

RESEARCH ARTICLE

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

Craig A. Radford^{1,2,*} and Allen F. Mensinger^{2,3}

ABSTRACT

In the underwater environment, sound propagates both as a pressure wave and as particle motion, with particle motion 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 neuron's 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 fibres 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: Sound detection, Acoustic stimuli, Lateral line, Fish

INTRODUCTION

An underwater acoustic stimulus has two components, both of which provide important information to fish. The 'near-field' is dominated by hydrodynamic flow and the 'far-field' 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 (near-field), 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 lateralphysic 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. carp and catfishes) (Chardon and Vandewalle, 1996; von Frisch, 1938), direct connections between the auditory bullae (swim bladder extensions) and the ear in Clupeiformes (e.g. herring) (Best and Gray, 1980; O'Connell, 1955), isolated air bubbles in the branchial chamber in Anabantoidae (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). Lateralphysic 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 lateralphysic 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* spp., also has a unique connection between the inner ear and trunk lateral line canal system (Bleckmann et al., 1991). The most well-described lateralphysic connection is in the family Chaetodontidae (butterflyfishes), which possess an association of the anterior bilateral swim-bladder horns with a medial opening in the supracleithral 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 lateralphysic 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 the opposite direction to the threat. Furthermore, fish with deactivated posterior trunk lateral lines 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

¹Leigh Marine Laboratory, University of Auckland, PO Box 349, Warkworth 0941, New Zealand. ²Marine Biological Laboratory, Woods Hole, MA 02543, USA.

³Biology Department, University of Minnesota Duluth, Duluth, MN 55812, USA.

*Author for correspondence (c.radford@auckland.ac.nz)

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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 1960s (Harris and van Bergeijk, 1962). However, very few studies since have addressed the contribution of the lateral line to acoustic sensitivity. Weeg and Bass (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 fibres of the lateral line and auditory nerves have features for cancelling self-generated noise to maintain peripheral sensitivity. Apart from three studies (Mirjany and Faber, 2011; Mirjany et al., 2011; Higgs and Radford, 2013), there is no other published work 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 (aLLns) to both pure tones and naturally relevant vocalisations in the oyster toadfish, *Opsanus tau* (Linnaeus 1766), to provide further insight into how fish localise sound.

Batrachoidid fishes (toadfish and midshipman) represent some of the best studied vocalising fishes (for reviews, see Amorim, 2006; Bass and McKibben, 2003). During late spring and early summer, male oyster toadfish establish territories in shallow water (1–3 m deep), 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 and 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 (near-field) 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 electrode implant 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 min. Breathing rates returned to pre-surgical levels with 60 min and toadfish gradually regained locomotory capability as the pancuronium bromide was metabolised. 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 h. Fig. 1 shows an example of the stimulus and raw spike data recorded in Spike2.

Temporal response patterns

The mean (\pm s.e.) spontaneous discharge rate of all the fibres was 48.0 ± 2.4 spikes s^{-1} with a range of 2.4 to 95.3 spikes s^{-1} (Fig. 2). Eighty-nine per cent (28/33) of the fibres were tonic and exhibited a sustained response throughout the 2 s stimulus presentation (Fig. 3A). At lower frequencies (100–150 Hz), type 1 fibres were strongly phase locked to the signal ($Z > 6.91$; $P < 0.001$; $R > 0.05$; Fig. 3D, Fig. 4B,C and Fig. 5) while responses to 250 and 300 Hz were weakly phased locked. The three phasic fibres only responded

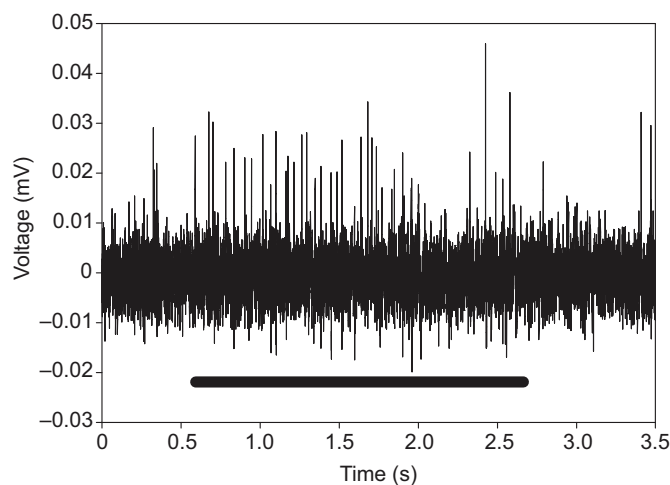


Fig. 1. An example of raw spike data. Data were recorded from the chronic electrode implanted into the anterior lateral line nerve (aLLn) exposed to a 100 Hz acoustic stimulus (horizontal black bar) at 90 dB re. 1 μ Pa.

to the onset of the stimulus and discharged at only one phase of the stimulus (Fig. 3B). However, they also displayed phase locking to low frequency (≤ 200 Hz) stimuli. The tonic fibres had a significantly higher (49 ± 2.4 spikes s^{-1} ; $t_{0.05(2),36} = 5.7$; $P < 0.01$) spontaneous activity than phasic fibres (5 ± 1.2 spikes s^{-1}). There was a third fibre 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. 3C).

