Anterior lateral line nerve encoding to tones and play back vocalisations in free swimming oyster toadfish, *Opsanus tau*

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Running Head: Lateral line nerve response to sound.
Summary

In the underwater environment, sound propagates both as a pressure wave and particle motion, with particle motions dominating close to the source. At the receptor level, the fish ear and the neuromast hair cells act as displacement detectors, and both are potentially stimulated by the particle motion component of sound. The encoding of the anterior lateral line nerve to acoustic stimuli in freely behaving oyster toadfish, Opsanus tau, was examined. Nerve sensitivity and directional responses were determined using spike rate and vector strength analysis, a measure of phase-locking of spike times to the stimulus waveform. All units showed greatest sensitivity to 100 Hz stimulus. While sensitivity was independent of stimuli orientation, the neurons ability to phase-lock was correlated with stimuli origin. Two different types of units were classified, Type 1 (tonic), and Type 2 (phasic). The Type 1 fibers were further classified into two sub-types based on their frequency response (Type 1-1 and Type 1-2), which was hypothesised to be related to canal (Type 1-1) and superficial (Type 1-2) neuromast innervation. Lateral line units also exhibited sensitivity and phase locking to boatwhistle vocalisations, with greatest spike rates exhibited at the onset of the call. These results provide direct evidence that oyster toadfish can use their lateral line to detect behaviourally relevant acoustic stimuli, which could provide a sensory pathway to aid in sound source localisation.

Key Words: anterior lateral line nerve, acoustic stimuli, vocalisations, toadfish
Introduction

An underwater acoustic stimulus has two components, both of which provide important information to fish. The “nearfield,” is dominated by hydrodynamic flow and the “farfield,” is dominated by a propagating pressure wave. Hydrodynamic flow is generated by the movement of water near the acoustic stimulus source, while sound pressure waves propagate from the acoustic source as a cyclic compression and rarefaction of the water (Higgs et al., 2006; Rogers and Cox, 1988). The fish mechanosensory lateral line is sensitive to hydrodynamic flow within one to two body lengths from the source (nearfield), and is not generally sensitive to pressure (Montgomery et al., 1995; Webb et al., 2008). The lateral line has two types of receptors: superficial neuromasts, which lie on the surface of the skin, and canal neuromasts, which are found in subdermal canals that open to the external environment via a series of pores. The inner ear is also sensitive to the particle movement of an acoustic field as a result of whole body accelerations (Montgomery et al., 2006; Rogers and Cox, 1988). Sound pressure can be detected by fish from pressure-induced oscillations of the walls of an air pocket, such as the swim bladder, that then are transduced into mechanical stimuli appropriate to sensors (Higgs et al., 2006), such as the hair cells of the inner ear (Montgomery et al., 2006).

Detection of the pressure component of sound waves typically results in increased sensitivity and/or hearing bandwidth (Higgs et al., 2006) and is accomplished via either otophysic or laterophysic connections. Four different patterns of otophysic connections, between the inner ear and the swim bladder, exist (Popper and Fay, 2011; Schellart and Popper, 1992): skeletal connections between the swim bladder and ear in Ostariophysi (e.g. carps and catfishes - Chardon and Vandewalle, 1997; von Frisch, 1938), direct connections between the auditory bullae (swim bladder extensions) and the ear in Clupeiformes (e.g. herrings – Best and Gray, 1980; O’Connell, 1955), isolated air bubbles in the branchial chamber in Anabantoidei (e.g. gouramis – Fletcher and Crawford, 2001; von Frisch, 1938), and extensions from the swim bladder to the ear in a variety of groups (e.g. Holocentrids – Chao, 1978; Coombs and Popper 1979; Ramcharitar et al., 2001; Webb et al., 2006).

Laterophysic structures connect the swim bladder and lateral line, and appear to occur less frequently than otophysic structures (Popper and Fay, 2011; Schellart and Popper, 1992; Webb et al., 2006). The first description of a laterophysic connection was in the clupeomorphs where the auditory bulla connects the swim bladder to the recessus lateralis of the lateral line (O’Connell, 1955). The catfish, Ancistrus sp. also has a unique connection
between the inner ear and trunk lateral line canal system (Bleckmann et al., 1991). The most well described laterophysic connection is in the family Chaetodontidae (butterflyfishes), which possess an association of the anterior bilateral swim-bladder horns with a medial opening in the supracleithal bone, linking swim bladder vibrations to lateral line canals (Webb, 1998; Webb and Smith, 2000; Webb et al., 2006).

