were located between the six experimental fish (~ 1 on each side of the toadfish head). Neuromast locations are represented by shapes on the toadfish head, and each different shape represents neuromasts found on different experimental fish.



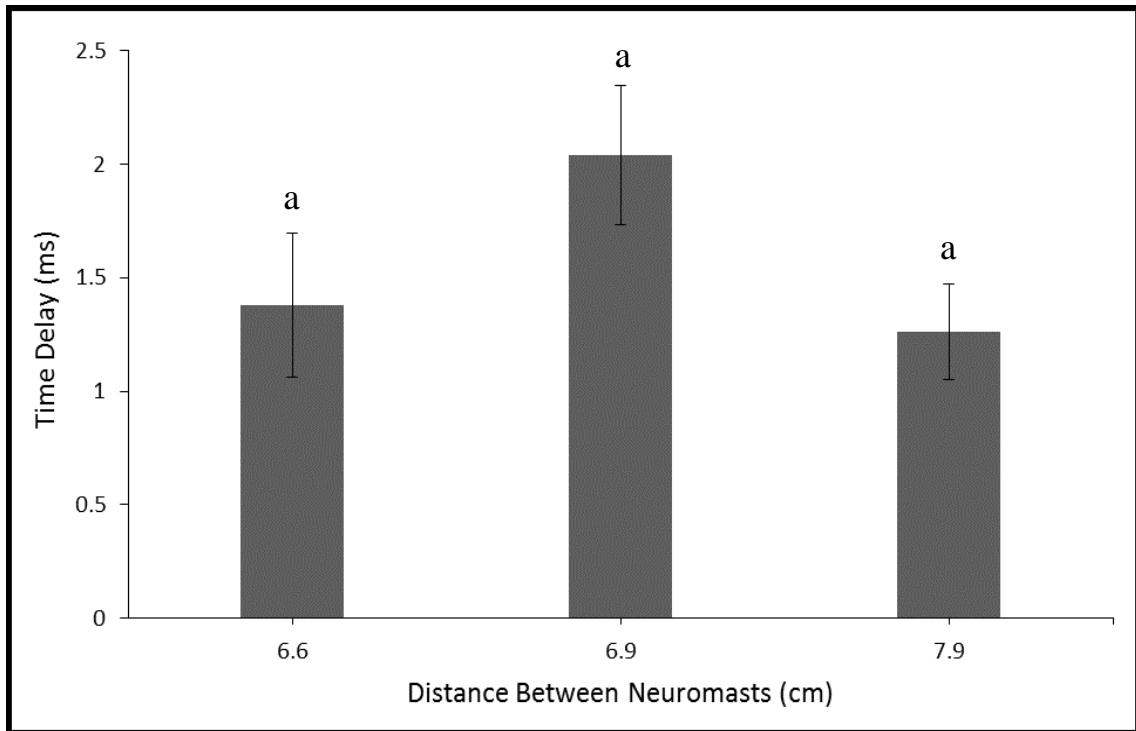
**Figure 5. Polar plots of neuromast tuning curves.** Polar plots were constructed using vector strength analysis to determine phase-locking responses of individual afferent fibers from both the left and right anterior lateral line nerves. Each point represents the R value at each angle ( $0^\circ - 180^\circ$ ) the stimulus was presented. R values greater than 0.5 represent strong phase-locked afferent fibers. Connecting lines are used for illustrative purposes only. The two colors on each plot represent two individual neuromasts. Top: Sharply tuned units (one angle  $> 0.5$ ). Middle: Intermediate units (two angles  $> 0.5$ ). Bottom: Broadly tuned (4 or more angles  $> 0.5$ ).