Anterior lateral line tuning curves

All fibres displayed maximum sensitivity at 100 Hz. Overall, the aLLn afferents were significantly more sensitive ($F_{5,165} = 2598.43$; $P < 0.001$) at the lower frequencies (< 150 Hz) than at the higher frequencies (> 200 Hz) (Fig. 6). There was also a significant difference between fibre type ($F_{2,165} = 50.78$; $P < 0.001$), with type 1 fibres being significantly more sensitive than type 2 fibres. The aLLn tonic responses were subdivided further into type 1–1 and type 1–2 fibres; the former responded to the entire frequency range

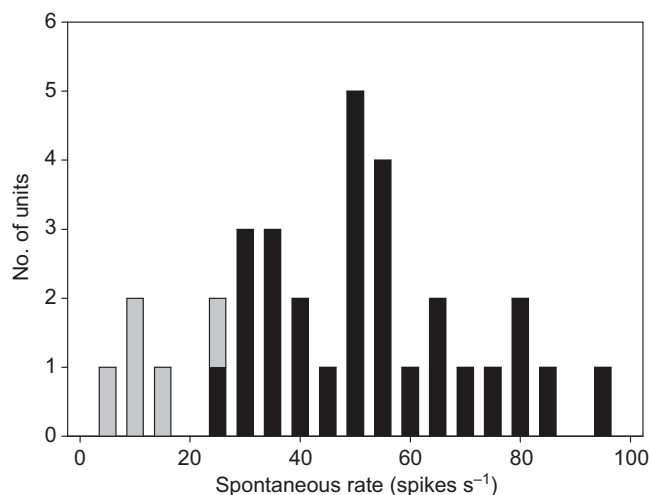


Fig. 2. The number of aLLn afferent units plotted versus spontaneous activity rate. Bin size = 5 spikes s^{-1} . Grey bars represent phasic fibres ($N=5$) and black bars indicate tonic fibres ($N=28$).