Recent experiments have demonstrated that the lateral line enables fishes without otophysic or laterophysic connections to determine sound direction (Mirjany and Faber, 2011; Mirjany et al., 2011). These studies employed an auditory evoked escape response (startle) to show that the lateral line was essential for fish to determine which direction the threat was coming from, as fish with deactivated lateral lines, fled in random directions. However, fish with impaired vision but intact lateral lines, successfully headed in an opposite direction to the threat. Furthermore, fish with posterior trunk lateral lines deactivated were still able to escape, indicating the anterior lateral line is essential for the fish to locate the source of the threat. Another study (Higgs and Radford, 2013) using auditory evoked potentials (AEPs), has shown that goldfish hearing sensitivity to low frequency (100 – 200 Hz) sound decreased after ablation of the lateral line neuromasts. However, there was no change in hearing sensitivity if only the superficial neuromasts were damaged. Therefore, it appears that canal neuromasts are the main factor in increasing hearing sensitivity.

The potential complications of near-field effects on the study of the acoustic-lateralis system, and the overlap of lateral line and inner ear stimuli were highlighted in the early 1960’s (Harris and van Bergeijk, 1962). However, very few studies since have addressed the contribution of the lateral line to acoustic sensitivity. Weeg and Bass (2002) examined the posterior lateral line nerve of the plainfin midshipman fish (Porichthys notatus) and provided evidence of acoustic sensitivity using near-field stimuli. A further study (Weeg et al., 2005) showed that the efferent fibers of the lateral line and auditory nerves have features for cancelling self-generated noise to maintain peripheral sensitivity. Apart from Mirjany et al (2011a; b,) and Higgs and Radford (2013), there are no other studies investigating the sensitivity of the lateral line to an underwater acoustic stimulus. Therefore, the focus of this study was to investigate the sensitivity of anterior lateral line nerves to both pure tones and naturally relevant vocalisations in the oyster toadfish, Opsanus tau to provide further insight into how fish localize sound.
Batrachoidid fishes (toadfish and midshipman) represent some of the best studied vocalizing fishes (see Amorim, 2006; Bass and McKibben, 2003 for reviews). During late spring and early summer, male oyster toadfish establish territories in shallow water (1 - 3 m), where they acoustically attract females by production of their characteristic boatwhistle call (Fine, 1978; Maruska and Mensinger, 2009; Thorson and Fine, 2002). The dominant frequency of the boatwhistle ranges between 90 - 250 Hz depending on season and geographical location (Fine, 1978). However, in the shallow waters, sound propagation of these low frequencies is limited because of their long wavelengths and constant interaction with the sea surface and floor resulting in rapid attenuation (Bass and Clark, 2002; Rogers and Cox, 1988). Additionally, females may encounter numerous male toadfish in close proximity indicating mate choice may be dictated by close range interactions (nearfield) where particle motions will dominate, providing support for the lateral line playing a major role in acoustic reception and localisation.

**Results**

**Tether**

Toadfish tolerated the procedures well and regained equilibrium within minutes of being placed in the experimental tank. Spontaneous neural activity was initially depressed but reached normal resting levels within 90 minutes. Breathing rates returned to pre surgical levels with 60 minutes and toadfish gradually regained locomotory capability as the pancuronium bromide was metabolized. However, as with non-experimental fish, the toadfish remained quiescent during the sound stimulus and therefore strain on, and/or entanglement with the tether was rarely observed. Units maintained strong activity for at least 36 hours. Figure 1 shows an example of the stimulus and raw spike data recorded in Spike2.

**Temporal response patterns**

The average spontaneous discharge rate of all the fibers was 48.0 ± 2.4 SE with a range of 2.4 to 95.3 spikes s⁻¹ (Fig. 3). Eighty-nine percent (33/37) of the fibers were tonic and exhibited a sustained response throughout the 2 sec stimulus presentation (Fig. 4A). At lower
frequencies (100 to 200 Hz), Type 1 fibers were strongly phase-locked to the signal (Z > 6.91; P < 0.001; R > 0.05 Fig. 4D, 5, & 6) while responses to 250 and 300 Hz were weakly phase locked. The three phasic fibers only responded to the onset of the stimulus and discharged at only one phase of the stimulus (Fig. 4B). However, they also displayed phase locking to low frequency (≤ 200 Hz) stimuli. The tonic fibers had a significantly higher (49 ± 2.4 spike s⁻¹; t₀.₀₅(2), 36 = 5.7; P < 0.01) spontaneous activity than phasic fibers (5 ± 1.2 SE spikes s⁻¹). There was a third fiber type isolated (n = 1), whose spontaneous firing rate (3 spikes s⁻¹) decreased during sound presentation before rebounding to resting rates following stimulus cessation (Fig. 4C).