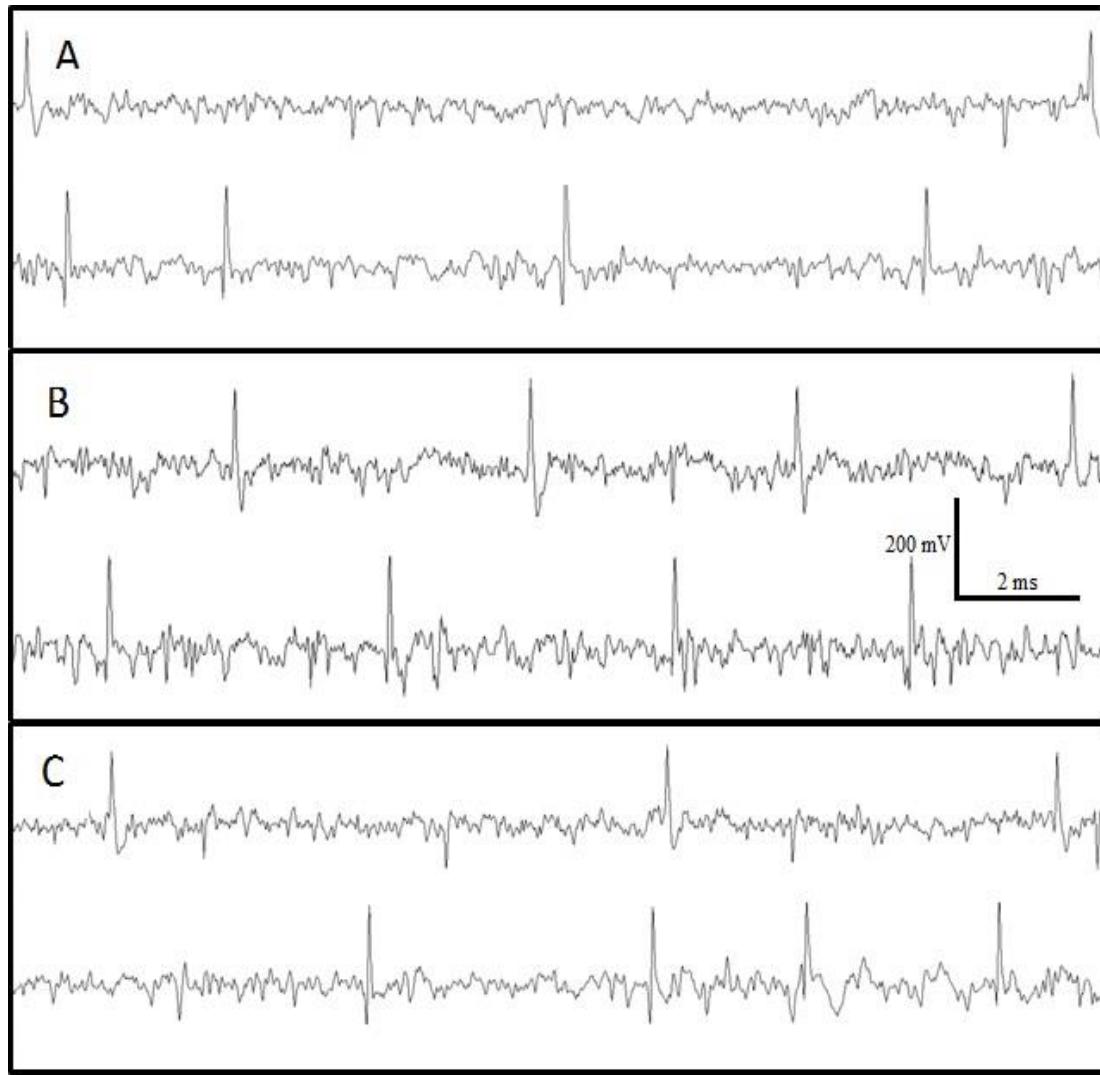
**Figure 6. The effect of distance on the average coefficient of synchronization (R).** R represents the degree of phase-locking, with higher values associated with stronger phase-locking ( $R > 0.5$ ). Box and whisker plots display median R values at near (0.5 cm), mid (4.7 cm), and far (8.9 cm) distances from the fish when the 60 Hz stimulus was present. The horizontal line in each box displays the median R value, the top and bottom of the box show 1<sup>st</sup> and 3<sup>rd</sup> quartiles, and the error bars indicate the 10<sup>th</sup> and 90<sup>th</sup> percentiles.