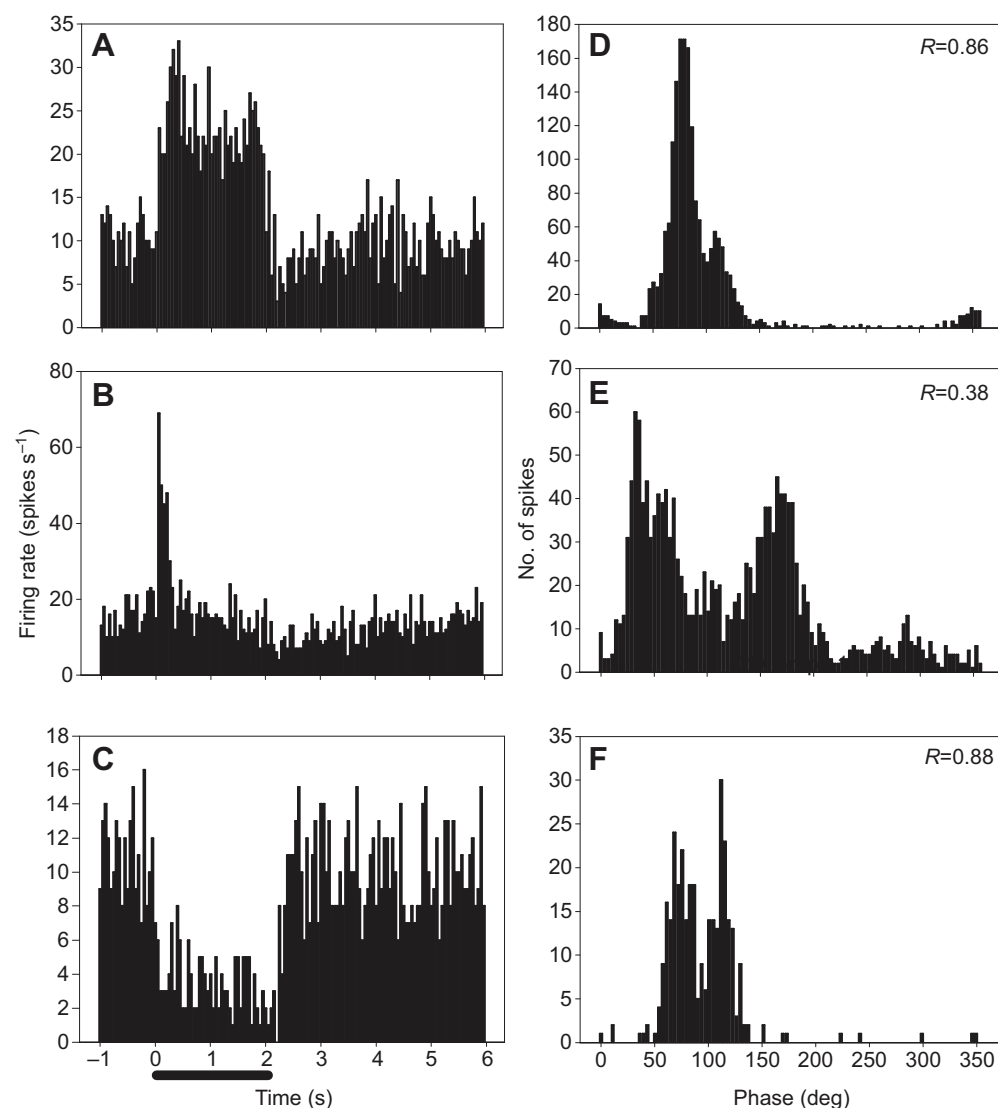


Fig. 3. Peristimulus time histograms (PSTH) and phase histograms of three classes of toadfish aLLn fibres.

Graphs on the left display the firing rate of the fibre binned in 50 ms increments, in response to acoustic stimulus intensity. Phase histograms on the right are for the same fibres binned in 3 deg increments (graphs A and D, type 1–1 tonic fibre; B and E, type 2 phasic fibre; and C and F, inhibitory fibre. R is the coefficient of synchronisation and represents the degree of phase locking.

and were significantly more sensitive to the lower frequencies than the latter (Fig. 6). The type 1–1 responses exhibited greatest sensitivity at 100 Hz (-50 dB re. 1 m s^{-2}), followed by 80 Hz (-38 dB re. 1 m s^{-2}) and 150 Hz (-34 dB re. 1 m s^{-2}), and were least sensitive at 300 Hz (-19 dB re. 1 m s^{-2}). The type 1–2 fibres also had greatest sensitivity at 100 Hz. The third fibre type isolated showed a similar shape in the turning curve to type 1 and 2 fibres, and in 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 their orientation to the speaker; however, phase-locking ability was significantly influenced by fish position. All recorded aLLn responses were directionally sensitive to the 100 Hz pure tone stimulus ($F_{36,224}=152.38$; $P<0.001$). Fig. 7 shows examples of directional responses from three different fish. Three afferents were recorded from supra-orbital neuromasts on fish 22 (Fig. 7A) with two fibres most sensitive along the anterior–posterior axis (0 – 180 deg) with the sensitivity of the third shifted 45 deg to the right. In contrast, fish 18 (Fig. 7C) had three sub-mandibular fibres that exhibited three different directional sensitivities (0 – 180 , 90 – 270 and 135 – 315 deg).

Responses to playback of the boatwhistle vocalisation

ALLn fibres were less sensitive to toadfish calls (boatwhistle), with the threshold (-23 dB re. 1 m s^{-2}) sitting between the 250 and 300 Hz thresholds (Fig. 6B), which were the least sensitive of the pure tones tested. The type 1–1 fibre response to the vocalisation exhibited a highly phasic response (Fig. 8C,D), with strong modulation at the onset of the vocalisation. Modulations of the fibre following ~ 40 ms of stimulus onset were reduced substantially. There was also evidence that the fibre modulation was reduced after each consecutive vocalisation was presented (Fig. 8C). However, the responses showed remarkably strong phase locking to the fundamental frequency (195 Hz) of the call, with the spike responses clustered around 210 deg ($Z=920$; $P<0.001$; $R=0.08$) (Fig. 8E).

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 fibres exhibiting directional sensitivity. The response range of the anterior lateral line includes