Anterior lateral line tuning curves

All fibers displayed maximum sensitivity at 100 Hz. Overall, the anterior lateral line afferents were significantly more sensitive (F₅, 165 = 2598.43; P < 0.001) at the lower frequencies (< 150 Hz) compared to the higher frequencies (> 200 Hz) (Fig. 7), there was also a significant difference between fiber type (F₂, 165 = 50.78; P < 0.001), with Type 1 fibers being significantly more sensitive than the other Type 2s. The anterior lateral line tonic responses were subdivided further into Type 1-1 fibers which responded to the entire frequency range and were significantly more sensitive to the lower frequencies than Type1-2 fibers (Fig. 7). The Type 1-1 responses exhibited greatest sensitivity at 100 Hz (-50 dB re 1 ms⁻²), followed by 80 (-38 dB re 1 ms⁻²) and 150 Hz (-34 dB re 1 ms⁻²), and were least sensitive at 300 Hz (-19 dB re 1 ms⁻²). The Type 1-2 fibers also had greatest sensitive at 100 Hz. The third fiber type isolated showed a similar shape in the turning curve to Type 1 and 2 fibers and the thresholds at which there was a decrease in firing rate during the stimulus playback.

Directional response

All fish tended to respond to the same threshold level independent of orientations to the speaker, however phase locking ability was significantly influenced by fish position. All recorded anterior lateral line responses were directionally sensitive to the 100 Hz pure tone stimulus (F₃₆, 22₄ = 152.38; P < 0.001). Figure 8 shows examples of directional responses from 3 different fish. Three afferents were recorded from supra orbital neuromasts on Fish
22 (Fig. 7A) with two fibers most sensitive along the anterior/posterior axis (0-180°) with the sensitivity of the third shifted 45° to the right. In contrast, Fish 18 had three sub mandibular fibers that exhibited three different directional sensitivities (0-180°, 90-270° and 135-315°.

Reponses to play-backs of the boatwhistle vocalisation

Anterior lateral line fibers were less sensitive to toadfish calls (boatwhistle), with the threshold (-23 dB re 1 ms^2) sitting between 250 and 300 Hz thresholds(Fig. 7), which were the least sensitive of the pure tones tested. The Type1-1 fiber response to the vocalisation exhibited a highly phasic response (Fig. 9C & 9D), with strong modulation at the onset of the vocalisation. Modulations of the fiber following approximately 40 msec of the stimulus onset were reduced substantially. There was also evidence that the fiber modulation was reduced after each consecutive vocalisation was presented (Fig. 9C). However, the responses showed remarkably strong phase-locking to the fundamental frequency (195 Hz) of the call, with the spike responses clustered around 210° (Z = 920; P < 0.001; R= 0.08) (Fig. 9E).

Discussion

The objective of this study was to assess the ability of the anterior lateral line to respond to both pure tones and naturally relevant bioacoustical stimuli. The procedure allowed underwater sound presentation to a free swimming, naturally behaving toadfish under controlled conditions. The oyster toadfish could detect boatwhistles and pure tones from 80 to 300 Hz, with peak sensitivity at 100 Hz, with the majority of the recorded nerve fibers exhibiting directional sensitivity. The response range of the anterior lateral line includes the frequency range of both toadfish grunts and boatwhistles, and strongly suggests that in the acoustic near-field, the acoustico-lateralis system has the potential to play a crucial role in sound detection and localisation in fish.

Spontaneous activity

Patterns of spontaneous activity were similar to the irregular type of lateral line fibers, while tonic fibers exhibiting similar discharge rates to those reported previously for the oyster.
toadfish (Palmer et al., 2005; Tricas and Highstein, 1991). Although silent fibers that
previously have been isolated in the toadfish lateral line were not isolated (Palmer et al.,
2005), several fibers of relatively low frequency were characterized and found to have phasic
properties. These types of fibers have also been reported in a closely related toadfish, the
midshipman (Weeg and Bass, 2002) and in other teleosts (Coombs and Janssen, 1990; Kroese
and Schellart, 1992; Montgomery et al., 1988; Munz, 1985; Wubbels et al., 1990).