**Figure 7. Phase-locking comparison between male and female toadfish.** Gray bars represent the average coefficient of synchronization (R) for female and male toadfish. R represents the strength of phase locking. Only R values calculated from the near distance (0.5 cm) stimulus position were used for each neuron ( $n = 24$ ). Error bars represent  $\pm 1$  standard error. Asterisk denotes significant differences between means (t-test,  $p < 0.01$ ). Black bars represent the percent of neurons phase-locked in female ( $n = 16$ ) and male ( $n = 8$ ) toadfish.



**Figure 8. Average time delays between neuromasts of the left and right anterior lateral line system.** Time delays were quantified based on time between phasic firing of two strongly phase-locked neurons ( $R > 0.5$ ;  $p \leq 0.05$ ;  $Z \geq 3.1$ ). Distances between neuromasts were measured (6.6, 6.9, and 7.9 cm) for three toadfish exhibiting strong phase-locking, simultaneously between the left and right anterior lateral line nerves, to a 60 Hz vibratory stimulus. Fifteen recorded time delays were averaged. Error bars represent  $\pm 1$  standard error.



**Figure 9. Time delays between afferent fibers of the anterior lateral line nerve.**

Strong phase-locking ( $R > 0.5$ ) was found simultaneously between the left and right anterior lateral line during a 60 Hz vibrational stimulus. (A) Raw neural activity from two afferent fibers exhibiting no phase-locking before vibrational stimulus presentation. (B) Raw neural activity from simultaneously phase-locked afferents for a time delay comparison. (C) Raw neural activity displaying no phase-locking trend after the presentation of the vibrational stimulus.

## **Chapter 3: Future Directions for Determining How Fish Localize Sound**

Through past behavioral experiments on the inner ear and lateral line system of fish, it is obvious that both systems aid fish in localizing sound sources. The long standing question in the field of neuroethology is how do fish localize sound? The exact mechanism of how these systems aid in localizing sound is still unknown. However, the directionally oriented hair cells of both systems is a pertinent starting point. Therefore, a combined use of these systems is hypothesized to aid in sound localization, but the potential of the lateral line system to exhibit sufficient time delays that the otolithic inner ear cannot has not been quantified. Knowing the differences between these receptive systems may give insight into how fish overcome obstacles such as self-generated movement impacts on the auditory system and the effect of anthropogenic noise on communication and predation.

Sensory information about self-generated movement is provided through the vestibular system. The utricle and sacculus potentially play a role in the vestibular as well as the auditory system. With this dual role, self-generated movement may decrease hearing sensitivity of the otolithic endorgans. However, the lateral line is not known to have a vestibular function and therefore, may provide relevant auditory information for localizing sound sources during movement. While male toadfish remain stationary during courtship calling, females need to locate and swim to the males. With the otolithic endorgans potentially responding to this movement, the lateral line may provide the information necessary for locating the mating calls at near distances. To determine the

effect of self-generated movement on toadfish mating behavior, an electrophysiology study recording neural activity in both the inner ear and lateral line system during female trajectory to the males would be beneficial.

Another obstacle associated with localizing mating calls is the anthropogenic noise found in shallow waters near nesting sites. Since males move to shallow waters during mating season, boat traffic may be more common. It is unknown the impact of boat traffic on the ability for females to locate mating calls, but if the otolithic endorgans potentially respond to pressure waves (indirectly through vibrations off the swim bladder), the lateral line may have a better hearing sensitivity for successfully locating mating calls through solely particle motion vibrations at near distances. Therefore, comparing the differences in auditory perception of external sound sources between the inner ear and lateral line would provide insight into adaptations for sound localization in fish.

To better determine temporal differences in sound arrival time between neuromasts of the lateral line, the frequency of maximum sensitivity (100 Hz) could be the stimulus to look at the compared response between the left and right anterior lateral lines. This may elicit a clear phase-locking and/or spike rate trend so these mechanisms could be further used in measuring time delays. Additionally, to determine what further information the lateral line system can provide that the inner ear cannot, bilateral electrodes can be implanted into both the auditory and lateral line nerve to compare differential responses during sound stimuli presentation. The function range, physical properties received, and overall response of each system could be determined.

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