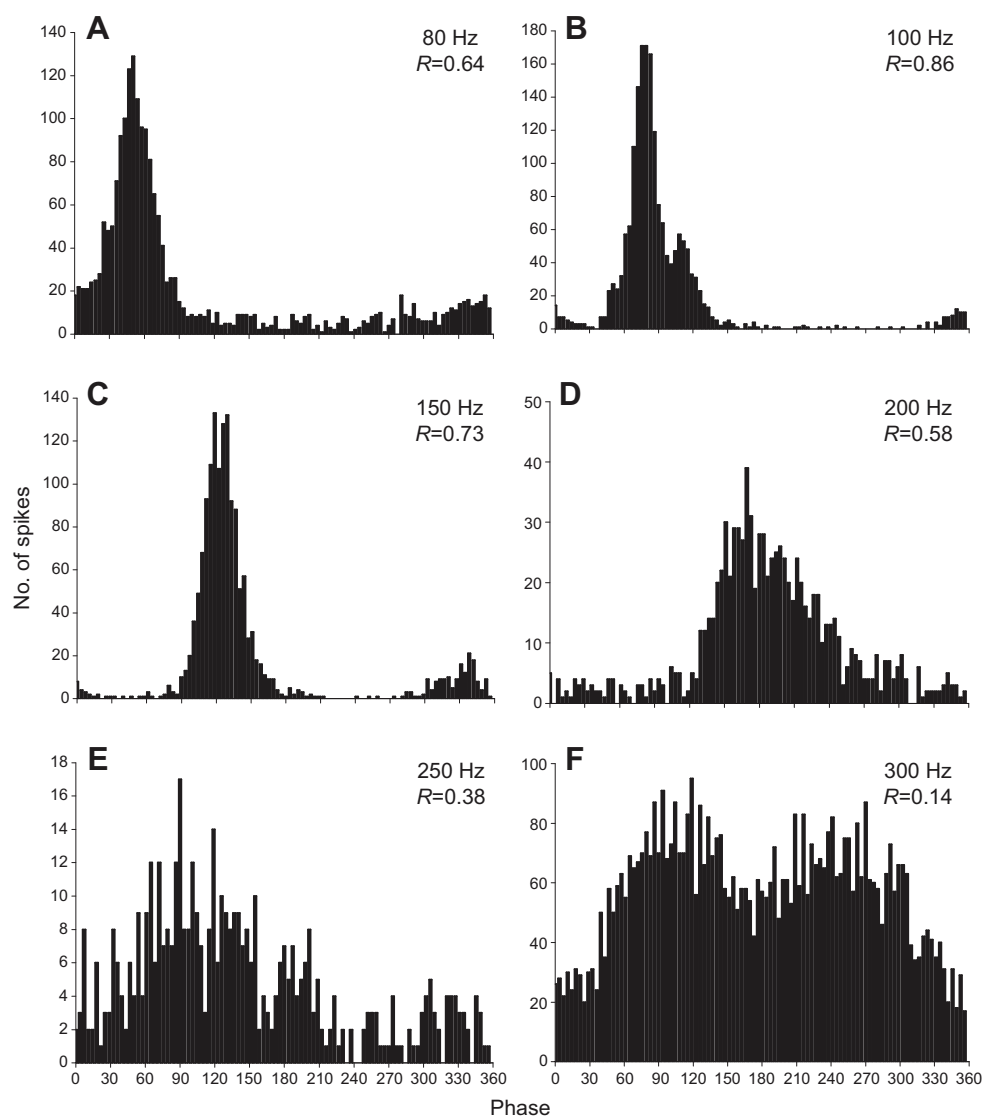


Fig. 4. Phase histograms of a type 1 fibre. Responses to pure tone stimuli from 80 to 300 Hz were recorded from the aLLn of the toadfish and binned in 3 deg increments. R is the coefficient of synchronisation and represents the degree of phase locking.

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 that of the irregular type of lateral line fibres, while tonic fibres exhibited similar discharge rates to those reported previously for the oyster toadfish (Palmer et al., 2005; Tricas and Highstein, 1991). Although silent fibres that previously have been isolated in the toadfish lateral line were not isolated here (Palmer et al., 2005), several fibres of relatively low frequency were characterised and found to have phasic properties. These types of fibres 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 with superficial neuromasts. Using this definition, the type 1–1 fibres appeared to respond to canal neuromast stimulation and the type 1–2 fibres were modulated to superficial neuromast 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 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 because of differences in stimulus duration. Other studies (Coombs et al., 1998; Montgomery et al., 1996; Weeg and Bass, 2002) have used shorter stimulus cycles (<1 s) compared with the current study which had a 2 s duration.