Although the approximate neuromast location could be determined, it was difficult to
assess which types of neuromasts were responsible for the observed neural activity. Previous
studies (Montgomery et al., 2000; Voigt et al., 2000) have shown that superficial and canal
neuromast afferents have different response properties. Typically, canal neuromasts are more
sensitive and respond to a wider range of frequencies compared to superficial neuromasts.
Using this definition, the Type 1-1 fibers appeared to respond to canal neuromast stimulation
and the Type 1-2 fibers were modulated to superficial neuromasts stimulation.

The pool of afferent responses of the oyster toadfish had a sustained (tonic) non-
adapting response to the stimulus that consisted of an increase in spike rate, synchronisation
of the spike times to the stimulus waveform, or more commonly a combination of the two.
These types of responses have been commonly observed in lateral line afferents of other
toadfish (Weeg and Bass, 2002) and teleosts in general. A highly phasic afferent response
was also observed, for three anterior lateral line nerve (aLLn) afferents, in which they only
responded to the onset of the stimulus similar to that observed for saccular afferents in the
sleeper goby (Dormitor latifrons) (Lu et al., 1998). These types of phasic responses have not
been seen before for primary lateral line afferents, but have been observed in the crest cells of
the midbrain (Montgomery et al., 1996). It is postulated that the phasic response could be
caused by down regulation of the afferents by the efferent system. It is also possible that this
type of response was not observed previously due to differences in stimulus duration. Other
studies (Coombs et al., 1998; Montgomery et al., 1996; Weeg and Bass, 2002) have used
shorter stimulus cycles (< 1 sec) compared to the current study which had a 2 sec duration.

There was one fiber that exhibited an inhibitory type of response to the stimulus.
Weeg and others (2005) showed that the efferent system exhibits an increase in activity
during fictive vocalisation in the midshipman which reduces the sensitivity of the lateral line
and inner ear to self-generated noise. Putative efferent fibers in the vestibular system of the
toadfish exhibit very low spontaneous firing (< 0.5 Hz) and rarely respond to external stimuli
(Weeg and Bass, 2002). However, unlike the midshipman efferents, this fiber decreased firing rate during sound presentation, indicating that it may be receiving efferent modulation but is probably not an efferent fiber. Although nearly 90% of the characterized units were tonic, the current preparation was unable to assess if this was a representative sampling of the population or if the results were biased due to the implant site. However, the existence of two distinct fiber classes provides a wide response range for lateral line fibers.

**Frequency sensitivity**

The anterior lateral line system of the oyster toadfish responded between 80 – 300 Hz with best sensitivity at 100 Hz. Comparisons with other lateral line studies are difficult because most have reported physiological responses in terms of displacement or acceleration in response to a unidirectional vibrating sphere in contrast to the present study which reported the physiological responses in terms of the magnitude of particle acceleration from an omnidirectional underwater speaker. Additionally, many of these studies examined frequency and threshold response properties below 80 Hz (Bleckmann et al., 1989; Montgomery et al., 1988; Weeg and Bass, 2002), which were below the capabilities of the underwater speakers. However the underwater speaker allows the generation of naturally relevant sound in addition to a pressure component in comparison to highly directional particle motion of a vibrating sphere. The shapes of frequency response curves are dependent on the stimulus parameters (Kalmijn, 1988; Kalmijn, 1989) and translating the frequency response between different stimulus components is not appropriate if the responses are not linear (Coombs and Montgomery, 1994; Montgomery et al., 1988). However, the frequency of the toadfish anterior lateral line is well within the range that has been reported for other teleost fish (Webb et al 2008) and the natural vocalizations of the toadfish.

**Directional response properties**

Even though there is considerable knowledge on the directional response properties of saccular afferents of the inner ear (Fay, 1984; Fay and Edds-Walton, 1997; Fay and Edds-Walton, 2000; Lu et al., 1998). To the authors’ knowledge, this is the first study to investigate the directional response properties of the lateral line in an intact, naturally behaving fish using an underwater sound stimulus. Lateral line neuromasts are known to be
directionally sensitive and in teleosts the axis of maximum sensitivity corresponds with the long axis of the sensory strip (Coombs et al., 1988; Coombs and Montgomery, 1994; Janssen et al., 1987; Webb, 1989).