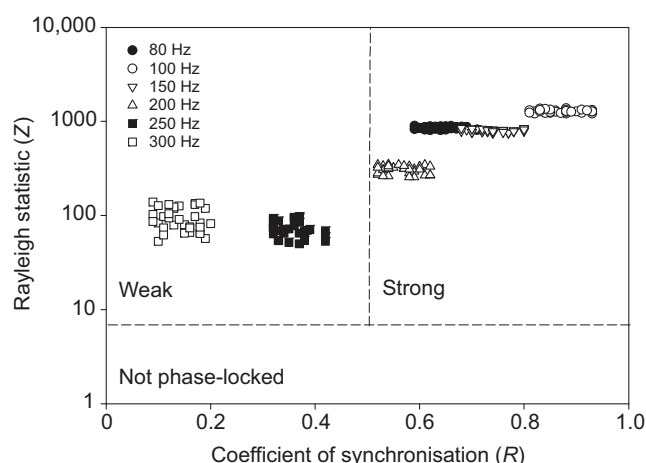


Fig. 5. The Rayleigh statistic plotted versus the coefficient of synchronisation for anterior lateral line type 1 fibre stimulation. Fibres were stimulated at frequencies of 80 to 300 Hz; $N=33$ for all frequencies except 300 Hz ($N=25$) as type 1–2 fibres did not respond at this frequency. The horizontal dashed line, $Z=6.91$, distinguishes significantly phase-locked afferents, which are located above the line. The vertical dashed line at $R=0.5$ divides strongly phase-locked afferents ($R>0.5$) from weakly phase-locked afferents.

There was one fibre that exhibited an inhibitory type of response to the stimulus. Weeg et al. 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 (Weeg et al., 2005). Putative efferent fibres 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 fibre decreased firing rate during sound presentation, indicating that it may be receiving efferent modulation but is probably not an efferent fibre. Although nearly 90% of the characterised units were tonic, the current preparation was unable to assess whether 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 fibre classes provides a wide response range for lateral line fibres.

Frequency sensitivity

The anterior lateral line system of the oyster toadfish responded between 80 and 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 used here. However, the underwater speaker allows the generation of naturally relevant sound in addition to a pressure component in comparison to the 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

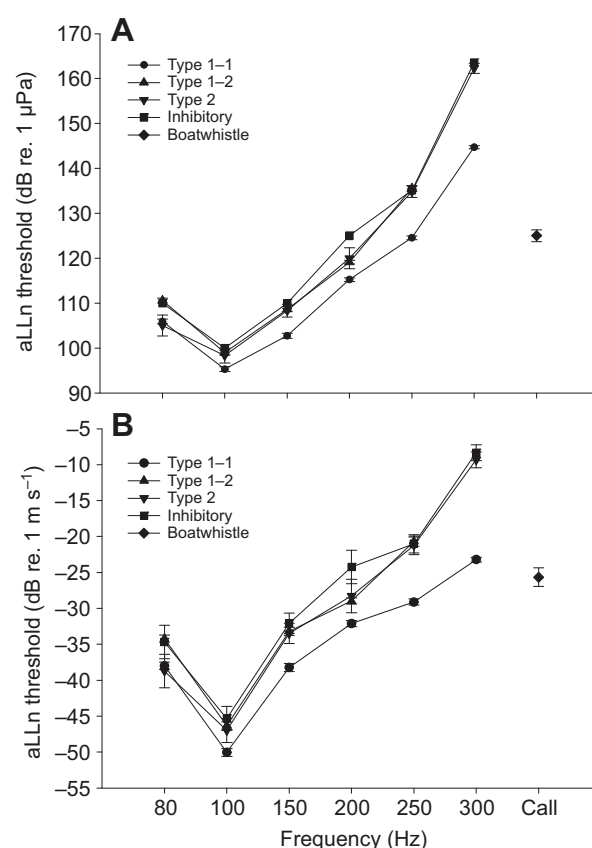


Fig. 6. Sound pressure and particle acceleration thresholds for the three fibre types. Responses to (A) pressure and (B) particle acceleration are plotted. Each point represents the mean (± 1 s.e.) threshold for each fibre. The type 1 response is split into two: type 1–1 responded to the entire frequency 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 boatwhistle ($N=37$) responses are also shown.

within the range that has been reported for other teleost fish (Webb et al. 2008) and the natural vocalisations 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 whether aLLn afferents were directionally sensitive to the stimulus at a statistically significant level. If there was a significant difference observed among responses (Z and R values) at different angles, it would indicate that the afferent 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 *Aplocheilichthys lineatus* (Bleckmann et al., 1989) and the amphibian *Xenopus laevis* (Görner and Mohr, 1989), in which the response magnitude increases as the

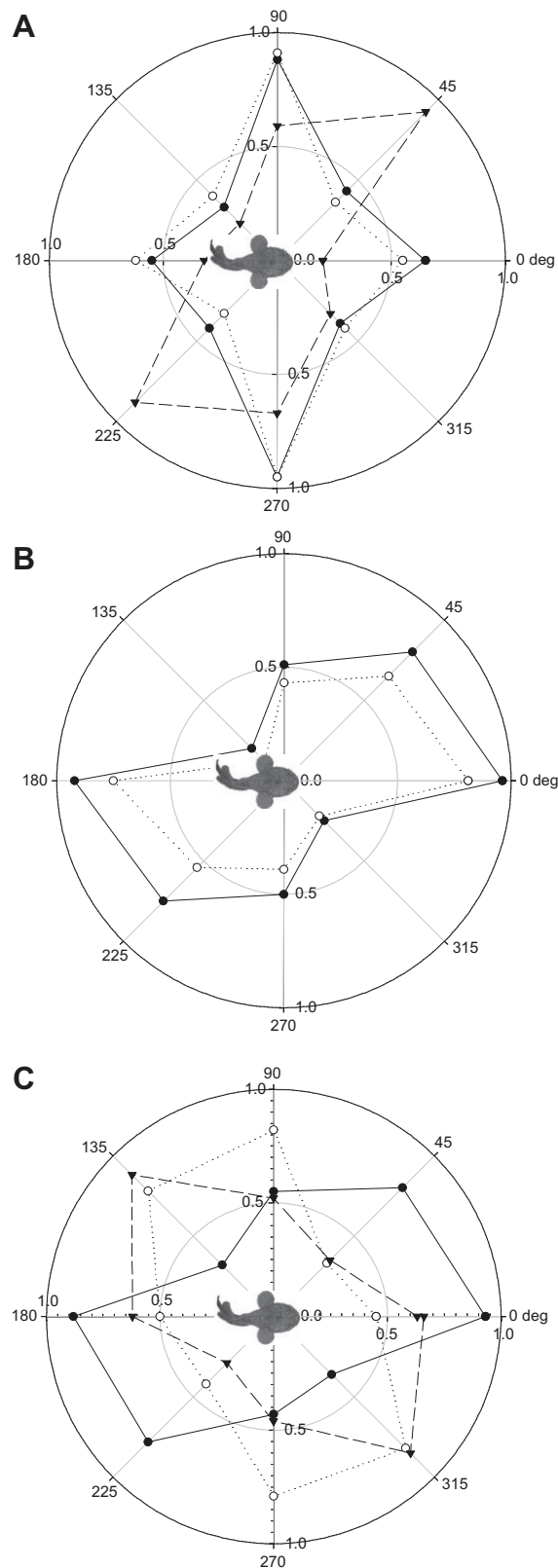


Fig. 7. Directional response properties of the anterior lateral line type 1 fibres. $N=3$ individual fish. Each symbol represents an R value for the response of that fibre to 100 Hz stimulus. The lines connecting each symbol are for illustrative purposes only. Each symbol represents the response of a single fibre. The grey lines on the axis represent the angle to the speaker at which the fish were placed and each line represents the different fibres isolated from the fish.