In the present study, the $F$ statistic was used as a criterion to determine if afferent’s were directionally sensitive to the stimulus at a statistically significant level. If there was a significant difference observed among responses ($Z$ & $R$ values) at different angles, the afferents response was directional. It was shown that 100% of the afferents recorded from the aLLn were directional, with different afferents showing different strengths and degrees of directionality. Directional sensitivity has also been demonstrated in the surface feeding fish, *Aplocheilus lineatus* (Bleckmann et al., 1989) and the amphibian, *Xenopus laevis* (Görner and Mohr, 1989) in which the response magnitude increases as the stimulus direction becomes more aligned with the axis of maximum sensitivity.

As suggested above, it is highly probable that the majority of the tonic responses were responses from superficial neuromasts. Superficial neuromasts distributed over the head in many teleosts show variable orientations (Janssen et al 1987; Song & Northcutt 1991; Coombs & Montgomery 1994), while the orientation of the phasic canal responses depends on the orientation of the canals themselves. One interesting aspect of the morphology of at least a portion of these superficial neuromasts is that they are surrounded by paired finger like projections. One function of these appendages may be to protect the hair cells from suspended sediment common in the toadfish habitat. However, the axis of hair cell orientation is perpendicular to the projections, which would channel water currents in specific directions over the cupula. This may be analogous to what occurs in the killifish (*Aplocheilus lineatus*), where the fleshy ridges on the fishes head block the propagating surface waves and direct the energy of waves along the axes of sensitivity of the neuromast (Schwarz et al., 2011). Additionally, individual neuromasts are arrayed in a myriad of different orientation which may allow directionally sensitivity (Marranzino et al., 2013). Considering the variability in the directionality observed in the tonic and phasic aLLn responses, it seems the anterior lateral line system has a population of hair cells in different orientations to respond to particle motions generated by a sound stimulus in many different directions.

*Sound detection and localisation*
The role of the lateral line in sound detection has long been debated (see Braun et al 2002 for review). The acoustic field around a sound source consists of a particle motion nearfield and a pressure dominated farfield (Rogers and Cox, 1988). The effective stimulus of the lateral line is water movement relative to the fish, subsequently nearfield particle motion is the only part of the sound stimulus the lateral line is capable of detecting. Therefore, it is likely that the acoustic stimulation of the lateral line system of teleosts is confined to close proximity to the source.

Male oyster toadfish produce loud (~140 dB re 1µPa – Tavolga, 1971) reproductive vocalisations with fundamental frequencies ranging between 90 – 250 Hz depending on season and geographical location (Fine, 1978). The results show that the aLLn response was well within this range, plus there was significantly phase-locking to the fundamental frequency (195 Hz) of the call. The nearfield dominates the acoustic field up to a distance of $\lambda/2\pi$ from the source (Bass and Clark, 2002) and is on the order of 1 – 3 m for the fundamental frequency range observed for oyster toadfish reproductive vocalisations. During the reproductive season, male toadfish produce advertisement calls (“boatwhistles”) to attract females and “grunts” and “growls” to deter conspecific males during territory defense (Fine, 1978; Maruska and Mensinger, 2009). These interactions typically occur in shallow water (1 – 3 m) and often within several body lengths and place the receiver well within the regions of the nearfield, where stimulation of the lateral line would occur.

The lateral line system might also be stimulated indirectly through the swim bladder (Braun et al., 2002; Sand, 1981). Within the farfield, the swim bladder will oscillate in response to the pressure stimulus and reradiate the sound creating an indirect nearfield signal. Sand (1981) demonstrated that the displacement thresholds of the roach (Rutilus rutilus) lateral line are below the particle displacements of reradiated pressure waves, suggesting that the indirect stimulus is detectable by the lateral line system of these fish. Therefore, it is reasonable to assume that the oyster toadfish lateral line potentially could indirectly detect conspecific vocalisations beyond the range of direct lateral line stimulation.

Alternatively, determining flow direction may be all the fish needs for sound source localisation at these short distances and hence the ears may be redundant in this respect. Theoretically, flow direction could be determined by comparing the response of differently orientated neuromasts within the anterior lateral line population (Sand, 1981; Webb, 1989). Neuromasts that are orientated with their axis of maximum sensitivity parallel to the flow will
respond maximally, while neuromasts orientated orthogonally to the flow will respond minimally. The anterior neuromasts of oyster toadfish in the present study displayed directional tuning, therefore they have the potential to determine source direction of nearfield particle displacements produced by a vocalising fish using only the lateral line system.