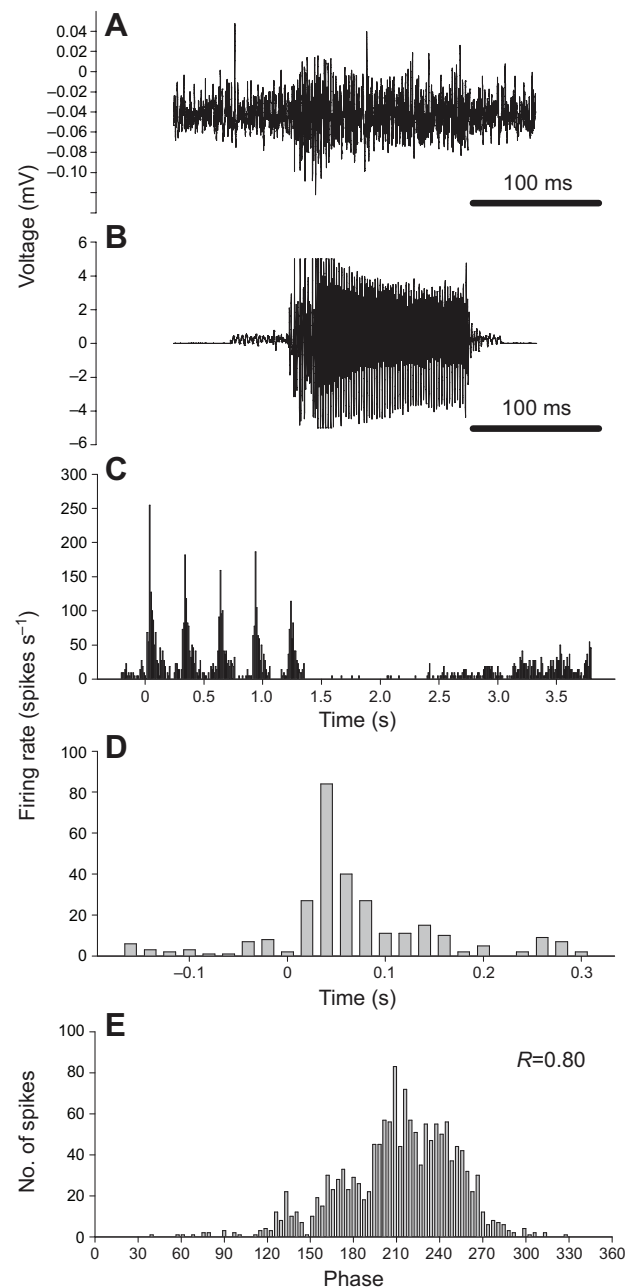


Fig. 8. Anterior lateral line type 1 fibre responses to the toadfish boatwhistle vocalisation. (A) Raw aLLn spike data recorded with Spike2. (B) The waveform of the toadfish boatwhistle call. (C) An example of a PSTH of the type 1–1 fibre response to the vocalisation stimulus. The stimulus was presented five times consecutively (500 ms between each presentation) followed by 3 s silence. There was a graded response to the stimulus such that there was strong fibre 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 fibre response to one presentation of the boatwhistle stimulus. (E) A phase histogram showing that the aLLn was phase locked to the toadfish boatwhistle call.

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 and Northcutt, 1991; Coombs and 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 (*A. lineatus*), where the fleshy ridges on the fish's 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 orientations, 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 motion 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 (for review, see Braun et al., 2002). The acoustic field around a sound source consists of a particle motion near-field and a pressure dominated far-field (Rogers and Cox, 1988). The effective stimulus of the lateral line is water movement relative to the fish; subsequently, near-field 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. 1 μ Pa) (Tavolga, 1971) reproductive vocalisations with fundamental frequencies ranging between 90 and 250 Hz depending on the season and geographical location (Fine, 1978). Our results show that the aLLn response was well within this range, plus there was significant phase locking to the fundamental frequency (195 Hz) of the call. The near-field dominates the acoustic field up to a distance of $\lambda/2\pi$ from the source (Bass and Clark, 2002) and is of 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 defence (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 near-field, 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 far-field, the swim bladder will oscillate in response to the pressure stimulus and re-radiate the sound, creating an indirect near-field signal. Sand demonstrated that the displacement thresholds of the roach (*Rutilus rutilus*) lateral line are below the particle displacements of re-radiated pressure waves (Sand, 1981), 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 aLLn 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 near-field 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 is capable of encoding frequencies within the range of natural vocalisations. The question still beckons of what further information the lateral line can 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 probably used to guide the female to an advertising male (Winn, 1972); however, the final decision may be occurring in the near-field, especially with high densities of males within metres of each other. Additionally, the effects of calling on hearing sensitivity remain unknown and the lateral line could provide additional sensitivity to detect conspecific boatwhistles and subsequently adjust calling rates. Therefore, it is suggested that all components of the octavo-lateralis system (inner ear and lateral line) can contribute to the crucial role of sound localisation in fish.

MATERIALS AND METHODS

Animal husbandry

Adult toadfish ($N=17$; 25 ± 2.7 cm standard length) of either sex were obtained from the Marine Biological Laboratory (MBL, Woods Hole, MA, USA). 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 no. 12-07F-IACUC).

Microwire electrode

Microwire electrodes consisting of three insulated 20 μ 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 μ m diameter, New England Wire) with conductive silver paint. The multistranded wire was attached to silver wire (320 μ m) that terminated into a multipin underwater connector. The anterior portions of the microwires were threaded through a 1 cm length of polyimide tubing (180 μ m 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 M Ω were used.

aLLn potential measurements

Fish were anaesthetised by immersion in 0.005% tricaine (MS-222; 3-aminobenzoic acid ethyl ester) in seawater and paralysed with an intramuscular injection of 0.01% pancuronium bromide (600 μ g kg⁻¹). The fish was then placed in a custom-designed, Plexiglas 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 aLLn. Using a micromanipulator, each electrode was lowered into the right aLLn just prior to its exit from the braincase. Potentials were differentially amplified (Dagan) and monitored on a portable computer using Spike2 for windows software (Cambridge Electronic Design Ltd). 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 fibre 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 waterproof connector to a 2.5 m long, flexible tether that terminated into the differential amplifier.

Experimental set-up, stimulus generation and calibration

The experimental tank consisted of an aquarium (1.2×50×40 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), which was positioned at one end of the acoustically isolated aquarium. Acoustic stimuli were synthesised and generated using RPvdsEX software [Tucker Davis Technology (TDT)], digitised (RP 2, TDT) attenuated (PA5, TDT) and amplified (Speco PAT20TB) 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.) and a B&K 4524 triaxial accelerometer (Bruel & Kaer) positioned at the location of the fish's head during the experiments. Relative sound pressure and particle motion were calculated using an oscilloscope and adjusted with the attenuator to ensure that the sound pressure and particle motion 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 (Eqn 1) is reported in the current study:

$$\text{Acceleration magnitude} = \sqrt{(x^2 + y^2 + z^2)}. \quad (1)$$

Auditory stimuli consisted of 30 repetitions of single tones, 2 s 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 s (i.e. there was 4 s between the end and the start of the next repetition) for pure tones, and five repetitive call bursts (500 ms intervals) followed by 3 s silence for the boatwhistle call. Anterior lateral line thresholds from aLLn fibres 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 m s^{-2} (80 dB re. $1 \mu\text{Pa}$). It was determined that *O. tau* was most sensitive to 100 Hz, and therefore this frequency was used to determine whether the aLLn fibres showed different responses to different placement of the acoustic source. The fish was rotated around the centre axis of an imaginary line through the transverse crest in 45 deg increments to maintain a constant distance between the fish head and speaker. At each position (e.g. 45 deg), 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 two to three units per implant. Pure tone stimulation at 400 Hz failed to modulate any units and, therefore, this frequency was excluded from statistical analysis. Resting spike rate for each fibre 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. 9). From these, auditory tuning curves were created for the aLLn fibres.

Based on the profiles of the PSTH for stimuli 10 dB above threshold, anterior lateral line responses were classified into two groups: tonic or

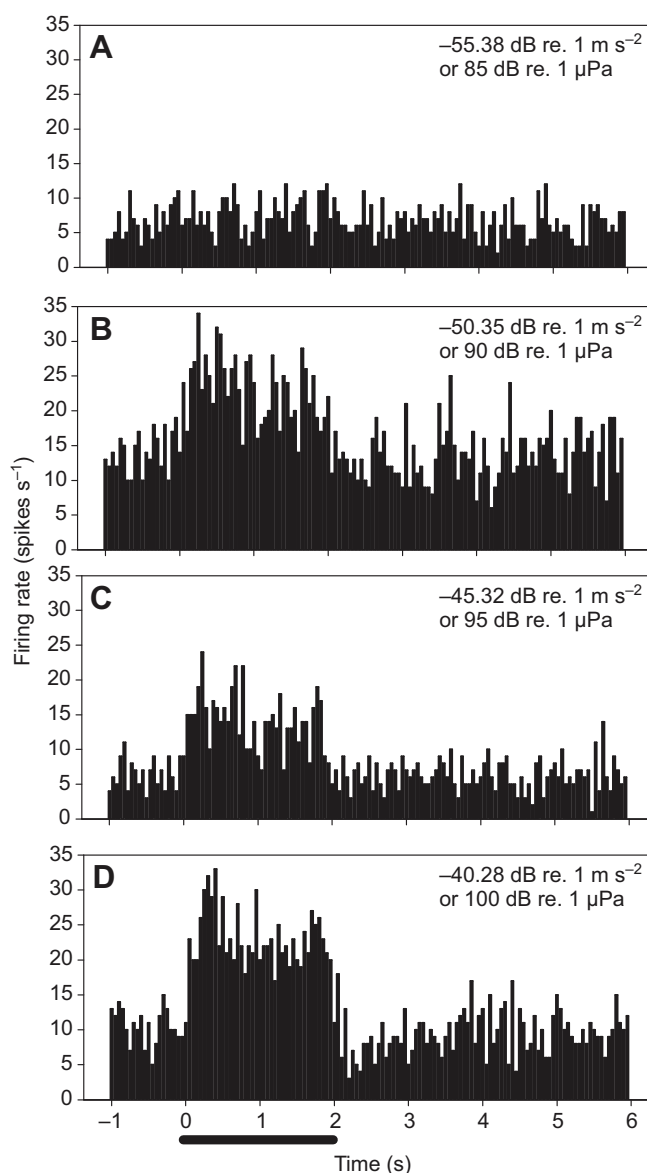


Fig. 9. PSTHs of toadfish aLLn fibres responding to 100 Hz stimulation.

The response is from a type 1 fibre with plots A–D displaying the firing rate of the fibre, binned in 50 ms increments, to increasing stimulus intensity. The horizontal black bar represents the onset and duration of the signal.

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 s) over the total number of spikes was equal to 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 (80–300 Hz) and type 1–2 only modulated from 80 to 250 Hz.

To determine whether 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 $N \times R^2$, where *N* is the total number of spikes (Batschelet, 1981), and represents the response magnitude of the aLLn 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, 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 ($R < 0.5$). All phase-locking analysis was done in Matlab using the CircStat toolbox (Berens, 2009).

To determine directional sensitivity, R and Z were calculated for each isolated afferent at each 45 deg 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 \pm s.e.

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Competing interests

The authors declare no competing financial interests.

Author contributions

C.A.R. and A.F.M. designed the experiments. C.A.R. conducted the experiments and analysed the data. C.A.R. and A.F.M. wrote the paper.

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