**Conclusions**

The present study demonstrates that the oyster toadfish anterior lateral line system was capable of encoding frequencies within the range of natural vocalisations. The question still beckons, what further information can the lateral line provide that the inner ear cannot? It is unknown what cues female toadfish use for mate selection, especially in the nocturnal and shallow murky habitats they inhabit. The auditory system is most likely used to guide the female to an advertising males (Winn, 1972), however the final decision may be occurring in the nearfield, especially with high densities of males within meters of each other. Additionally, the effects of calling on hearing sensitivity remain unkown and the lateral line could provide additional sensitivity to detect conspecific boatwhistles subsequently adjust calling rates. Therefore, it is suggested that all components of the octavolateralis system (inner ear and lateral line) can contribute to the crucial role of sound localisation in fishes.

**Methods**

*Animal Husbandry*

Adult toadfish (n = 17; 25 ± 2.7 SE cm standard length) of either sex were obtained from the Marine Biological Laboratory (MBL, Woods Hole, MA). The fish were maintained in large flow through seawater tanks and maintained at local ambient seawater temperatures (19-21 °C). All experimental procedures conformed to institutional animal care protocols (Approval MBL # 12-07F-IACUC).

*Microwire electrode*

Microwire electrodes consisting of three insulated 20 µm-diameter 10% platinum/iridium wire (Sigmund Cohn) were custom fabricated for each implant. Each microwire was fixed to
hard silver-plated copper multistrand wire (25 µm diameter, New England Wire) with conductive silver paint. The multistranded wire was attached to silver wire (320 µm) that terminated into a multipin underwater connector. The anterior portions of the microwires were threaded through a 1 cm length of polymide tubing (180 µm outer diameter) to maintain the recording sites in proximity. Any exposed wire/connectors were encased in medical device adhesive and cured with ultraviolet light. The impedance of each electrode channel was determined with an impedance-test unit (FHC) and only electrodes with impedances between 0.5 and 1.5 MΩ were used.

Anterior lateral line nerve potential measurements

Fish were anaesthetized by immersion in 0.005% tricaine (3-aminobenzoic acid ethyl ester) in seawater and paralyzed with an intramuscular injection of 0.01% pancuronium bromide (600 µg kg⁻¹). The fish was then placed in a custom designed, plexiglass holding tank. An incision was made through the dorsal musculature overlying the sagittal crest, and the muscle retracted. A small craniotomy was performed to the right of the sagittal crest and posterior to the transverse crest to expose the anterior ramus of the anterior lateral line nerve. Using a micromanipulator, each electrode was lowered into the right anterior lateral line nerve just prior to its exit from the braincase. Potentials were differentially amplified (Dagan, USA) and monitored on a portable computer using Spike2 for windows software (Cambridge Electronic Design Ltd, UK). Once extracellular neural activity was detected, a small brush was run over the head to approximate neuromast location. The fish was left undisturbed for 30 min to ensure fiber stability.

Cyanoacrylic gel was used to affix the electrode to the skull and seal the craniotomy. The muscle was restored to its original position, and the muscle, fascia and epidermis were individually sutured to provide a watertight seal over the craniotomy and around the transdermal electrode lead. The fish was then transferred to the experimental tank and allowed to recover for 90 min which is the time necessary for the MS-222 to lose its efficacy (Palmer and Mensinger, 2004). Following the recovery period, the electrode implant was attached with a water proof connector to 2.5 m long, flexible tether that terminated into the differential amplifier.
The experimental tank consisted of an aquarium (1.2 cm × 50 cm × 40 cm) that was lined on the four walls with extruded polystyrene (Foamular 150 XPS; 5 cm thick) to acoustically isolate the tank. Water depth was maintained at 50 cm and the tank was constantly provided with flow through ambient seawater (18 – 20 °C) except during sound presentation. The fish was positioned in the centre of the tank such that the head of the toadfish was 25 cm away from the underwater loudspeaker (UW30, Lubell, USA), which was positioned at one end of the acoustically isolated aquarium. Acoustic stimuli were synthesised and generated using RPvdsEX software (Tucker Davis Technology (TDT), USA), digitised (RP 2, TDT, USA) attenuated (PA5, TDT, USA) and amplified (Speco PAT-20TB) before being played through an underwater loudspeaker. The frequency response of the underwater loudspeaker was measured using a calibrated HTI-96-MIN hydrophone (High Tech Inc, USA) and a B&K 4524 triaxial accelerometer (Bruel & Kaer, Denmark) positioned at the location of the fish’s head during the experiments. Relative sound pressure and particle motions were calculated using an oscilloscope and adjusted with the attenuator to insure that the sound pressures and particle motions at all frequencies were of equal amplitude (± 2 dB). The lateral line senses particle acceleration (canal) or velocity (superficial) (Webb et al., 2008) which is a three-dimensional vector and therefore accelerations were calculated for the x-, y- and z-planes and the acceleration magnitude (1) is reported in the current study.

\[ \sqrt{x^2 + y^2 + z^2} \] (1)

Auditory stimuli consisted of 30 repetitions of single tones 2 sec in duration, with 100 ms rise and fall times or a pre-recorded 350 ms boatwhistle (fundamental frequency 195 Hz). Each repetition was presented at a rate of every 4 sec (i.e. there was 4 sec between the end and the start of the next repetition) for pure tones, and five repetitive call bursts (500 msec intervals) followed by 3 sec silence for the boatwhistle call. Anterior lateral line thresholds from anterior lateral line fibers were determined in response to pure tone frequencies of 80, 100, 150, 200, 250, 300, and 400 Hz at increasing sound levels from -60 to 0 dB re 1 ms⁻² (80 dB re 1µPa). It was determined that O. tau was most sensitive to 100 Hz, and therefore this frequency was used to determine if the anterior lateral line fibers showed different responses to different placement of the acoustic source. The fish was rotated around the center axis of an imaginary line through the transverse crest in 45° increments to maintain constant distance.
between the fish head and speaker. At each position (e.g. 45°), 100 Hz tones were played in increasing sound levels till a response was clearly seen in Spike2.

Data analysis

Spikes were discriminated using Spike2 software (Cambridge Electronic Design Ltd) allowing differentiation of 2 to 3 units per implant. 400 Hz pure tone stimulation failed to modulate any units and therefore, this frequency was excluded from statistical analysis. Resting spike rate for each fiber was measured for 1 min while the fish was unperturbed. Neural responses were measured in terms of average evoked spike rate and vector strength of synchronisation. Spike rate was determined for each stimulus and averaged across the 30 presentations. Anterior lateral line auditory thresholds were defined as the level at which evoked spike rates first showed an increase in firing rate towards the stimulus, and increased firing rates were maintained with the increase in sound level (Fig. 2). From these, auditory tuning curves were created for the anterior lateral line fibers.

Based on the profiles of the PSTH for stimuli 10 dB above threshold, anterior lateral line responses were classified into two groups: tonic, or phasic. The response was classified as “phasic”, if the ratio of the number of spikes occurring during the second half of the stimulus presentation (1-2 sec) over the total number of spikes was equal or less than 0.1, and “tonic” if the ratio was greater than 0.1 (Lu and Fay, 1995). The tonic responses were subdivided based on the auditory tuning curve with Type 1-1 modulated across the full frequency range (80 to 300 Hz) and Type 1-2 only modulated from 80 to 250 Hz.

To determine if the anterior lateral line responses were phase-locked, phase histograms were generated for each unit. The coefficient of synchronisation (R) was calculated from the phase histograms to represent phase-locking strength (Anderson, 1979; Goldberg and Brown, 1969). However, R is likely to 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 anterior lateral line afferents. An afferent was significantly phase-locked to the tone or call stimulus if Z > 6.91 (P < 0.001). To describe the strength of phase-locking of the afferents, the same criterion as Lu and Fay (1993; 1995;
1998) was applied to distinguish strongly phase-locked afferents (R ≥ 0.5) from weakly
phase-locked afferents (R < 0.5). All phase-locking analysis was done in Matlab using the
CircStat toolbox (Berens, 2009).

To determine the directional sensitivity, R and Z were calculated for each isolated
afferent at each 45° increment once the threshold level was determined (see above).
Subsequently, polar-plots of R were constructed to characterise an afferent’s directionality,
with maximum R values indicating the direction of best sensitivity for that particular afferent.

Statistical analysis

To determine the effects of afferent type and frequency on the lateral line tuning curves, a
two-way ANOVA was conducted. Following a significant result, a Tukey’s honestly
significant difference (HSD) test was conducted to determine differences. One-way ANOVA
were conducted on the R values followed by Tukey’s HSD test to determine directionality.
All data conformed to normality and homogeneity tests and statistical analysis was conducted
in SigmaPlot (v11, Systat Software). Data are reported as means ± standard error.

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References


Figure 1: An example of raw spike data recorded from the chronic electrode implanted into the aLLn exposed to a 100 Hz acoustic stimulus (black line) at 90 dB re 1 µPa.

Figure 2: Peristimulus time histograms (PSTH) of toadfish anterior lateral line fibers responding to 100 Hz stimulation. The response is from a Type 1 fiber with plots A through D, displaying the firing rate of the fiber (spikes s⁻¹) binned in 50 ms increments, to increasing stimulus intensity. The horizontal black bar represents the onset and duration of the signal.

Figure 3: The number of anterior lateral line afferent units is plotted versus spontaneous activity rate (spikes s⁻¹). Bin size = 5 spikes s⁻¹. Black bars represent phasic fibers and grey bars tonic fibers.

Figure 4: Peristimulus Time Histograms (PSTH) and phase histograms of three classes of toadfish anterior lateral line fibers. Graphs on the left display the firing rate of the fiber (spikes s⁻¹) binned in 50 ms increments, in response to acoustic stimuli intensity. Phase histograms (on right) of the same fibers binned in 3 ° increments (Graphs A and D – Type 1-1; B and E – Type 2; and C and F – inhibitory. R is the coefficient of synchronisation and represents the degree of phase locking.

Figure 5: Phase histograms of Type 1 fiber recorded from the anterior lateral line nerve of the toadfish in response to pure tone stimuli from 80 to 300 Hz and binned in 3 ° increments. R is the coefficient of synchronisation and represents the degree of phase locking.

Figure 6: The Rayleigh statistic (Z) is plotted versus the coefficient of synchronisation (R) for anterior lateral line Type 1 fibers at each frequency 80 to 300. N= 33 for all frequencies except 300 Hz (N=25) as Type1-2 fibers did not respond at this frequency. The dotted horizontal line, Z = 6.91, distinguishes significantly phase-locked afferents which are located above the line. The dotted vertical line at R = 0.5, divides strongly phase-locked afferents (> 0.5?) from weakly phase-locked afferents.

Figure 7: Toadfish aLLn thresholds pressure (A – dB re 1 µPa) and particle acceleration (B - dB re 1 ms⁻²) are plotted versus frequency for the three fibers types; Each point represents the mean threshold ± 1 SE for each fiber. The Type 1 response is split into 2 responses Type 1-1 (●) responded to the entire frequency range (n=25) and Type 1-2 (■) responded to a maximum frequency of 250 Hz (n=8). Phasic (○ Type 2, n=4), inhibitory (▼, n = 1), and the boatwhistle (♦, n = 37).
**Figure 8:** Polar plots showing the directional responses curve of anterior lateral line Type 1 fibers from 3 individual fish. Each symbol represents an R value for the response of that fiber to 100 Hz stimulus. The lines connecting each symbol are for illustrative purposes only. Each symbol represents the response of a single fiber. The gray lines on the axis represent angle to the speaker the fish were placed in and each line represents the different fibers isolated from the fish.

**Figure 9:** A: Raw aLLn spike data recorded with spike2. B: The waveform of toadfish boatwhistle call presented. C: An example of a PSTH of the Type 1-1 fiber response to the vocalisation stimulus. The stimulus was presented 5 times (arrows indicate onset of stimulus) consecutively (500 msec between each presentation) followed by 3 sec silence. There was a graded response observed in response to the stimulus where there was strong fiber modulation in response to the first part of the vocalisation, with little modulation to the second part. D: An example of a PSTH of the Type 1-1 fiber response to one presentation of the boatwhistle stimulus. E: A phase histogram showing that the aLLn was phase locked to the toadfish boatwhistle call.
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- 80 Hz: R = 0.64
- 100 Hz: R = 0.86
- 150 Hz: R = 0.73
- 200 Hz: R = 0.58
- 250 Hz: R = 0.38
- 300 Hz: R = 0.14

Spikes vs. Phase for different frequencies.

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